A common European approach to the regulatory testing of nanomaterials

NANoREG

Grant Agreement Number 310584

Deliverable D 1.09

NANoREG final report with (elements of) answers to selected issues/questions

Due date of deliverable (DoW): *2016/02/29*

New due date: *2016/06/30*

Actual submission date: *2017/04/10*

Author(s) (Beneficiary(ies)):	JRC (editor), RIVM, TEMAS, IOM, FOPH, AIT, EPFL, SINTEF, TUKES
Work package/task:	WP1 / Task 1.3
Document status:	draft / final
Confidentiality:	confidential / restricted / public
Key words:	Questions Answers Regulatory Safety Assessment Nanomaterial

This project has received funding from the European Union Seventh Framework Programme (FP7/2007-2013) under grant agreement no 310584

DOCUMENT HISTORY

Versio	*Date*	*Reasons for changes*
1	2016/01/28	First draft edited by JRC starting from D1.3, after a refinement (deletion of irrelevant sections) and inclusion of feedback and notes from 6th Consortium Meeting (Den Haag, WP7-WP1 meeting).
1	2016/02/01	Version 1 of D1.9 has been circulated to the NANoREG partners by PO for their comments and inputs. Request sent on 01/02/2016 –
2	2016/03/02	JRC: Inclusion of input and feedback received from partners until 02/03/2016 (36, 44, 11, 43, 32, 41, 54, 51, 18, 45, 22, 39, 46, Univ Bern
3	2016/05/03	JRC: inclusion of input and feedback received by the LCPs from relevant task leaders and partners after the third round of consultation – until 29/04/2016
4	2016/08/31	JRC updated the document including feedback form the CM5 'T1.3 poster session'
5	March 2017	JRC update to align with outcome of ProSafe-OECD conference issued by OECD in March.
6	2017/04/10	Final version submitted to MC
7	2017/04/18	Project Office harmonized lay-out
8	2017/06/01	Project Office harmonized pictures turning black in PDF/A

Lead beneficiary for this deliverable: Joint Research Centre, JRC, 02

Owner(s) of this document	
Owner of the content	JRC, 02
Co-Owner 1	RIVM, 05
Co-Owner 2	TEMAS, 10
Co-Owner 3	IOM, 05
Co-Owner 4	FOPH, 40
Co-Owner 5	AIT, 08
Co-Owner 6	EPFL,
Co-Owner 7	SINTEF, 44
Co-Owner 8	TUKES, 54
Co-Owner 9	BioNanoNet, 36

Table of Contents

A. SUMMARY OF ANSWERS AND ACHIEVEMENT OF D1.9 IN NANOREG 6

A.1 DESCRIPTION OF TASK 1.3 6

A.2 BACKGROUND OF TASK 1.3 AND STRUCTURE OF THE ELEMENTS OF ANSWERS IN CHAPTER B 6

A.3 SUMMARY OF THE FINDINGS AND ELEMENTS OF ANSWERS 10

A.4 EVALUATION AND CONCLUSIONS. 14

A.5 DEVIATIONS FROM THE WORK PLAN 14

B. ELEMENTS OF ANSWER TO THE QUESTIONS OF REGULATORY RELEVANCE 15

1. QUESTION 1 16

1.1. NANOREG (ELEMENTS OF) ANSWER TO THE QUESTION 16

1.2. IMPACT / IMPLICATIONS FOR THE STAKEHOLDERS 17

1.3. OVERALL ASSESSMENT / CONCLUSIONS 17

1.4. ANY OTHER RELEVANT ISSUES? 17

2. QUESTION 2 19

2.1. NANOREG (ELEMENTS OF) ANSWER TO THE QUESTION 19

2.2. IMPACT / IMPLICATIONS FOR THE STAKEHOLDERS 20

2.3. OVERALL ASSESSMENT / CONCLUSIONS 20

2.4. ANY OTHER RELEVANT ISSUES? 20

3. QUESTION 3 21

3.1. NANOREG (ELEMENTS OF) ANSWER TO THE QUESTION 21

3.2. IMPACT / IMPLICATIONS FOR THE STAKEHOLDERS 23

3.3. OVERALL ASSESSMENT / CONCLUSIONS 23

4. QUESTION 4 24

4.1. NANOREG (ELEMENTS OF) ANSWER TO THE QUESTION 24

4.2. OVERALL ASSESSMENT / CONCLUSIONS 26

4.3. ANY OTHER RELEVANT ISSUES? 26

5. QUESTION 5 28

5.1. NANOREG (ELEMENTS OF) ANSWER TO THE QUESTION 28

5.2. IMPACT / IMPLICATIONS FOR THE STAKEHOLDERS 30

5.3. OVERALL ASSESSMENT / CONCLUSIONS 31
5.4. ANY OTHER RELEVANT ISSUES? 31
6. QUESTION 6 32
6.1. NANOREG (ELEMENTS OF) ANSWER TO THE QUESTION 32
6.2. OVERALL ASSESSMENT / CONCLUSIONS 35
6.3. ANY OTHER RELEVANT ISSUES? 36
7. QUESTION 7 37
7.1. NANOREG (ELEMENTS OF) ANSWER TO THE QUESTION 37
7.2. OVERALL ASSESSMENT / CONCLUSIONS 38
7.3. ANY OTHER RELEVANT ISSUES? 39
8. QUESTION 8 40
8.1. NANOREG (ELEMENTS OF) ANSWER TO THE QUESTION 40
8.2. OVERALL ASSESSMENT / CONCLUSIONS 41
8.3. ANY OTHER RELEVANT ISSUES? 41
9. QUESTION 9 42
9.1. NANOREG (ELEMENTS OF) ANSWER TO THE QUESTION 42
9.2. OVERALL ASSESSMENT / CONCLUSIONS 43
9.3. ANY OTHER RELEVANT ISSUES? 43
10. QUESTION 10 44
10.1. NANOREG (ELEMENTS OF) ANSWER TO THE QUESTION 44
10.2. OVERALL ASSESSMENT / CONCLUSIONS 45
- HARMONIZED PREPARATION AND EXPOSURE CHARACTERIZATION PROCEDURES (NANOREG SOPS IN WP2) WERE DEVELOPED TO REDUCE UNCERTAINTY FOR HAZARD CHARACTERIZATION. 45
- IT IS RECOGNIZED THAT BOTH *IN VIVO* AND *IN VITRO* METHODS NEED TO BE ADAPTED IN ORDER TO BE APPROPRIATE FOR DETERMINATION OF THE HAZARD POTENTIAL OF NANOMATERIALS. 45
- FOR COMPARATIVE TESTING, BOTH SUBMERGED AND AIR-LIQUID INTERFACE (ALI) CONDITIONS WERE USED IN NANOREG STUDIES. THE POTENTIAL CROSSING OF SEVERAL MNMS HAS BEEN EVALUATED USING DIFFERENT *IN VITRO* BARRIER MODELS, INCLUDING BLOOD-BRAIN BARRIER, INTESTINAL EPITHELIUM, LUNG EPITHELIUM, ORAL MUCOSA AND BIOMIMETIC LIPID MEMBRANES. 45
- THERE IS A NEED FOR AN INTRACELLULAR MNM DOSE DEFINITION ALSO FOR HAZARD IDENTIFICATION. 45

10.3. ANY OTHER RELEVANT ISSUES? 45

11. QUESTION 11 46

11.1. NANoREG (ELEMENTS OF) ANSWER TO THE QUESTION 46

11.2. OVERALL ASSESSMENT / CONCLUSIONS 47

11.3. ANY OTHER RELEVANT ISSUES? 48

12. QUESTION 12 49

12.1. NANoREG (ELEMENTS OF) ANSWER TO THE QUESTION 49

12.2. OVERALL ASSESSMENT / CONCLUSIONS 50

12.3. ANY OTHER RELEVANT ISSUES? 50

13. QUESTION 13 51

13.1. NANoREG (ELEMENTS OF) ANSWER TO THE QUESTION 51

13.2. OVERALL ASSESSMENT / CONCLUSIONS 52

13.3. ANY OTHER RELEVANT ISSUES? 52

14. QUESTION 14 54

14.1. NANoREG (ELEMENTS OF) ANSWER TO THE QUESTION 54

14.2. OVERALL ASSESSMENT / CONCLUSIONS 56

14.3. ANY OTHER RELEVANT ISSUES? 56

15. QUESTION 15 57

15.1. NANoREG (ELEMENTS OF) ANSWER TO THE QUESTION 57

15.2. OVERALL ASSESSMENT / CONCLUSIONS 64

15.3. ANY OTHER RELEVANT ISSUES? 64

I. SVCCS1 65

I.1. NANoREG (ELEMENTS OF) ANSWER TO THE QUESTION 65

I.2. IMPACT / IMPLICATIONS FOR THE STAKEHOLDERS 65

I.3. OVERALL ASSESSMENT / CONCLUSIONS 66

I.4. ANY OTHER RELEVANT ISSUES? 66

A. Summary of answers and achievement of D1.9 in NANoREG

A fast-reading summary of the elements of answers is provided in section A.3

A.1 Description of task 1.3

(According to the DoW, version 30 May 2016)

"Task 1.3: Interaction with WP 2-6 on the scientific answers to the issues/questions related to regulatory needs for nanomaterials safety assessment and management.

This task is a key outcome of NANoREG and namely answers the prioritized questions and issues from Task 1.1 and the gaps identified in Task 1.2. Working groups will be established to prepare chapters/sections of a grant report/paper/website in which each issue/question will be addressed. Working groups will be supported by members of WP2-6. A working group will start by providing detailed specifications of the regulatory questions and demands and help translate these into actions to be carried out by WPs 2 – 6. It will review the proposals to ensure coherence with the overall project objectives and relevance of the anticipated results. Interim reports and resulting answers/tools (databases/strategies/tests/approaches) will be discussed with the advisory boards.

This task, led by JRC in collaboration with RIVM, AIT, IOM, TEMAS and TUKES, involves continuous feed-back work and includes evaluation and decision on which proposals from WPs 2 to 6 are coherent with the project. As such, those proposals may arise at any stage of development of the project, even almost at the end. On month 20 a draft report on selected questions/answers will be delivered. All WP1 partners will contribute to defining answers to the prioritized questions from Task 1.1 based on the results provided by the other WPs. NRCWE and VN-Ecamricert will participate in the working groups, collaborate in writing the appropriate reports and will act as interfaces similarly as in other tasks of WP1. In addition, ENEA will in particular participate to/coordinate the working groups on exposure scenarios and focus on framework expansion for including LCA methodology. INRS will help to guide the research work and collecting results related to occupational safety and health issues, in collaboration with other partners, in particular OSH institutes (NRCWE, IOM, ISS) participating in this task.

(Lead: JRC with contributions from all partners)"

A.2 Background of task 1.3 and structure of the elements of answers in Chapter B

Task 1.3 implemented the link between the scientific WPs 2-6 and WP1. It helped to identify the crucial aspects in regulatory research for nanomaterials safety assessment that NANoREG needed to address (better) and fuelled the dialogue between WP1 and the other scientific WPs, helping to oversee how NANoREG actually works on (partially) answering the questions of regulatory relevance (see Table 5 of D1.1). Information generated in T1.6 during the implementation of the safety in the value chain case studies (SVCCSs) was also taken into account.

The whole consultation process of partners conducted by the T1.3 core group is described in paragraph A.2.3 of the interim version of the present deliverable: D1.3.
Further consultations that took place after the production of D1.3 included: 2 rounds of written consultation of all partners / task leaders of the project (in February 2016 and in April-May 2016), and a live T1.3 'Poster discussions' event at CM7 in June 2016, along the lines of the previous event in May 2015 at CM5.

This project has received funding from the European Union
Seventh Framework Programme (FP7/2007-2013)
under grant agreement no 310584

Due to the relevant similarities between this NANoREG task and the work of the H2020 ProSafe Task Force, which also identified key issues or question of regulatory relevance, and the discussions held with a wide community at the ProSafe-OECD Conference 30 November – 2 December 2016, which included most of the NANoREG project outputs as background information, the conclusions of that conference were also integrated in this deliverable question by question.

In order to make D1.9 lighter and more readable than D1.3, and also considering that D1.9 is subsequent to D1.3 and remains strictly connected to it, the sections included in the 'question master documents' of D1.3 have been removed/refocused, namely:
"*'NANoREG (elements of) answer to the question*", *"Impact and implications for stakeholders"*, *"Overall assessment/conclusions"* and "*Any other relevant issues*" have been maintained, since they can contribute to reaching (partial) answers to the questions and may provide hints at needed future work. D1.9 is thus directly linked to D1.3 regarding the sections that have been removed from D1.9.

Overall, it has not been easy for the T1.3 Core group to harvest concrete or definitive elements of answer, despite the many rounds of consultation that were organised. Often, the feedback received was too brief of generic, and raising more questions, rather than providing much needed elements of answer.

Also and unfortunately, the overall production of R&D knowledge in the project suffered delays and some deliverables could not be ready before the contents of section A.3 and chapter B were harvested (September 2016). They are hence compiled / edited to the best of the available NANoREG (and OECD) information received.

The output of T1.3 fed directly into the development of the NANoREG Framework for the safety assessment of nanomaterials (D.1.11 of T1.4) and the related NANoREG Toolbox (D1.12 of T1.7).

Table 1 provides an overview of the NANoREG experts that were involved in collection and editing the elements of answers to the various NANoREG questions.

Table 1: List of lead contact persons (LCPs) per NANoREG question of regulatory relevance or SVCCS (updated from D1.3)

Q#	Question theme	Keywords	Lead Contact Person(s) (LCPs)	E-mail addresses of LCP(s)
1	Measurement and characterisation - Identification	According to the EC definition	Hugues Crutzen (JRC) Sara Totaro (JRC)	hugues.crutzen@ec.europa.eu sara.totaro@ec.europa.eu
2	Measurement and characterisation	Characterisation strategy, measurements protocols		
3	Characterisation / Transformation	Surface modifications, dissolution, transformation, agglomeration		
4	Metrology and dose metrics	Dose, mass, particle numbers, surface area, metrics	Flemming Cassee (RIVM) Heinrich Hofmann (EPFL)	flemming.cassee@rivm.nl heinrich.hofmann@epfl.ch
5	Extrapolation and grouping	Bulk/nanomaterials, other forms, read-across (RX), categorisation, grouping inter/extrapolation	Hugues Crutzen (JRC) Sara Totaro (JRC)	hugues.crutzen@ec.europa.eu sara.totaro@ec.europa.eu
6	Fate, persistence and long-term effects	Accumulation, surface modification, biopersistence	Carlos Rey-Castro Adrienne Sips (RIVM)	carlos.rey@quimica.udl.cat adrienne.sips@rivm.nl
7	Kinetics and fate, determination	Absorption, deposition, , accumulation, uncertainty biodistribution, route of exposure		
8	Kinetics and fate, extrapolation	Possible links to grouping/RX, triggering, waiving, PCC-toxicity links, extrapolation		
9	Mode of action	Toxicity bulk/NM, nano-biointeractions, PCC driving (eco)toxicity, life cycle	Mats-Olof Mattsson (AIT) 2nd LCP - TBD	mats-olof.mattsson@ait.ac.at 2nd LCP - TBD
10	Hazard	Methods for EH toxicity,	Mats-Olof Mattsson (AIT) 2nd LCP - TBD	mats-olof.mattsson@ait.ac.at 2nd LCP - TBD
11	Exposure	Occupational and consumer exposure, exposure duration	Rob Aitken (IOM) Juergen Hoeck (TEMAS)	rob.aitken@iom-world.org juergen.hoeck@temas.ch
12	Exposure	EH exposure assessment, scenario, uncertainty, background, standards, validation		
13	Exposure and life cycle analysis	Exposure scenario, release, recycling, end of life ,LCA		
15	Risk Management	PPE, control banding tools, 'zero' exposure	Christoph Studer (FOPH) Juergen Hoeck (TEMAS)	christoph.studer@bag.admin.ch juergen.hoeck@temas.ch
14	Risk Assessment	Long/short-term exposure links, low dose/(sub-)acute,	1st LCP - TBD Paula Jantunen (JRC)	1st LCP - TBD anna-paula.jantunen@ec.europa.eu

16	Health surveillance	Triggers, signals for biological monitoring, intelligent testing	1st LCP - TBD 2nd LCP - TBD	1st LCP - TBD 2nd LCP - TBD

VCS1	Gallant	TiO2 leaching, pharmaceutical solutions, SOPs	Mats-Olof Mattsson (AIT) Andy Booth (SINTEF)	mats-olof.mattsson@ait.ac.at andy.booth@sintef.no andreas.falk@bionanonet.at
VCS2	CNTs in electronic goods	Batteries, production, end-of-life, exposure scenarios, recovery	Mats-Olof Mattsson (AIT) Rob Aitken (IOM)	mats-olof.mattsson@ait.ac.at rob.aitken@iom-world.org

A.3 Summary of the findings and elements of answers

SUMMARY OF THE FINDINGS AND ELEMENTS OF ANSWERS

Identification, characterisation and transformation, including metrics.

IDENTIFICATION

- The work performed in NANoREG has highlighted electron microscopy (EM), in particular TEM, as the best technique for primary particle size distribution determination. Given the small amount of NMs that is used to perform TEM imaging, particular attention should be adopted during the subsampling phase, in order to obtain a sample that is as representative as possible of the whole.
- Wherever possible, results of TEM analysis should be checked against other techniques, such as SEM, sp-ICP-MS, DLS, PTA, etc.
- Methods to support the substance identification were developed and presented in D2.04, D2.08, D2.10, and D2.11.
- As discussed in NANoREG and in the OECD context, EM methods for automated size distribution of primary particles are promising, but the preparation methods need to be standardized. In that respect NANoREG has worked hard on validated dispersion protocols (see NANoREG deliverable D2.6).
- The evaluation and verification of VSSA as possible identifier for nanomaterials was addressed both in NANoREG, in deliverable D2.11, and the other relevant EU-funded project, NanoDefine. Results show that VSSA, as identifying parameter for NMs, is not an adequate alternative to TEM for particle size distribution measurements. It can complement TEM and be a useful supplementary identifier within certain boundaries. The determination of VSSA requires a precise measure of the material density, which was addressed in D2.9.
- Deliverable D2.10 has established a useful set of SOPs for the quantitative size and shape analysis of manufactured nanomaterials using TEM.
- Aggregates evaluation depends on the method used for sample preparation.
- The identification of fibres is best done using TEM (TEM was used on HARNs and fibrous NMs in D4.13 and D4.16).

MEASUREMENT, CHARACTERISATION and TRANSFORMATION

- Aspect ratio has been identified as key parameter for the characterisation of fibres.
- Based on studies conducted in NANoREG (deliverables D2.12 and D6.3), a list of relevant (meaningful) physicochemical properties to be measured for characterisation includes: particle size distribution, shape, chemical composition and impurities, surface chemistry, specific surface area and porosity (and VSSA), solubility (rate of dissolution and equilibrium solubility), zeta potential, dustiness, aggregation / agglomeration state, dispersion stability, dustiness, crystalline phase and crystallite size, photo-catalytic activity, redox potential and radical formation potential.

- Solubility, and in particular the dissolution rate, is considered as an important descriptor. The evaluation of this parameter allows in principle to understand the NM behaviour when it enters in contact with the environment or it is uptaken into the body. The solubility of a NM and the related solubility rate will allow to possibly set a limit (time of dissolution or rate of dissolution) for which the "nano-effects" are negligible with respect to the ionic (or other "decomposition products") ones.
- Different methods to evaluate the solubility for a given NM (both in environmental and biological media) have been identified (see details in Q3). The methods are not validated yet, one is in the process at OECD as TG.
- Reactivity of a NM deserves attention. However, there is first the need to clearly define what *reactivity* means when considering NMs.
- Concerning the extensive work done by NANoREG on the assessment of key characterisation methods, the project has i) found in D2.3 that none of the analysed OECD TGs were suitable for characterization of MNM, and ii) revisions of several of the OECD TGs were proposed or proposed to be replaced with alternative or new methods and presented in NANoREG D2.9.

METRICS

- The metric to express the biological effective dose largely depends on the exposure pattern. There are no scientific reasons why mass as a metric cannot be applied to assess the dose-effect relationships in the context of regulatory toxicology (oral, inhalation, dermal etc.). This statement is underlined by the fact that, for granular NMs, a transfer to *surface* or *number* and, finally, to *deposited* dose is possible if the size distribution and density are known.
- For rigid biopersistent fibrous materials, which are falling within the range advised by WHO, fibre number concentration is the adequate dose metrics.
- In order to extrapolate toxicity data for granular biopersistent NM with varying size or different chemical identity, particle agglomerate volume seems to be the best applicable metric to describe longer-term toxicity. This seems to include non-rigid fibrous materials. Acute effects may be better explained by surface area and should therefore be considered as one of the possible and applicable alternative metrics to express the dose in regulatory context.
- For MNM with low aspect ratio all the mentioned metrics can be calculated knowing few properties like size distribution, density of primary particles, agglomerate size distribution and agglomerate density.
- Data sets to allow systematic evaluation of physical parameters and their effect on the dose-effect relationships, and the applicability of other dose metrics than mass, are key missing information so far.
- Additional measures may be requested to support ‘read-across’ and extrapolation of data on NMs with the same chemical identity, but different physical aspects (e.g. size, phase/crystallinity), since the combination of chemical composition and mass is insufficient to predict the risk of particles and fibres over a wide size range, including mixtures of NMs and larger-sized non-nanoparticles.

Risk assessment, grouping and read-across

- Grouping and read-across approaches are expected to deliver the most important contribution to more efficient ways to evaluate the large numbers and varieties of nanomaterials.
- 'High-quality', well-structured and reliable datasets are needed to justify the grouping and read across. And agreement with regulators needs to be reached on what high-quality means.
- In the process of reading-across and grouping, efficiency is very important. What do we lose (in terms for instance of data) when grouping?
- The most important physicochemical properties that need to be considered for read-across and grouping, as well as a stepwise approach to come to a justified grouping or read-across, have been identified and proposed by NANoREG in D5.1. Further development is still needed to establish values of specific physicochemical properties that set the boundaries of a group.
- Only for some groups of nanomaterials (e.g. ion release from certain metal-based nanomaterials) sufficiently reliable data appear to be available to justify the boundaries of a group.
- Linking physicochemical and (eco)toxicological endpoints is essential for grouping and reading-across.
- Work performed in NANoREG does not conclusively answer Q14. It contributes to an eventual understanding of the relationship of short-term and long-term toxicity of nanomaterials and to identify information and methodology that facilitate prediction of exposure levels connected with long-term toxicity in practical contexts. Production of chronic ecotoxicological data, and/or standardized methodology for this purpose, remains a large gap in the context of this question.
- Looking at grouping of NMs in relation to identification, reflection work has been done in NANoREG, leading to a proposal for a categorisation protocol for NMs based on substance identification (D2.5)

Hazard assessment and kinetics

- Methods to support the analysis of NM fate issues were developed and presented in D2.04, D2.08, D2.10, and D2.11.
- Confirmation that kinetics of nanoparticles gives essential information to risk assessment of nanomaterials. Kinetics cannot be deduced from microparticle or molecular or ionic form.
  - Kinetic studies are supportive in the evaluation of long-term toxicity when this can be considered as probable.
  - Information on dissolution rate is urgently needed.
- Nanoparticles tend to end up in organs, in particular those with phagocytotic capacity, thereby implying that especially distribution to these organs need attention in test designs.
  - Barrier crossing is also very important. That is where the NPs enter the organ. The fate of NPs when they enter the cells needs to be understood. *In vitro* barrier models were addressed in NANoREG D5.3.
- Absorption and accumulation potential is relevant to the likelihood or long-term effetcs and, thereby, to long-term toxicity testing.
  - If there is low exposure, this has to be considered if it is happening, for instance, on a daily basis and possibly also with a low body clearance is, thus leading to accumulation.

- Clearance is often related to dissolution, gastro-intestinal transformations, accumulation in the lungs. Dissolution, whether it takes place before or after uptake, has an impact on the risk assessment.

- Information on dissolution is pivotal for estimation of both absorption and accumulation. Dissolution testing in biological media like macrophages fluid or other relevant fluids, like simulating dissolution media used in pharmacopeia, is an important source for estimating kinetics.
- In dissolution testing dissolution rate is especially important.
- This latter application is in line with the questions to reduce uncertainty and to come up with affordable tests. However, it needs to be stressed that the information retrieved by this tests does not dismiss a producer from obligations for studying kinetics as laid down in various regulations.
- It is recognized that both *in vivo* and *in vitro* methods may need to be adapted in order to be appropriate for determination of the hazard potential of nanomaterials. Several examples of adapted methods are present within NANoREG.
- Currently, *in vitro* methods are not suitable yet to determine kinetic parameters of MNMs, thus animal tests should still be used to generate information on absorption, deposition, biodistribution and internal exposure. Environmental persistence cannot be determined through the partition coefficient of MNMs, as partition coefficients cannot be determined for these particles. A new approach is therefore necessary.
- Extrapolation of kinetic parameters is not possible from the bulk form and hardly possible from other nanoparticle-forms, implying this information will require substantial testing.

Exposure and exposure/risk management

- The main output from NANoREG in relation to this question (Q11) is new data about determinants, emissions and exposures which adds to the existing data collected and collated (as part of the review process).
- This data is itself valuable for both industry and regulators and will provide them with increased confidence in their exposure assessments and selection of controls and in the validity of the CSAs.
- Data for consumer exposure remains sparse and, specifically, information on transfer factors is lacking. The situation regarding consumer use of products is made more challenging by there being insufficient knowledge (in terms of NMs) about these products.
- The improved and validated occupational exposure models of release, exposure, dispersion and transfer (D3.8) provide greater confidence for industry in their exposure assessments and selection of controls. Use of these models within REACH CSA provides industry and regulators with increased confidence in the validity of the CSAs.
- The mesocosm platform approach (D3.5) appears to offer interesting possibilities for the testing of environmental exposure. Whether it is sufficiently validated or robust enough at the remains to be determined. NANoREG did not test environmental release models.
- While the ongoing problem about the lack of access to field sites exposure and exposure determinant data remains an issue, the researchers have attempted to overcome this through simulation studies which will add significantly to the body of data available.
- The lack of consumer exposure data is acknowledged as are the difficulties in collecting new field data.
- Deliverable D3.9 is now a very valuable source of information on reviewed RMMs and PPE for regulators and industry, and a notable output of NANoREG.

- It includes for instance decision flow charts for respiratory protective equipment, gloves and protective clothing.

A.4 Evaluation and conclusions.

Notwithstanding the difficulties the T1.3 partners faced in collecting the scientific information from within NANoREG to compile elements of answers to the questions of regulatory relevance (D1.1), this deliverable, which was preceded by the interim overview of D1.3, shows that:

i) Several important conclusions and elements of answer have been directly or indirectly produced by this large FP7 project, as can be seen by going through the "Summary Of The Findings And Elements Of Answers " of section A.3.

ii) In several cases, procedures (SOPs) and a way to tackle an issue have been identified, developed and published, but the verification/validation process ('testing the tests') requires more time and resources than what the project had to offer.

iii) The findings generally came to light toward the very end of the project. Nevertheless, all partners involved in WP1 were able to ensure that the output of T1.3 fed directly into the development of the NANoREG Framework for the safety assessment of nanomaterials (D.1.11 of T1.4) and the related NANoREG Toolbox (D1.12 of T1.7).

iv) The information now included indirectly in the Framework and the Toolbox is an asset for other on-going nanoEHS initiatives at EU level, such as the ProSafe White Paper drafting, the development of a knowledge/database by former FP7 eNanoMapper project, and feeds into work at OECD level and into the US-EU scientific collaborations.

A.5 Deviations from the work plan

A few months delay in the progress of the task and the delivery of the present D1.9 was in line with the overall lag of the project, in particular the scientific work, which delayed D1.3 and hence D1.9, from June 2016 to about October 2016.

The task leader took more time than expected to conclude the editing of all the accumulated information, also because it seemed wise to wait until the OECD-ProSafe Conference to take stock of the findings by the various panel there and, in early 2017 cross-check them with outcomes / recommendations of this deliverable.

All (core) partners of T1.3 have done their best to collect from elsewhere in NANoREG supporting scientific evidence that would provide elements of answers to the questions and issues. The general level of participation and response to T1.3's multiple rounds of consultation (general emails, targeted emails, 'Poster sessions' at 2 general Consortium meetings, etc.) was much lower than expected, and this has impacted the objective quality of some answers in this document.

B. Elements of answer to the questions of regulatory relevance

ELEMENTS OF ANSWER TO THE QUESTIONS OF REGULATORY RELEVANCE

Contributing LCPs by question

Sara Totaro / Hugues Crutzen (JRC – Q1, Q2, Q3)

Flemming Cassee / Heinrich Hofmann (RIVM / EPFL – Q4)

Carlos Rey-Castro / Adriënne Sips (UDL / RIVM – Q6, Q7, Q8)

Mats-Olof Mattsson (AIT – Q9, Q10)

Rob Aitken / Jürgen Höck (IOM / TEMAS – Q11, Q12, Q13)

Paula Jantunen (JRC – Q14)

Christoph Studer / Jürgen Höck (FOPH / TEMAS – Q15)

Mats-Olof Mattsson / Andy Booth / Andreas Falk (AIT / SINTEF / BioNanoNet – SVCCS)

This project has received funding from the European Union Seventh Framework Programme (FP7/2007-2013) under grant agreement no 310584

1. Question 1

NANoREG Question of Regulatory Relevance number 1
Measurement and characterization – Identification

T1.3 – Information-gathering master document

Question theme	*Measurement and characterisation - Identification*
Keywords	*According to the EC recommended definition*
Regulatory context:	*How can MNMs be identified according to the EC recommendation for a definition of MNMs and for regulatory purposes (i.e. the implementation of the EC definition in e.g. REACH, CLP, cosmetics, novel food, etc.), including other jurisdictions (global harmonisation)? Can we develop robust measurement protocols which enable assessment of whether a NM falls under, or not, the EC definition? Are there robust measurement protocols available (and for which matrices) that enable identification? – from D1.1*
Owner(s):	*Lead Contact Person(s): Sara Totaro, Hugues Crutzen – JRC*

1.1. NANoREG (elements of) answer to the question

- The work performed in NANoREG has highlighted Electron Microscopy (and in particular TEM) as the best technique for primary particle size distribution determination. Given the small amount of NMs that is used to perform TEM imaging, particular attention should be adopted during the subsampling phase, in order to obtain a sample that is as representative as possible of the whole.
- SOPs for the quantitative size and shape analysis of near-spherical, near-monomodal colloidal materials, as well as for fractal-like and aggregated powder NMs have been produced (D2.10). The developed methodologies are validated on reference and representative nanomaterials for application for regulatory use, focusing on the EC recommended definition of nanomaterials.
- Results of TEM analysis should be checked against other techniques (e.g. SEM, sp-ICP-MS, DLS, PTA, etc.). Coupling these techniques (higher amount of samples) can help in overcoming potential issues related to representative samples (first bullet above). There is growing data/evidence that *a priori* knowledge is required to make a meaningful interpretation of the results of alternative methods (DLS and PTA: aggregation + agglomeration state?, sp-ICP-MS: chemical composition, aggregation + agglomeration state, shape, BET: drying, monoconstituent, shape?). This *a priori* knowledge can often (only/ most efficiently) be obtained by (a preliminary) EM analysis.

This project is funded by the EU Framework 7 Programme, contract no 310584.

- As discussed in NANoREG and in the OECD context, EM methods for automated size distribution of primary particles are promising, but the preparation methods need to be standardized. In that respect NANoREG has worked hard on validated dispersion protocols (see NANoREG deliverable D2.6).
- The measurement of the density of the investigated material is essential in order to obtain an accurate determination of the VSSA. See WP2 evaluation of the OECD TG for the determination of density (D2.9).
- The evaluation and verification of VSSA as possible identifier for nanomaterials was addressed both in NANoREG, in deliverable D2.11, and the other relevant EU-funded project, NanoDefine. Results show that VSSA, as identifying parameter for NMs, is not an adequate alternative to TEM for particle size distribution measurements. It can complement TEM and be a useful supplementary identifier within certain boundaries.
- A systematic approach has been developed and proposed in WP2 (D2.5) to enable quick identification of which type of NM is under investigation. This descriptive approach is also translated into a categorisation approach.

1.2. Impact / Implications for the stakeholders

- The established SOPs for the quantitative size and shape analysis of manufactured nanomaterials using TEM will provide number-based size distributions of the primary particles of MNM. These are fundamental to identify MNM according to the recommended EC-definition of NM. The accompanying inter- and intra-lab validation dossiers will facilitate their introduction in CEN and/or ISO guidelines and standards.
- VSSA, as identifying parameter for NMs, is not an adequate alternative to TEM for particle size distribution measurements, but it can complement TEM and be a useful supplementary identifier within certain boundaries.
- It is possible to implement methods and estimate uncertainties with respect to the identification of NMs as such.
- From a regulatory point of view, to build a usable and reliable system for identification, it is recommended to make use of the following 'approach':
    - 1A→ Automation (cheap, fast, many),
    - 2T→ True (certified reference materials), Traceable (SI vs method-defined),
    - 3S→ Size, Shape, Surface,
    - 4M→ Multi-layered, Multi-component, Mixtures, Matrix.

1.3. Overall assessment / conclusions

- The work performed in NANoREG has highlighted Electron Microscopy (and in particular TEM) as the best technique for primary particle size distribution determination. Other methods for consideration will need to undergo to a verification or validation process.
- VSSA use as identifying parameter for NMs, has been assessed (see .1.1 above).
- Aggregates evaluation depends on the method used for sample preparation.
- The identification of fibres is best done using TEM (TEM was used on HARNs and fibrous NMs in D4.13 and D4.16).

1.4. Any other relevant issues?

- TEM is quite expensive, and some other techniques possibly more at hand should be also foreseen/recommended in addition to TEM.

- 'Complicated techniques' require a certain degree of expertise for full applicability. It is not always possible to have dedicated experts, especially in SMEs.
- Comparing various preferred and alternative techniques as to their applicability for specific purposes and their feasibility (e.g. cost and availability) for companies of various sizes is a valid concern. However, since NANoREG was only designed to "test the tests" and not to evaluate their price and/or feasibility, the T1.3 partners could not perform any comparison and draw any conclusion.

-oOo-

2. Question 2

NANoREG Regulatory question number 2
Measurement and characterisation

T1.3 – Information-gathering master document

Question theme	Measurement and characterization
Key words	*Characterisation strategy, measurements protocols*
Regulatory context	*What is a minimal set of physical (and/or chemical) characteristics that should be available for risk assessors within the context of regulatory toxicology? What are the relevant features to characterise MNMs, e.g. size, form, aspect ratio, rigidity, flexibility and coating? What methods (SOPs) should be developed / used to determine the physical chemical characteristics of MNMs throughout their different life cycle stages within the context of regulatory toxicology?* *These questions (closely related to Q1) refer to developing cost-effective standard methods, detailed protocols and reference materials both for calibration and analysis of both pristine materials and materials in relevant media or complex matrices throughout the complete life cycle of the nanomaterial. They also refer to whether different categories of characterisation methods (varying e.g. in precision and accuracy) can be defined: Could an "intelligent characterisation strategy" be defined?*
Owner(s)	Lead Contact Person(s): *Sara Totaro, Hugues Crutzen – JRC*

2.1. NANoREG (elements of) answer to the question

- For a proposed minimum list of physicochemical characteristics, see "*Table 2. Minimum list of physicochemical information requirements to be used* […]", in D2.12 and, in the same deliverable, section 3.3.2 on "*NANoREG Procedures for characterization of endpoints*".
- The properties identified, and for which procedures are described in D2.12 are: particle size distribution, shape, chemical composition and impurities, surface chemistry, specific surface area and porosity (and VSSA), solubility: rate of dissolution / equilibrium solubility, zeta potential, dustiness, aggregation/agglomeration state, water solubility, dispersion stability, dustiness, crystalline phase and crystallite size, photocatalytic activity, redox potential and radical formation potential.
- On the basis of the characteristics of a NM, a systematic approach is being developed in WP2 (and included in D2.5) that should in principle allow a simplification in understanding which kind of

This project is funded by the EU Framework 7 Programme, contract no 310584.

material is under investigation. This descriptive approach should also be translated in a categorisation approach.

- Identifying a set of physicochemical properties that are related to the (eco)toxicity of nanomaterials is not easy. NANoREG has developed in T6.3 a relational database that contains information retrieved from peer reviewed scientific literature on nanomaterial's physicochemical characteristics and (eco)toxicity (D6.5). This information can used to identify physicochemical properties related to the fate and toxicity of nanomaterials.
- Solubility should be considered as a combination of intrinsic (e.g. chemical composition) and extrinsic phys-chem properties, since it is highly dependent on the test medium characteristics (pH, composition, stirring, etc).

2.2. Impact / Implications for the stakeholders

Through this question, the identification of a set of characteristics that can be made available for risk assessors within the context of regulatory toxicology shall be identified. Those characteristics should in principle be those needed as a minimum characterisation requirement for a NM. Their measurement shall be also coupled with SOPs and/or agreed protocols in order to harmonise the testing methods and allow the comparability of results.

2.3. Overall assessment / conclusions

From the work performed so far in NANoREG (and as a result of the workshop in Bern), it is already possible to derive some preliminary highlights:

- Size is the main characteristic, that defines a NM as such – also in relation to Q1;
- Crystallinity and morphology are very important;
- Solubility, and in particular the dissolution rate, has to be considered as an important descriptor, and it is addressed in WP2-WP5;
- For fibres, aspect ratio and rigidity are a key characteristics to be measured;

2.4. Any other relevant issues?

-oOo-

3. Question 3

NANoREG Question of Regulatory Relevance number 3
Characterisation/Transformation

T1.3 – Information-gathering master document

Question theme	*Characterisation / Transformation*
Keywords	*Surface modifications, dissolution, transformation, agglomeration*
Regulatory context:	*What testing should be performed to identify surface modifications that occur once a MNM has been released into the environment or taken up into the body? How can transformation, including agglomeration surface modification, dissolution and incineration, be determined and considered in the exposure and hazard assessment and how do they change the intrinsic toxic properties and bio-distribution Do we need to know the details of such surface modifications or of what is bound, or do we need some simple test systems that actually determine the behaviour and transformation of MNM in relevant media throughout all life cycle stages? Is a nano-derived material still nano when it becomes agglomerated? Take into account relationship with questions 7-9.*
Owner(s):	Lead Contact Person(s): Sara Totaro, Hugues Crutzen – JRC

3.1. NANoREG (elements of) answer to the question

- Dissolution rate, agglomeration behaviour and transformation are key to understand and characterise the environmental fate of nanomaterials (also reported at OECD conference).
- Development of solubility/dissolution SOPs are underway. The analytical requirements were reviewed by T2.3 and T5.2 and published recently[1]. No single analytical technique is at present able to meet the requirements and provide all the necessary information for an accurate evaluation of the solubility, so that a combination of methods is recommended.
- NANoREG D5.2 provides a report on the development of a solubility testing procedure.

[1] Tantra, R., H. Bouwmeester, E. Bolea, C. Rey-Castro, C. A. David, J.-M. Dogné, J. Jarmann, F. Laborda, J. Laloy, K. N. Robinson, A. K. Undas and M. van der Zande (2016). "Assessing Suitability of Analytical Methods to Measure Solubility for the Purpose of Nano-Regulation." Nanotoxicology **10**(2): 173-184

This project is funded by the EU Framework 7 Programme, contract no 310584.

- As recognised in WP5 D5.2, when measuring dissolution in-depth knowledge on the material and the matrix is of importance when using Single particle (SP) ICP-MS measurement, ultrafiltration (UF) or ultracentrifugation (UC) methods. Processing protocols (i.e. sonication, elemental detection method and procedures etc.) were shown to influence nanomaterial dissolution and it is therefore recommended to further standardise these procedures.
- Previous research results have shown that temperature, pH, particle primary size, hydrodynamic conditions, and agglomeration state are relevant for both dissolution kinetics and "equilibrium" (steady-state) solubility[2]. Also hydrochemical reactivity of NM in relation with the testing medium composition is very important for dissolution and fate of the NM, and may in some cases lead to transformation into concomitant/new solid materials such as metal carbonates/phosphates etc. in the case of e.g. ZnO in DMEM medium[3].
- NANoREG WP2 has developed guidelines / SOPs to describe dissolution/solubility of MNM in biological and environmental systems as well as their ability to interact with specific biomolecules (a.o. curosurf, LDH, IL6, IL8). These protocols and test results are reported in D2.9 and D2.12.
- NANoREG has produced and reported in D2.8 a TGD for ecotoxicology studies where a minimum level of MNM characterisation in ecotoxicity studies is implemented in order to ensure that MNM transformation is adequately monitored and available for use in interpretation of ecotoxicity data generated. However D2.8 does include any experimental validation of the recommendations.
- Related to the previous paragraph and the tasks performed on WP2, the 'Technical guidance document on procedures for the quantification of manufactured nanomaterials exposure and fate in dispersions for aquatic ecotoxicological studies' propose techniques such as DLS, SEM, ICP (MS, OES or AES), and UV-vis spectroscopy, as appropriate approaches for the determination of MNM aggregation, sedimentation, and quantification. This document provides a useful input to define procedures for quantification of MNM exposure and fate in dispersions for aquatic ecotoxicological studies, and has been distributed and implemented in WP4 ecotoxicity tests (Task 4.6 Biokinetics and toxicity in aquatic organisms).
- D6.4 provides an assessment of the use of dissolution test as a regulatory tool for safe-by-design innovation process
- Effective density measured for aerosol or suspensions is important to consider as it may influence:
  - The regional lung deposition,
  - The cellular dose for in-vitro testing (ALI or submerged).
- Methods for testing dissolution/metal ion release of metal and meta-oxide NPs inside macrophages should be developed and validated.
- For certain NMs there is a need to further develop analytical detection techniques in order to measure dissolution in biological media.

---

[2] David, C. A., J. Galceran, C. Rey-Castro, J. Puy, E. Companys, J. Salvador, J. Monné, R. Wallace and A. Vakourov (2012). "Dissolution Kinetics and Solubility of ZnO Nanoparticles Followed by AGNES." The Journal of Physical Chemistry C **116**(21): 11758-11767.

[3] Mu, Q., C. A. David, J. Galceran, C. Rey-Castro, Ł. Krzemiński, R. Wallace, F. Bamiduro, S. J. Milne, N. S. Hondow, R. Brydson, G. Vizcay-Barrena, M. N. Routledge, L. J. C. Jeuken and A. P. Brown (2014). "Systematic Investigation of the Physicochemical Factors That Contribute to the Toxicity of ZnO Nanoparticles." Chemical Research in Toxicology **27**(4): 558-567.

- Reactivity of a NM deserves attention. However, there is first the need to clearly define what reactivity means when considering NMs.

3.2. Impact / Implications for the stakeholders

- The development and implementation of SOPs for the reproducible preparation of nanomaterial dispersions is essentially for establishing a common 'starting point' for all for human toxicity studies and for environmental fate and effects studies. This means that knowledge generated and reported from one experiment can directly be used to inform about likely outcomes in another experiment conducted with different materials or toxicological endpoints. When combined with the TGD on procedures for the quantification of MNM exposure and fate in dispersions for aquatic ecotoxicological studies, a more complete understanding of MNM effects will be achievable. Importantly, the TGD will set a benchmark for the minimum level of characterisation required in aquatic ecotoxicity testing in order to characterise MNM transformation during ecotoxicity tests. This information would then be used for improved interpretation of ecotoxicity study data, and subsequently in the risk assessment of MNMs.
- SOPs and data developed on the biological fate and interaction potential with biological compartments and the environment can be used to develop or test hypothesis about the influence of different parameters on the hazard and fate of NANoREG MNM.
- An OECD TG on dissolution rate is currently undergoing standardisation process.
- A first set of characteristics, and related analytical/instrumental techniques that can be useful to describe and evaluate the transformation a NM can undergo during its life cycle have been highlighted. SOPs and/or testing guidelines have also been produced as a result of the NANoREG experimental work (deliverables reported in 3.1 above).
- Since size is expected to be a key characteristic in the NM toxicity, agglomeration or the NM tendency to agglomerate can play a very important role.

3.3. Overall assessment / conclusions

- Dissolution rate, agglomeration behaviour and transformation are key to understand and characterise the environmental fate of nanomaterials (also reported at OECD conference).
- Different suitable methods to evaluate the solubility for a given NM have been identified, but no conclusions have been reached so far. Furthermore, the methods could be not be validated within NANoREG due to limited reosources.

-oOo-

4. Question 4

NANoREG Question of Regulatory Relevance number 4
Metrology and dose metrics

T1.3 – Information-gathering master document

Question theme	Metrology and dose metrics: Which metrics (metrology) should be used for MNMs in regulatory toxicology?
Keywords	Metrology, dose metrics
Regulatory context:	*Estimating the effective dose is a key for conducting risk assessments. In particular, in in vitro assays it is difficult to determine the real dose and to transfer it to in vivo results (and vice versa). Having the same metrics for MNM characterization in exposure and hazard assessment is a necessity for regulatory risk assessment.*
Owner(s):	Lead Contact Person(s): Flemming R Cassee & Heinrich Hofmann

4.1. NANoREG (elements of) answer to the question

- *Mass* is an appropriate metric to establish dose-effect relationship for risk assessment. Mass can (still) be used as dose metric for MNMs in regulatory toxicology. There is no scientific support for deviations from the current regulatory requirements for particles and fibres, assuming that size and surface area are taken into account as unique substance identifiers.
- As shown in WP2's review of TGs and SOPs, such as granulometry, density, dispersion stability and dose (D2.9, D2.10), mass metric is repeatedly adequately used as metric.
- As reported in NANoREG *in vivo* studies, for combined chronic/carcinogenicity whole body inhalation studies to verify the hypothesis that, based on quantifying particle mass concentration, particle agglomerate volume seems to be the best applicable metric to describe longer-term toxicity for a highly relevant category of nanomaterials. These nanomaterials are called poorly soluble, low toxicity particles (PSLT), poorly soluble particles of low cytotoxicity (PSP) or respirable granular biodurable particles without known significant specific toxicity (GBP)
- Also from NANoREG R&D work on dosimetric studies, intracellular concentration expressed as a mass concentration, ng/cm2 or ppm, can be considered as a toxicologically relevant truly effective dose with high relevance for risk assessment.
- Comparison of toxic effects *in vitro* and *in vivo* on the basis of knowledge on intracellular effective dose in culture cells and tissues provides a tool for estimating the relevance of *in vitro* data for *in vivo* predictions.

This project is funded by the EU Framework 7 Programme, contract no 310584.

- Acute effects may be better explained by *surface area* and should therefore be considered as one of the possible and applicable alternative or additional metrics to express the dose in a regulatory context.
- *Particle agglomerate volume* seems to be the best applicable metric to describe longer-term toxicity. This seems to include non-rigid fibrous materials.
- For rigid biopersistent, i.e. poorly soluble, fibrous materials (length > 5 µm, fibre diameter < 3 µm and aspect ratio (FL/FD) > 3), the *fibre number concentration* is the adequate dose metrics. However, the assessment of dose-effect relationships for high aspect ratio material, which is not rigid and biopersistent, is not fully understood yet. As there is no method to determine rigidity, number counts should be applied for all fibres in addition to mass as dose metric.
- For granular MNMs, a transfer to surface or number and, finally, to *deposited dose* is recommended, if both size distribution and density are known.
- For ecotoxicity, the dosage is *mass/volume*, because surface/volume can be estimated if the NM is characterised.
- Mass/cm^2 is the only metrics applicable for *in vivo* and *in vitro* approaches (figure 1).

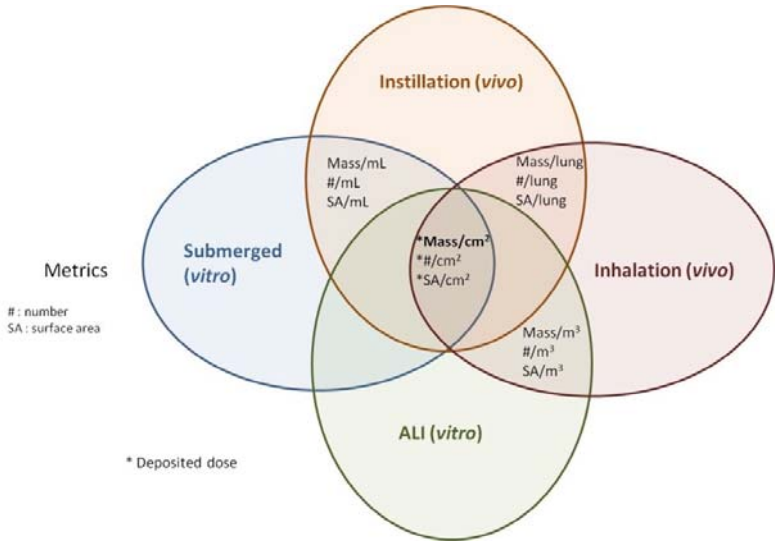


Fig1. Metrics used in in vivo and in vitro approaches

- The mass metrics (mass/cm^2) is not easy to determine *in vitro*, because it is a "deposited dose".
- For NMs with low aspect ratio all the mentioned metrics can be calculated knowing few properties, in the medium of interest, like size distribution (measured by TEM and given as a number distribution), density of primary particles, agglomerate size distribution (estimated from DLS) and agglomerate density (in a recent work of Glen DeLoid et al., an easy and cheap method to measure the agglomerate density was reported (Glen DeLoid et al., NATURE COMMUNICATIONS | DOI: 10.1038/ncomms4514).

4.2. Overall assessment / conclusions

- Based on the experience in NANoREG, there are is no need to deviate from currently used dose metrics (mass) in a regulatory context, when assessing the health risk on a case-by-case basis.
- For fibres, number count is an essential additional metric. For rigid biopersistent fibrous high aspect ratio nanomaterials, fibre number concentration is the adequate dose metrics.
- For granular NM a transfer of the metric from mass to surface or number and, finally, to deposited dose is possible if the size distribution and density are known.
- Additional measures may be requested to support 'read-across' and extrapolation of data on NMs with the same chemical identity, but different physical aspects (e.g. size, phase/crystallinity), since the combination of chemical composition and mass is insufficient to predict the risk of particles and fibres over a wide size range, including mixtures of NMs and larger-sized non-nanoparticles.
- Extrapolation between in vitro and in vivo data can be done using both mass and number counts per surface area, provided that models such as MPPD can be used.
- Surface area and number of MNMs are advised metrics to facilitate extrapolation across a range of sizes of NMs with the same chemical composition. The crystalline phase, for instance 'rutile' versus 'anatase', is an essential characteristic that has to be provided.
- Data sets to allow systematic evaluation of physical parameters and their effect on the dose-effect relationships, and the applicability of other dose metrics than mass, are key missing information so far.
- SOPs for the determination of the different metrics need to be established.

4.3. Any other relevant issues?

The table below reports the unofficial occupational exposure limits (OELs) proposed by different organizations all over the world. They are based on *number concentration and/or mass concentration*.

For the evaluation of asbestos in terms of number concentration the source is OSHA document1910.1001 Toxic and Hazardous Substances- Asbestos; similar guidance has been released by the UK HSE (Control of asbestos regulation 2012).

With respect to OEL setting in Germany, a reference value has been published for respirable nanoscaled granular biopersistent dusts ((http://www.baua.de/en/Topics-from-A-to-Z/Hazardous-Substances/TRGS/Assessment-criteria.html; document nanoscaled GBP). According to this document, nanoscaled granular biopersistent dusts are fourfold more potent with respect to lung inflammation compared to microscaled granular biopersistent dusts on mass basis. Announcement on Hazardous Substances 527 concludes on this basis that an OEL of 0,5 mg/m² should not be exceeded (http://www.baua.de/en/Topics-from-A-to-Z/Hazardous-Substances/TRGS/Announcement-527.html). Announcement on Hazardous Substances 527 also concludes that in case of working with rigid nanofibres in WHO dimension, an asbestos-like mode of action and similar toxic potency has to be assumed. In this case, the targeted air concentration is 0.01 WHO-F/ml, 0.1 F/ml may not be exceeded.

Table 1. Proposed workplace exposure limits for different ENPs (all data refer to concentration per cubic metre, when not otherwise specified)

ENPs	BSI (UK)	IFA (Germany)	DMSAE (The Netherlands)	NIOSH (USA)	SWA (Australia)	AIST (Japan)	KML (Korea)
Fibre-like ENPs							
Rigid, biopersistent CNTs	10^4 f	10^4 f	10^4 f	0.007 mg	10^5 f	0.03 mg	
Fibre-like metal oxides	10^4 f				10^5 f		
CNTs with explicitly excluded asbestos-like effects		4×10^7 p	4×10^7 p				
Biopersistent granular ENPs with a density <6000 kg/m³							
Titanium dioxide	0.066× WEL*	4×10^7 p	4×10^7 p	0.3 mg	0.03× Australian inhalable or 0.1× Australian respirable WEL	0.61 mg	
Carbon black	0.066× WEL* or 2×10^7 p	4×10^7 p	4×10^7 p		3 mg		3.5 mg
Silica	0.066× WEL* or 2×10^7 p	4×10^7 p	4×10^7 p		2 mg (fumed silica)		
Fullerene	0.066× WEL* or 2×10^7 p	4×10^7 p	4×10^7 p		0.03× Australian inhalable or 0.1× Australian respirable WEL	0.39 mg	
Zinc oxide, aluminium oxide, dendrimers, polystyrene, nanoclay	0.066× WEL* or 2×10^7 p	4×10^7 p	4×10^7 p		0.03× Australian inhalable or 0.1× Australian respirable WEL		
Biopersistent granular ENPs with a density >6000 kg/m³							
Cerium oxide, gold, iron, iron oxide, silver, cobalt, lanthane, lead, antimony oxide, tin oxide	0.066× WEL* or 2×10^7 p	2×10^7 p	2×10^7 p		0.03× Australian inhalable or 0.1× Australian respirable WEL		
Insoluble ENPs for which WEL* is not available					0.3 mg		
CMAR ENPs							
Nickel, cadmium containing quantum dots, chromium VI	0.1× WEL*	2×10^7 p	2×10^7 p		0.1× WEL*		
Beryllium, arsenic, zinc chromate	0.1× WEL*	4×10^7 p	4×10^7 p		0.1× WEL*		
CMAR ENPs for which WEL* is not available					0.003 mg		
Liquid and soluble ENPs							
Fat, hydrocarbons, syloxane		Same WEL*	Same WEL*				
Sodium chloride	0.5× WEL*	Same WEL*	Same WEL*		0.5× WEL*		
Other soluble ENPs	0.5× WEL*	Same WEL*	Same WEL*		0.5× WEL*		
Soluble ENPs for which WEL* is not available					1.5 mg		

f, fibres; p, particles; WEL*, work exposure limit of coarse material.

Workplace exposure limits are also relevant with respect to metrics, like i) NRVs (nano reference values) "*Exposure Limits for Nanoparticles: Report of an International Workshop on Nano Reference Values*" Broekhuizen et al. 2012; ii) UE- SCOEL/SUM/171 MAK (2013) Recommendation from the Scientific Committee on Occupational Exposure Limits for Copper and its inorganic compounds.

-oOo-

5. Question 5

NANoREG Question of Regulatory Relevance number 5
Extrapolation and Grouping

T1.3 – Information-gathering master document

Question theme	*Extrapolation and grouping: What guidance can be provided on how to decide when information from different forms of manufactured nanomaterials (MNMs) (or from the bulk material) can be "re-used" in the sense of read-across, categorisation and grouping? Should / could guidance be based exclusively on physico-chemical properties or could exposure related (eco)toxicological and mechanistic information (as Mode of Action) be used as well and how? Take into account the relation with the following questions.*
Keywords	*Bulk/nanomaterials, other forms, read-across (RX), categorisation, grouping inter/extrapolation*
Regulatory context:	*Similar nanomaterials may be grouped according to their adverse outcome pathways (AOPs) which enables moving away from case by case assessments towards smart testing strategies of smaller efforts. Nanospecific effects should be determined with the associated AOPs. In the end, testing key events for new MNMs might be enough to connect the resulting data to existing knowledge on hazardous effects.*
Owner(s):	*Lead Contact Person(s): JRC*

5.1. NANoREG (elements of) answer to the question

Work done in NANoREG and elsewhere, referred to by D5.1, show that it is possible to pursue grouping strategies on nanomaterials using different combinations of properties.

'High-quality' data is a pre-requisite for reliable grouping or read-across. This underlines the importance of

Identification of properties

Within D5.1 (Report on identification and setting of categorization, read-across, and extra/intrapolation criteria), the most important properties that need to be considered for read-across purposes, are described. These properties can tentatively be placed in four categories:

- *Substance identity*, including chemical composition, crystal structure, surface coating, functionalization and capping agents, impurities, all of which influence surface charge and reactivity;

This project is funded by the EU Framework 7 Programme, contract no 310584.

- *Particle characteristics*, including size (distribution), surface area (which depends on particle size and porosity), surface roughness, shape and aspect ratio, all of which generally influence mobility and transport;
- *Transport behaviour*, which reflects characteristics of the nanoparticle that are (partly) influenced by the surrounding medium, such as solubility/dispersibility (rate of dissolution and equilibrium concentration, both size-related), surface charge, tendency to agglomerate, dustiness.
- *Activity and reactivity*, including redox potential.

In addition, in the NANoREG project also the different reasons why grouping is pursued were evaluated. In general, three different main purposes for grouping have been identified: i) physico-chemical properties, ii) exposure potential, iii) (eco)toxicological effects, fate and transport.
For each of these aims, a different combination of parameters to evaluate the similarities of the MNM can be used.

- Physicochemical grouping

  The purpose of physicochemical grouping is to provide an indication of the general hazardousness of MNM in relation to inherent properties. This grouping should be independent from exposure, target, and environmental compartments. Examples of parameters can include high-aspect-ratio, chemicals inherent toxicity, reactivity, redox potential, solubility rate, (bio)degradability. Data from non-nanochemicals can be used in this stage for some parameters (a degradable chemical is likely degradable in every form, non-nano or nano). Benchmarks can be available for some parameters, as provided by CLP and literature.

- Exposure grouping

  The exposure grouping is linked to the evaluation of a MNM bioavailability (how likely it is that the MNM reaches its toxicological target). Therefore, it includes both external (e.g. concentration in the environment) and internal exposure (i.e. absorption, distribution, metabolism, and excretion), from the point of release to the point of action. The scope of this grouping could be the estimation of the environmental behaviour, the exposure pathway or the likelihood of release from a product of a given MNM. Specific exposure-related parameters can be: dustiness, chemical/physical stability, (bio)persistence, (bio)accumulability, release potential (from products, during production steps, during life cycle steps).

- (Eco)Toxicity grouping

  The (eco)toxicity grouping goal is to use relevant physico-chemical parameters to identify or estimate toxicological endpoints and general toxic mode of action of target MNM. The grouping can be based on a similar mode of action (MoA), such as: inflammation, genotoxicity, protein denaturation, altered cell cycle alteration, cytotoxicity, ROS generation, cellular uptake. The physico-chemical parameters accompanied by relevant benchmarks can allow a preliminary grouping, which then should be demonstrated via appropriate testing, and testing results comparison. The testing has to be linked to the target organ, which in turns can be influenced by the exposure route.

  To identify the MoA, basic physico-chemistry should be linked to higher level parameters (e.g. reactivity, cellular uptake, interaction with proteins), or higher level parameters (with respect to the basic physicochemistry as required by REACH) should be directly used among the grouping criteria. For an effective grouping, it should be possibly to measure the similarity among the components of a group. For traditional chemicals, benchmarks are sometimes measured via statistical approaches (e.g. cluster analysis), or through a clear-cut value, a function linking the distance from the nearest neighbour to a property (or set of properties), or qualitative, based on literature findings about the effects of change of some properties. It is difficult to identify benchmarks for all relevant MNM properties, but for parameters where this is possible, it should be implemented already.

Stepwise approach and case studies

Referring to work done by RIVM, JRC and ECHA, D5.1 explores the scientific aspects of justifying when and how to use test data from an (eco)toxicity study on one nanoform to cover other nanoforms of the same substance. The steps composing this approach are:

- Identification of the nanoform,
- Initial grouping of nanoforms,
- Identification of available data and data gaps,
- Identification of potential source materials,
- Identification of potential source materials,and
- Assess any new data for the impact on the hypothesis.

Within D5.1, this stepwise approach was further evaluated by using a few case studies/examples, based on available external data to NANoREG:

- Initial grouping based on shape and solubility: HARNs

Even though more knowledge is still needed (quantitative data for biopersistence, cut-off for length etc), it is likely that the fibre paradigm can be useful for grouping of nanofibres, such as CNTs, which is already done so in several control banding tools.

- Read-across of non-nanoforms based on solubility/metal ion release: Nickel compounds

In the case of metal-containing substances, it can often be the case that the metal ion is the responsible entity for the observed toxicity of the compounds. Thus, bioavailability data can be used to perform readacross assessments for metal substances.

- Read across/grouping of nanoforms based on chemical identity and photocatalytic activity: TiO_2 nanoparticles
- Read across/grouping of nanoforms based on particle size, shape and surface treatment: Silver nanoparticles

Data and Tools

The necessary tools for the generation and collection of reliable high quality data to perform and justify grouping and read-across are developed and tested. These tools include standard operating procedures (SOPs) and a database in which experimental data (characterisation and other experimental conditions) are stored in a well-structured manner with exhaustive metadata describing the conditions under which the data of an assay was generated (e.g. ISA-TAB-Nano approach in NANoREG and other initiatives – see D1.4).

5.2. Impact / Implications for the stakeholders

Grouping and read-across approaches are expected to deliver the most important contribution to more efficient ways to evaluate the large numbers and varieties of nanomaterials in a regulatory context for hazard assessment, risk assessment and/or risk management.

There are good hopes, based on the studies done in NANoREG (D5.1) that grouping and read-across strategies will help simplify NM safety assessment under REACH.

Limitations in the current grouping and read-across approaches are based on limitations in the available reliable experimental data that these approaches are built on.

The results of the EU projects NANoREG, GUIDEnano and NanoDefine will provide SOPs for reliable and reproducible physico-chemical characterisation of nanomaterials, followed by reproducible preparation of nanomaterial dispersions and experimental conditions within (eco)toxicity assays. This will allow the generation of well-structured ' high quality' data sets for relevant endpoints

(with known inter- and intra-laboratory uncertainties) that can provide case examples to test and justify grouping and read-across approaches (including predictive modelling tools). This will need to be taken up by other projects like NANoREG II, using the NANoREG dataset available the end of the project.

5.3. Overall assessment / conclusions

The most important physico-chemical properties that need to be considered for read-across and grouping, as well as a stepwise approach to come to a justified grouping or read-across, have been discussed in NANoREG (D5.1).

Further development is still needed to establish justified values of specific physico-chemical properties that set the boundaries of a group, i.e. benchmarks that determine whether a nanomaterial belongs within a specific group or not.

Reliable, well-structured and qualitatively good data availability is probably the critical point for the implementation of read-across and grouping.

5.4. Any other relevant issues?

ECHA's development of a Guidance Document for REACH including grouping and read-across is of relevance to stakeholders.

The European Centre for Ecotoxicology and Toxicology of Chemicals (ECETOC) "Decision-making framework for the grouping and testing of nanomaterials (DF4nanoGrouping)" (Arts et al., 2015), including the conduction of case studies is the only practical attempt at grouping, as also discussed at the November 2016 OECD-ProSafe conference.

-oOo-

References

Arts JHE, Hadi M, Irfan M-A, Keene AM, Kreiling R, Lyon D, Maier M, Michel K, Petry T, Sauer UG, Warheit D, Wiench K, Wohlleben W and Landsiedel R, 2015. A decision-making framework for the grouping and testing of nanomaterials (DF4nanoGrouping). Regul. Toxicol. Pharmacol. 71: S1–S27.

6. Question 6

NANoREG Question of Regulatory Relevance number 6
Fate, persistence and long-term effects

T1.3 – Information-gathering master document

Question theme	*Fate, persistence and long-term effects: Can effective in vitro and alternative models to understand long-term effects be developed? Will MNMs accumulate in humans, the environment, environmental species and the food chain and what are the driving forces? Is this mechanistically different from bulk materials? Will nanomaterials present long-term and/or cause deferred effects? How will coatings or surface modifications or the bio-based nature of the MNM affect biopersistence / biodegradability rates?[source D1.1]*
Keywords	*accumulation, surface modification, biopersistence*
Regulatory context:	*From a regulatory perspective, applicants with new MNMs have to report on the toxicity of their materials with the help of standardized test guidelines. Regulators provide validated guidelines and if possible, an integrative testing strategy. In vitro models can play an important role. Nanospecificity has to be guaranteed and hence, certain existing test guidelines need amendments, and in few cases, might be developed from scratch.*
Owner(s):	*Lead Contact Person(s): Adriënne Sips (RIVM)*

6.1. NANoREG (elements of) answer to the question

The title of Q6 'fate, persistence and long-term effects' seems to aim at the importance of investigating persistence and long-term effects. Wording as fate and persistence are merely used for environmental exposure and ecotoxicology. The context of this question seems to aim both at human and environmental safety testing. For this reason it is important to add the equivalents for fate and persistence in studying human kinetics, i.e. distribution and accumulation.

In the regulatory context, focus seems to be on the applicability of *in vitro* testing and other alternative testing for long-term effects. This is however not a nanospecific issue; this is about application domains of *in vitro* testing. Nevertheless, for MNMs long-term toxicity seems of utmost importance to study, as acute toxicity has hardly been reported.

For this question, the focus should be on nanospecific testing for fate/distribution, persistence/accumulation and long-term toxicity.

A. NANoREG merely focused on human hazard and safety. For that reason not much input on fate and persistence could be gathered.

This project is funded by the EU Framework 7 Programme, contract no 310584.

B. In a NANoREG workshop on biokinetics (May 2014, Berlin/organized by BfR/BAUA), RIVM presented a list of kinetic aspects that are different for nanoparticles, compared to conventional (molecular) substances. This list is given in table 1

Table 1. Kinetic aspects which distinguish nanoparticles.

Aspect	**Conventional (molecular) substances**	**Nanoparticles/ microparticles**
Type of kinetics	Dissolved substance kinetics	Particle kinetics
Substance form	Uniform	Pluriform, also during internal exposure
Linearity	<, >, or = dose-proportional	< dose-proportional due to agg. is seen at higher doses
Barrier transport	Gradient driven	Against gradient is seen
	0-100%	Mainly low (<10%)
Proteins	Protein binding decrease free fraction, free fraction determines activity	Corona formation which (may) affect kinetics
Metabolism (enzymatic degradation)	0-100%	Not important for metal; for organic-metal combinations??
Conjugation	Yes, aids excretion	Probably not
Distribution	Flow and extraction ratio dependent	Uptake by macrohpages, thus distribution mainly to tissues with phagocytic capacity
Uptake into tissue	Diffusion driven, carrier mediated	Active processes driven, some cases passive??
Excretion	Renal, hepatic, etc.	Clearance from tissues in general very low
	Renal, hepatic transporters	Mechanism of clearance not fully understood
Accumulation	Possible, both in plasma and tissues	Possible, merely in tissues, hardly in plasma
Mechanism of accumulation	Hydrophobic or bound to cellular structures or proteins	In vesicles
Interactions	Mechanisms known	???
Route-to-route extrapolation	Basic understanding	??, route-dependent kinetics seem plausible related to changes in phys-chem or protein corona
Interspecies differences	Basic understanding	???, some indications
PBPK models	Physiological parameterization is understood	Physiological parameterization is under development

C. Dissolution rate is important to study, in various media, like in the medium of exposure or in macrophages fluid. Kinetic models for dissolution rate in (stirred) exposure media (which reflect the influence of particle size) are already available in literature; see e.g. David, C. A. et al. (2012), "*Dissolution Kinetics and Solubility of ZnO Nanoparticles Followed by AGNES.*", The Journal of Physical Chemistry C 116(21): 11758-11767. These models can be used to extrapolate differences in dissolution rates between micro and nanoparticles. Also, there is abundant information on dissolution kinetic models for micro and macroparticles in the pharmaceutical context. Intracellular dissolution rates are still very poorly understood.

D. D2.3 and D2.9 highlight the need for kinetic data on dissolution rates, but there is no consensus yet on how to implement the specific modifications of the corresponding testing guidelines.

E. NM dissolution rates have been found to be extremely sensitive to experimental variables of the testing protocol, among others NM dispersion procedure, primary and agglomerate/aggregate size distributions, core and coating chemical composition, temperature, pH, composition of the test medium, hydrodynamic conditions (stirring, etc.). This sensitivity is significantly larger than with conventional chemicals.
Furthermore, there is still no consensus on which is the most suitable combination of solid-liquid separation step (UF, UC...) and elemental analysis technique (atomic spectrometry, voltammetry, etc.) nor which dissolved fraction (free ions, low MW dissolved complexes, metal bound to macromolecules, etc.) is the most relevant for toxicology purposes. Therefore, NM dissolution rate in in vivo compartments seems to be still an ill-defined endpoint from a regulatory point of view. Further development of test methods for dissolution rate is therefore necessary.

F. To understand and predict effects of Nanomaterials (NMs) in organisms, it is essential to determine how these NMs will distribute in an organism. The crossing of biological barriers is a crucial aspect in that context.

   D5.3 reports on the evaluation in NANoREG of potential penetration and translocation of several NANoREG NMs characterized by different physico-chemical properties into different *in vitro* barrier models.

   Hitherto no new 'nanomaterial toxicology' was evident in regulatory testing under NANoREG in WP4. Established test methods were used with some adaptations for human toxicity. They found no evidence that the established regulatory test are inadequate for nanomaterials.

G. The existing methods for ecotoxicity testing do not have any information about how to standardise NM dispersion preparation. WP2 has shown that this is possible using newly developed dispersion standard operating procedures (SOPs), which have been harmonised and benchmarked. Furthermore, there is a lack of guidance in existing ecotoxicity standard tests for what physicochemical characterisation should be conducted for NMs being studied in ecotoxicity tests and also how this should be determined. For this, WP2 has developed a technical guidance document (TGD) to guide ecotoxicologists through experimental design and characterisation of NM dispersion exposure systems. Finally, the TGD was applied in actual aquatic ecotoxicity exposure studies conducted in WP4. By implementing the TGD, the characterisation could be improved, at the same time trying to make it targeted in order to reduce associated time and analysis costs. This allowed to gain a much better overview of the actual exposure the organisms in the ecotoxicity tests were undergoing – in many cases this was nothing like a simple mass-based dosimetry associated with existing standard methods. Instead, it was possible to see aggregation during the ecotoxicity test, settling/sedimentation, NM concentration and dissolution behaviour. These helped to interpret the toxicity endpoint data generated. Finally, it was found that some of the standard ecotoxicity test methods have limitations for NMs. For example, the classic freshwater algae test needs modification. The traditional way this test is conducted involves measuring algal

growth (cell replication), but the NMs in the system cause interference with the measurement. Proposed modifications to the quantification of algal growth method have been investigated with some proposed in the literature also ultimately being unsuitable. Further work is needed to tailor the test methods for NM assessment. Finally, a decision is necessary about which standard tests are really relevant for NM assessment. The work in WP2 and presented in the TGD shows that in some cases certain NMs behave in such a way in aquatic ecotoxicity tests that there seems little point in conducting them.

H. NANoREG activities did not specifically focusing on accumulation of nanomaterials in the food chain.

6.2. Overall assessment / conclusions

- It is clear that nanoparticles have some nano-specific kinetic behaviour, depending in part on physical-chemical characteristics such as size, surface charge and the resulting protein corona. The kinetics of a NP can therefore not be deduced from microparticle form or molecular form.
- Dissolution rate is important and methods to determine it appear to be extremely sensitive to the procedure and environment, thus a strict protocol needs to be developed for this endpoint for each matrix.
- Nanoparticles tend to end up, in particular, in organs with phagocytotic capacity, thereby implying that especially distribution to these organs needs attention in test designs.
- Absorption and accumulation potential is relevant for likelihood for long-term toxicity and thereby relevance for long-term toxicity testing
- The only clearance pathway identified so far for nanoparticles is by dissolution into molecular form, thereby implying that risk assessment of the molecular form remains also important.
- Together with some, though low absorption observed for various NPs, absence of clearance for non-dissolving NPs means accumulation is possible. This has also been observed, e.g. for TiO_2, SiO_2 and CeO. This implies that long-term toxicity assessment is of high importance for NPs with low or no dissolution, and that the determination of the exact toxicokinetic behaviour is then also relevant.
- To assess toxicokinetics, suitable chemical analytical methods need to be available. This has been found to need further development for NPs, especially in biological environments such as tissues or culture media.
- Methods to study translocation over barriers (i.e. absorption) have been found to need further improvement, as the insert membranes separating the two compartments appears to be a barrier for NPs itself. A method without such membranes, or better membranes needs to be found.
- Non-animal methods to study long-term toxicity are lacking overall, not only for NPs. Further development in this area is therefore highly necessary, 3D-models and organs-on-a-chip seem promising for this, as they reach longer viabilities.
- Immunotoxic effects are embedded in OECD test guidelines to a limited extent, biochemical and haematological parameters for immunotoxicity are e.g. not standard in repeated dose testing. With the major uptake into macrophages, this endpoint deserves more attention in the long-term toxicity testing of NPs.

Elements of answer

- It is still uncertain whether useful *in vitro* or alternative models for long-term toxicity testing can be developed, this is a problem for the testing of conventional substances as well. The reason

is that the possible long-term effects are diverse and may include multiple tissues and organs, while *in vitro* models only encompass one small part of the human body. Additionally, *in vitro* tests have not been able to stay viable for longer than a week until recently, disabling exposure of longer duration. 3D models and organs-on-a chip, with the possibility to couple multiple tissues into possibly a human-on-a-chip are promising in alleviating these barriers. The development of these models thus deserves priority.

- Accumulation in mammals has been observed for some low-dissolving NPs, information about accumulation in the environment and food chain is scarce, but does not indicate a concern so far.
- The accumulation, and other kinetic features, is nano-specific, as the accumulation takes place in vesicles of macrophages, in tissues with high levels of monocytes (i.e. macrophages in tissues), with dissolution as the only known clearance pathway. In contrast, molecular (bulk/conventional) substances mainly accumulate due to high lipophilicity (leading to accumulation in fat tissue) or strong binding to cellular components in any cell.
- Because of the seen accumulation in mammals, long-term toxicity testing is relevant, as well as a precise kinetics assessment.
- Coatings and surface modifications can highly impact the kinetics, as this impacts e.g. the protein corona. The kinetics, such as absorption into the body, can subsequently impact the toxic effects caused by the MNM. The ultimate degradability is, however, not expected to change, as for ultimate degradation, the core will need to be degraded as well, and this is not affected by the coating.
- In dissolution testing especially dissolution rate is important. When reporting dissolution analysis, the protocol used must be reported in detail, including all experimental conditions in which the data were collected

To address the safety of MNM properly, at the current state of science, these findings underscore the necessity for:

- Determination of accumulation potential (incl. methods for this),
- Subchronic and chronic in vivo studies (incl. immunotoxicity), and
- Kinetic modelling for animal study-lifetime extrapolation.

Recommendations to improve assessments in the future:

- Invest in the development of chemical analytical method for NPs in biological matrices, in order to enable assessment of kinetics;
- Invest in the development of strict standard operating procedures for determining dissolution rates of NPs in different matrices;
- Invest in improvement of methods to determine translocation of NPs;
- Invest in methods for assessing immunotoxicity of NPs, as this is not strongly embedded in current tests;
- Invest in non-animal methods that may be useful for determining long-term toxicity, e.g. 3D models and organs-on-a-chip.

6.3. Any other relevant issues?

-oOo-

7. Question 7

NANoREG Question of Regulatory Relevance number 7
Kinetics and fate, determination

T1.3 – Information-gathering master document

Question theme	*Kinetics and fate, determination: How and when should information on absorption from the various routes of exposure, on deposition (e.g. lung burden), on biodistribution, on potential persistence and bioaccumulation, and on internal exposure (taking into account dose, duration, coating and interaction with biological systems) be generated and used? Relate the information with, for instance, the following objectives:* • *To perform more accurate risk assessment,* • *To decrease uncertainty (safety factors),* • *To select, if needed, a second route for acute toxicity testing,* • *To design additional tests – that are 'affordable' – or to relate to studies that involve exposed workers, such as in the silica industry,* • *To decide on a strategy for further testing (carcinogenicity, reproductive toxicity, etc.).[source D1.1]*
Keywords	*Absorption, deposition, , accumulation, uncertainty biodistribution, route of exposure*
Regulatory context:	*Depending on the regulatory regime, MNM falls into (e.g. cosmetics, food, plant protection agents, and chemicals) the determination of fate and kinetics looks different. We need tailored guidelines to assess the fate and kinetics of the MNMs in animals and different environmental media. Testing strategies are necessary to decide when and for what MNM such tests are needed.*
Owner(s):	*Lead Contact Person(s): Adriënne Sips (RIVM)*

7.1. NANoREG (elements of) answer to the question

- The role of kinetics in legislation has not been investigated in NANoREG.
- The conclusion from NANoREG is that kinetics information is needed just as much for MNMs as for molecular substances.

This project is funded by the EU Framework 7 Programme, contract no 310584.

- For environmental fate modelling, partition coefficients are key. Task 2.3 has led the physicochemical characterization of the nanomaterials and their conclusions is: NMs are a second phase (always thermodynamically unstable), so partition constants can never be determined. The fate calculation system therefore appears to need to be re-thought.
- D2.6 focused on the development of SOPs for the reproducible dispersion, characterisation and quantification of nanomaterials in aqueous media relevant for toxicity, ecotoxicity and environmental fate studies. Data from established and tested dispersion SOPs for toxicity and ecotoxicity testing have indicated that the generation of reproducible nanomaterial dispersions across different laboratories and using different equipment is achievable.
- D2.8 also focused on establishing SOPs for the quantification of MNM fate *in vitro* and ecotoxicity studies. With regards to ecotoxicity studies, SOPs to quantify uptake by organisms are in development. With regards to in vitro studies, most work focussed on quantification of the dissolution(-rate) of MNM in different cell mediums and the hydrochemical reactivity (pH, redox potential, ROS-formation capacity in water / cell mediums) of MNM. It was found that, when measuring dissolution, in-depth knowledge on the material and the matrix is of importance when using Single particle (SP) ICP-MS measurement, ultrafiltration (UF) or ultracentrifugation (UC) methods. Dissolution was measured in gastric juices (saliva, gastric, duodenal and bile) as well as in biological media DMEM and RMPI as well as in BSA used as control. Furthermore, processing protocols (i.e. sonication, elemental detection method and procedures etc.) were shown to influence nanomaterial dissolution and it is therefore recommended to further standardise these procedures.
- Finally, there is still no consensus neither on which is the most suitable combination of solid-liquid separation step (UF, UC...) and elemental analysis technique (atomic spectrometry, voltammetry, etc.), nor which dissolved fraction (free ions, low MW dissolved complexes, metal bound to macromolecules, etc.) is the most relevant for toxicology purposes. Therefore, it is justified to promote further research on the validation of tests.
- Task 5.3 revealed that in vitro barrier systems using inserts are not useful to test translocation of NPs across barriers as the inserts form the barrier instead of the cells. There is therefore a need to adapt these barrier systems
- A critical aspect rising from NANoREG is that the role and effect of higher generation NM is barely addressed in the current inhalation studies. It has been studied to some extent in the CNT instillation studies where important differences were observed. Maybe this is not a nanospecific toxic mechanism, but MNMs provide the ability to introduce complex chemistry at the nanoscale and cellular uptake of such MNM.
- D5.8 (decision tree for risk assessment) included requirements on information and advice on testing of kinetic parameters to be used within the decision tree.
- Information requirements and advice on testing of kinetic parameters in relation to various stages of innovation were described in D6.4.

7.2. Overall assessment / conclusions

The question was: how and when should information on absorption from the various routes of exposure, on deposition (e.g. lung burden), on biodistribution, on potential persistence and bioaccumulation, and on internal exposure (taking into account dose, duration, coating and interaction with biological systems) be generated and used? Relate the information with, for instance, the following objectives:

- To perform more accurate risk assessment,
- To decrease uncertainty (safety factors),

- To select, if needed, a second route for acute toxicity testing,
- To design additional tests – that are 'affordable' – or to relate to studies that involve exposed workers, such as in the silica industry,
- To decide on a strategy for further testing (carcinogenicity, reproductive toxicity, etc.)

The element of answer at present is: currently, *in vitro* methods are not suitable yet to determine kinetic parameters of MNMs, thus animal tests should still be used to generate information on absorption, deposition, biodistribution and internal exposure. Environmental persistence cannot be determined through the partition coefficient of MNMs, as partition coefficients cannot be determined for these particles. A new approach is therefore necessary.

7.3. Any other relevant issues?

-oOo-

8. Question 8

NANoREG Question of Regulatory Relevance number 8
Kinetics and fate, extrapolation

T1.3 – Information-gathering master document

Question theme	*Kinetics and fate, extrapolation: How and when can information on kinetics and fate be used to justify grouping / read across or testing triggering / waiving and for building knowledge on the relationship between physical-chemical properties and toxicity? In other words: to what extent are the kinetics and fate of MNMs (e.g. environmental distribution or deposition and biodistribution in the lung) different from the bulk material? Are there ways to extrapolate this information from the bulk material or from several forms (size, shape, coating, etc.) of the same chemical and how should this extrapolation be made? [source D1.1]*
Keywords	*Possible links to grouping/RX, triggering, waiving, PCC-toxicity links, extrapolation*
Regulatory context:	*Read across and grouping tools would definitely be helpful to minimise testing. If data on kinetics and fate are unavailable, they need to be tested.*
Owner(s):	*Lead Contact Person(s): TBC and Adriënne Sips (RIVM)*

8.1. NANoREG (elements of) answer to the question

- NANoREG has very limitedly addressed this question.
- Work on risk potentials, developed in D5.8 and in D6.2 may contribute to grouping on the basis of kinetic parameters. These potentials include dissolution, accumulation which both can be regarded as kinetic parameters.
- Dissolution is a physico-chemical parameter, but may also be seen as a kinetic parameter. Theoretical expressions relating dissolution rates with primary particle size, agglomerate/aggregate size, salinity, pH and temperature have been successfully tested with some NMs (e.g. ZnO) in synthetic solutions under thorough stirring conditions. Tests in more realistic conditions (e.g. systems mimicking actual conditions of in vivo internal compartments) are still missing and are certainly needed, since extrapolation is highly uncertain.

This project is funded by the EU Framework 7 Programme, contract no 310584.

8.2. Overall assessment / conclusions

- No specific input for this question was received. Within the regulatory question on grouping and read across, there is an eye for the possibility to group on the basis of kinetic parameters.
- When an MNM does not have a high solubility and does not have a high aspect ratio, it cannot be assigned to these specific groups, and then all kinetic information will be necessary for subgrouping under “passive MNMs” and “active MNMs”. Extrapolation of kinetic parameters is hardly possible, implying this information will require substantial testing.
- It should be taken into account that grouping or read across can be used from two different perspectives: 1) for materials already at market, so more safety information is already in place and applications are known. In this case grouping aims at gaining overview for which individual materials information is present or needs to be gathered. 2) For materials under development. In this case grouping criteria can give guidance to developers on how to proceed in toxicity testing strategies.
- When an MNM does not have a high solubility and does not have a high aspect ratio, it cannot be assigned to these specific groups, and then all kinetic information will be necessary for subgrouping under “passive MNMs” and “active MNMs”.
- Extrapolation of kinetic parameters is not possible from the bulk form and hardly possible from other nanoparticle-forms, implying this information will require substantial testing.

.

8.3. Any other relevant issues?

-oOo-

9. Question 9

NANoREG Question of Regulatory Relevance number 9
Mode of action

T1.3 – Information-gathering master document

Question theme	*Mode of action: What are the physical and chemical properties driving exposure and (eco)toxicity of MNMs at all stages of their life cycle? How is MNM interaction with biological systems affected? What are critical characteristics of MNMs that need to be considered and included / excluded when developing MNMs to ensure they are safe and which materials have a known increased toxicity in the nanoform vs. the bulk form, and why? How will this facilitate the regulatory safety assessment of new nanomaterials?*
Regulatory Importance urgency and context	*Context:* *Information on modes of action are important building blocks for AOPs. If key events, are known (e.g. for long-term effects), a testing strategy can be built based on a tiered approach.* *A robust assessment of how far in silico, in vitro and animal tests can be used for measuring key events of long-term effects would provide regulators and industry with the relevant information for further establishing alternative methods for hazard assessment.*
Keywords	*Toxicity bulk/NM, nano-biointeractions, PCC driving (eco)toxicity, life cycle*
Owner(s)	*Lead Contact Person(s): Mats-Olof Mattsson, AIT*

9.1. NANoREG (elements of) answer to the question

- Several of the *in vivo* and *in vitro* studies within the project are contributing data and experience to assess the mode of action (MOA), either directly or indirectly.
- MOA related knowledge is also part of the NANoREG decision tree in D5.8, where aspects such as solubility, stability, accumulation, inflammation/immunotoxicity, cancer potency/genotoxicity, ecotoxicity, and exposure potential are included.
- Experimental work was performed within several NANoREG tasks and has dealt with:
  - NM physical-chemical properties´ importance for developmental and reproductive toxicity

This project is funded by the EU Framework 7 Programme, contract no 310584.

- Chronic (and low dose) toxicity of nanosized Granular Biodurable Particles without know significant specific toxicity
- Development of SOPs for reproducible dispersion, assessment of dissolution/bio-durability, hydro-chemical reactivity in aquatic and simulated biological fluids/compartments
- Capacity of NM to absorb biomolecules (e.g. albumin, interleukins)
- Dustiness test for powder characteristics
- *In vivo* (90 days) study of tissue distribution and tissue effects correlated to physical-chemical properties
- *In vitro* high throughput screening involving various toxic endpoints on lung and intestine human cell models
- *In vitro* uptake following acute and chronic exposure in intestinal human cells
- PBPK modeling studies to better understand the uptake and biodistribution correlated to physical-chemical properties.
- The question of the importance of the presence of barriers in *in vitro* studies is highlighted. It is noted that co-culture *in vitro* models with e.g. mucus are available, but not used in NANoREG.

9.2. Overall assessment / conclusions

- Results have shown a decrease in the adverse effect of CeO_2 NPs and TiO_2 NPs on the unicellular green algae (Pseudokirchneriella subcapitata), after adding environmentally-realistic natural organic matter concentrations to the culture media. The results support the idea that the MNMs lead to generation of reactive oxygen species, which in turn cause toxicological effects.
- Concern has been voiced about the way the concentrations for in vitro testing are expressed, since the quantity of MNM in contact with the cells may vary with the volume, according to the operator and the test system used. This is especially relevant at concentrations in the µg/mL range.
- A Technical Guidance Document (TGD) for the quantification (aggregation, sedimentation, dissolution) of MNMs exposure and fate in dispersions for aquatic ecotoxicological studies has been developed and is being implemented in ecotoxicity tests ('Protocol for producing reproducible dispersions of manufactured nanomaterials in environmental exposure media', D2.6).
- The identification of differences between NMs and bulk materials is hampered by lack of studies, where bulk material and NM are tested together.

9.3. Any other relevant issues?

-oOo-

10. Question 10

NANoREG Question of Regulatory Relevance number 10
Hazard

T1.3 – Information-gathering master document

Question theme	*Hazard: Which methods should be used to assess the human and environmental toxicity? What is the applicability of conventional testing methods for nanomaterials? Is adaptation of the conventional methods needed, for example by including nano-specific endpoints or additional guidance on sample preparation? What testing is relevant at all stages of the nanomaterial life cycle?*
Keywords	*Methods for EH toxicity*
Regulatory Importance, urgency and context	*Context:* *Usually, OECD/EU test guidelines are used to evaluate the hazard potential of chemicals. Some of the test tests are not specific enough to measure nanoparticle toxicology. Therefore, sometimes, nanospecific adaptation is needed to the test guidelines. A nanospecific test strategy for the hazard assessment ideally relies on a small but sufficient set of such tests, which account for conventional and nanospecific effects. The results are evaluated by the regulators, according to clear rules and criteria.* *Further regulatory questions in this context are, whether and how the GHS/CLP and PBT criteria are applicable to nanomaterials.*
Owner(s)	Lead Contact Person(s): Mats-Olof Mattsson, TBD

10.1. NANoREG (elements of) answer to the question

- The decision tree D5.8 has identified which hazard information/tests are most important to prioritize NM and which info is most relevant for assessment.
- A number of studies and developments are employing adapted OECD Technical Guidelines. These activities include a pulmonary toxicity study using an adapted version of OECD 414; SOP development for dispersion and quantification protocols (OECD 453); a 90 day oral toxicity test of silica NM (OECD 407), which also is comparing *in vitro* and *in vivo* toxicity studies.
- Several existing OECD and ISO standard methods for ecotoxicity assessment have been adapted for MNM testing and developed into defined Standard Operational Procedures (SOPs). The specific organisms and guidelines adapted are the following:

This project is funded by the EU Framework 7 Programme, contract no 310584.

- Unicellular green algae *Pseudokirchneriella supcapitata*: OECD-Guideline 201
- Crustacean *Daphnia magna*: OECD-Guideline 202; ISO 6341:2012
- Soil nematode *Caenorhabditis elegans*: ISO 10872:2010

- The 'Protocol for producing reproducible dispersions of manufactured nanomaterials in environmental exposure media' has been applied. This method aims to produce a highly dispersed state of MNMs in Milli-Q water, which can subsequently be diluted into specific media necessary for different ecotoxicity tests. This SOP has been used to generate benchmark data on size distributions and stabilities of batch dispersions of some of the NANoREG core MNMs: SiO_2 NPs (JRCNM02000a/NM-200, JRCNM02003a/NM-203), CNTs (JRCNM04000a/NM-400, JRCNM04001a/NM-401,), Ag NPs (NM-300K and NM-302), TiO_2 NPs (JRCNM01000a/NM-100, JRCNM01001a/NM-101, and JRCNM01003a/NM-103), CeO_2 NPs (JRCNM02102a/NM-212), $BaSO_4$ NPs (NM-220) and ZnO NPs (JRCNM01100a/NM-110 and JRCNM01101a/NM-111).
- Further modified protocols include
  - Modifications of specific genotoxicity assays (micronucleus test and Comet assay) both *in vivo* and *in vitro*, assays for cytotoxicity and immunotoxicity, and also for use in high-throughput testing.
  - Mammalian mutagenicity tests (mouse lymphoma and HPRT gene mutation).
  - A chronic in vitro test model for intestinal barrier studies and for cellular uptake and toxicity endpoints.
- For two of the applied genotoxicity tests (comet and the micronucleus assays), OECD Test Guidelines are available (TG 489 and TG 474). The Pig-a gene mutation assay is currently undergoing international standardization. So far, no need has emerged for protocol adaptation to nanomaterials with respect to standard recommendations for these tests.
- The ECVAM protocol for Intestinal barrier crossing has been used. However, some concern has been raised regarding the role of the membrane insert for preventing the crossing of the nanoparticles, especially if aggregates/agglomerates are present.
- The *in vivo* sub-chronic oral toxicity study (90 days) with NM203 was coupled successfully with genotoxicity and immunotoxicity testing, suggesting that animal experimentation may be reduced to some level for NM testing.

10.2. Overall assessment / conclusions

- Harmonized preparation and exposure characterization procedures (NANoREG SOPs in WP2) were developed to reduce uncertainty for hazard characterization.
- It is recognized that both *in vivo* and *in vitro* methods need to be adapted in order to be appropriate for determination of the hazard potential of nanomaterials.
- For comparative testing, both submerged and air-liquid interface (ALI) conditions were used in NANoREG studies. The potential crossing of several MNMs has been evaluated using different *in vitro* barrier models, including blood-brain barrier, intestinal epithelium, lung epithelium, oral mucosa and biomimetic lipid membranes.
- There is a need for an intracellular MNM dose definition also for hazard identification.

10.3. Any other relevant issues?

-oOo-

11. Question 11

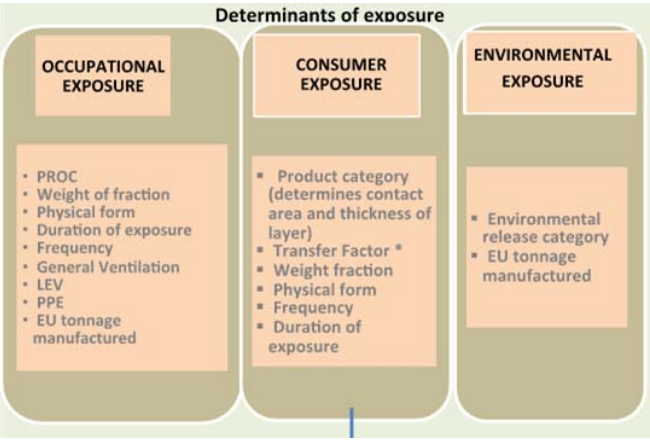
NANoREG Question of Regulatory Relevance number 11
Exposure

T1.3 – Information-gathering master document

Question theme	*Exposure: What are the main determinants for occupational and consumer exposure to MNM and what are the duration and type of exposure?*
Keywords	*Determinants for occupational and consumer exposure, level, duration, type*
Regulatory context:	*Independent of the matrix, MNM should be quantitatively measurable and characterisable. This would allow a robust exposure assessment.*
Owner(s):	*Lead Contact Person(s): Rob Aitken, Juergen Hoeck*

11.1. NANoREG (elements of) answer to the question

- Critical exposure scenarios (in terms of economic importance and regulatory gaps) across the three domains (occupational, consumer, environment), consider the data gaps and develop a program of data collection (measurement) have been analysed. An extensive evaluation of the state of the art for the core set of nanoparticles selected for the NANoREG project was performed, considering factors, such as volume of production, main market applications of ENM used in nano-enabled products, existing exposure data and previously reported data gaps. This included drawing together information regarding the determinants of exposure as identified in Figure 1 below. This is extracted from deliverable D3.1.



This project is funded by the EU Framework 7 Programme, contract no 310584.

- A series of simulation studies have been carried out to characterise the emissions (including both occupational and consumer scenarios). These comprise painting, sanding and drilling wood with wood-stain containing Ce_2O_3, drilling of concrete for emissions to the air and release to water through washing (washing machine). The methodology has been described in D3.3. Quantified release to air has been assessed using CPC, SMPS and ELPI. Analysis of physical samples collected by ELPI has provided quantitative assessment of the nature of the particles released. Analysis of the parameters of these simulations provides a comprehensive determinant data set for a group of important scenarios as well as information about exposure levels (D3.7).
- Existing test protocols for environmental release due to weathering were being tested by quantifying emissions from cement, packaging and wood samples looking to assess how much is coming out and how is characterised (e.g. size /aggregation/ speciation). A key issue is the degradation of the matrix and the project aims to establish the key parameters to measure seeking to establish a pre-standardisation of the protocols. These traditional approaches are being contrasted with new mesocosm based protocols. Small mesocosm systems (water, sediments and basic aquatic food chains) have been taken through a proof of concept (freshwater and marine environments). Same types of samples were being used and results (what is being released and where does it go in the food-chain) were analysed according to the input parameters and compared with the existing tests (D3.5).
- Field studies were carried out assessing occupational exposure (field access has been challenging so final numbers of studies remain uncertain). Particles include carbon based, SiO_2, TiO_2. Studies include collection of comprehensive contextual information, information about determinants as well as quantitative data from instrumentation including CPC, SMPS, and ELPI. This is reported in D3.7. Data will be stored in the NECID database.
- To generate data for the modelling task (T3.4) a large scale multi-instrument experiment assessing determinants, dispersion in a chamber and side by side comparison of instruments has been carried out (D3.4). Instruments used were CPC, FMPS, ELPI, OPS and NSAM. The emission source was varied including particle types (NaCl, Na Flourescein) olive oil, SiO_2), and source pattern (continuous, spiked). Effect of ventilation and time was assessed. This will generate a substantive data set to be used for the development of a tool from the “nano specific 2 box model”. This model, based on particle dynamics, will provide more detailed information on particle size distribution and dispersion. The data generated plus the tool will lead to greater understanding on the determinants of exposure.
- A detailed assessment of the effectiveness of RMM through laboratory simulations of scenarios for spraying, extrusion, and powder handling was conducted. This information reported in a rich D3.9

11.2. Overall assessment / conclusions

The three exposure questions (Q11, Q12 and Q13) are not mutually exclusive; there is substantial overlap and linkage between them. In the same way there is substantial overlap and linkage in the Exposure tasks in NANoREG.

The main output from NANoREG in relation to this question is new data about determinants, emissions and exposures which adds to the existing data collected and collated (as part of the review process). This new data comes from field studies and scenario based simulations in which determinants will be systematically varied. This will include data on the effectiveness of risk management measures. While the ongoing problem about the lack of access to field sites exposure and exposure determinant data remains an issue, the researchers have attempted to overcome this through simulation studies which will add significantly to the body of data available. The analysis of this data (in D3.7) will provide greater understanding in the linkage between determinants and exposure and will support the development and validation of improved models (Q12).

However, there will be some issues outstanding at the end of the project. Data for consumer exposure will remain sparse and specifically information on transfer factors will be almost completely lacking. The situation regarding consumer use of products is made more challenging by there being insufficient knowledge (in terms of nano) about these products.

11.3. Any other relevant issues?

An issue which runs through all of the exposure questions is a difficulty in obtaining field measurement data for occupational exposures and an (almost complete) lack of data for consumer exposure. Whilst exposure questions have been part of many EU projects, NANoREG has been no more successful than these other projects in gaining access to sites to collect this data. This suggests that other approaches need to be considered to facilitate collection of this critical data.

-oOo-

12. Question 12

NANoREG Question of Regulatory Relevance number 11
Exposure

T1.3 – Information-gathering master document

Question theme	*How should human and environmental exposure be assessed in practice (determining exposure scenario, quantify input parameters for models, assumptions and use of proxy indicators, background and uncertainty estimation)? Consider both measuring and specific modelling for nanomaterials and evaluate the needs for standardisation and validation.*
Keywords	*Exposure, scenario, modelling, methods*
Regulatory context:	*Exposure assessment is a critical part of the regulatory processes. Independent of the matrix, MNM should be measurable and characterizable. This would allow a robust exposure assessment. If modelling leads to similar results with less efforts, exposure modelling can replace lengthy experiments. Therefore, exposure modelling tools need to be developed and standardized for different, regulatory relevant, exposure scenarios.*
Owner(s):	*Lead Contact Person(s): Rob Aitken, Juergen Hoeck*

12.1. NANoREG (elements of) answer to the question

- Existing test protocols for environmental release due to weathering are being tested by quantifying emissions from cement and packaging samples looking to assess how much is coming out and how is characterised (e.g. size /aggregation/ speciation) (T3.2). These traditional approaches are being contrasted with new mesocosm-based protocols. The success of small mesocosm platforms (water, sediments and basic aquatic food chains) as proof of concept (freshwater and marine environments) has been reported in D3.5
- As part of D3.7 a literature review has collated and evaluated existing EA methods and SOPs. New techniques/SOPs have also been developed in WP3.
- A large-scale multi-instrument experiment assessing determinants, dispersion in a chamber and side by side comparison of instruments was conducted and reported in D3.4.
- A model validation exercise compared predicted with actual exposures, considered ranking and included an inter-user study. The dataset collected in D3.4 have allowed to investigate the performance of the new I-Nano tool in terms of temporal and spatial resolution (Near Field and various Far Field positions) under different source emission and ventilation conditions.

This project is funded by the EU Framework 7 Programme, contract no 310584.

The measurements collected at multiple FF points have allowed to estimate how different the FF concentrations at the different locations differed from each other and whether the single modelled FF concentration is justified for using in these scenarios.

The different source emission types (constant and burst/spike) as well as the different ventilation conditions allowed to explore the sensitivity of these models to changes in those variables and adds information on Q11 on the determinants of occupational exposure and Q12 on the how data should be collected for model input. The outcomes with recommendations for developers (on how the models could be improved) and guidelines for users of each model which will identify assumptions, limitations, range of application and adjustment factors were released in D3.8. In addition a further tool was developed (and test) based on the "I-Nano two-box model".

- Within Task 4.2 a satellite study was conducted, in collaboration by Partner 13 and Partner 18, as part of a 2-year chronic toxicity-carcinogenicity inhalation experiment. Specific aim of the satellite study was to assess systemic genotoxicity induced in hematopoietic system of rats exposed by inhalation for 3 or 6 months to 0.1, 0.3, 1 or 3 mg/m3 CeO2 NM-212 or 50 mg/m3 BaSO4 NM-220.

  The study evaluated two items belonging to the class of poorly soluble low toxicity nanomaterials with contrasting lung clearance characteristics: CeO2 nanomaterial showing high particle retention/slow pulmonary clearance and BaSO4 showing a remarkably fast pulmonary clearance. Data on lung burden, percent translocation from the lung to peripheral organs, long term retention were the most relevant input parameters used to assess the negative output obtained for genotoxicity in distal hematopoietic cells.

12.2. Overall assessment / conclusions

- The three exposure questions (Q11, Q12 and Q13) are not mutually exclusive, there is substantial overlap and linkage between them. In the same way there is substantial overlap and linkage in the Exposure tasks in NANoREG.
- The main output from the project in relation to this question is for occupational exposure, tested/validated exposure models (validated models, recommendations for developers (on how the models could be improved) and guidelines for users of each model which will identify assumptions, limitations, range of application and adjustment factors). Improved SOPs and measurement methods for the assessment were also produced. It also provided an assessment of the OECD tiered exposure assessment approach and how it can be used in the regulatory context (D3.6).
- The mesocosm approach appears to offer interesting possibilities for the testing of environmental exposure, but is at an earlier stage in the development process. Whether it is sufficiently validated or robust enough at the completion of the project remains to be determined.
- NANoREG did not test environmental release models.

12.3. Any other relevant issues?

- While good validated models can to some extent replace the need for data, validation of models requires good data so, as indicated in the response to Q11, difficulty in obtaining field measurement data for occupational exposures and an (almost complete) lack of data for consumer exposure is problematic, as indicated in other questions NANoREG has been no more successful than other projects in gaining access to access to sites to collect this data. This suggests that other approaches need to be considered to facilitate collection of this critical data.

-oOo-

13. Question 13

NANoREG Question of Regulatory Relevance number 13
Exposure and life cycle analysis

T1.3 – Information-gathering master document

Question theme	*Exposure and life cycle analysis: Which scenarios could denote potential exposure and what information do we have on them? Can we develop standardized and efficient testing procedures for estimating release of nanoparticles (NP) from powders and NPs in matrices? What are situations in which MNM exposure is expected to be negligible / high? Are the amount and the nature of releases of MNM similar to regular chemicals, when common recycling and end-of-pipe techniques are used?* *How to minimise and structure LCA to avoid ending up with a '1:1 model of the world'?* *In other words: what is the exposure probability throughout the different life cycle stages of the MNM: production process of the NM itself, releases during the production process of products in which MNM are used, waste treatment, consumer articles, wearing, abrasion, etc.? Do waste treatment / recycling processes lead to exposure to NMs that can be hazardous to health and environment? If so, are additional risk management measures required? Do the recycled product / residues lose some value /usefulness due to undesired characteristics?*
Keywords	*Life Cycle analysis, Life cycle assessment, value chain, release, emission*
Regulatory context:	*Life Cycle Analysis regarding the risk assessment at different stages in the life cycle of a MNM is of regulatory relevance (e.g. under REACH). Life Cycle Assessment (LCA, according to the ISO definition) are yet to be used in regulatory contexts. It is widely acknowledged that 1) MNMs have to be analysed on possible impacts along their life cycle, and that 2) new, nano-enabled products and services should be a better alternative in comparison to older, conventional counterparts. LCA allows doing that, once nano-specific amendments are made to the LCA method.*
Owner(s):	*Lead Contact Person(s): Rob Aitken, Juergen Hoeck*

13.1. NANoREG (elements of) answer to the question

- Formal LCA (i.e. as defined in international standards) was explicitly not being carried out in NANoREG. Rather the project focussed on the issues concerning risk assessment issues across the whole lifecycle and using lifecycle based thinking. Nevertheless, LCA is

This project is funded by the EU Framework 7 Programme, contract no 310584.

recognized as an important tool and framework in evaluating the negative and positive environmental implications of a product, process or technology and can also be employed to Nanomaterials. This consideration was proposed by the OECD in the Guidance Manual "Towards the Integration of Risk Assessment into Life Cycle Assessment of Nano-Enabled Applications" (2015), suggesting a complementary use of Risk Assessment (RA) and Life Cycle Assessment (LCA) for a fair evaluation of nano-enabled products. This is also mentioned in chapter 6 of the NANoREG Framework D1.11, published by JRC.
There is also a work package on LCA in the project NanoFASE.

- The NANoREG mapping approach has identified in a convincing way which scenarios could denote potential exposure, i.e. situations in which MNM exposure is expected to be negligible / high, and has collected and added to the information on them. New exposure data relating to about 20 of these scenarios has been collected and will support additional analysis to verify this approach (D3.1, D3.7).
- Lack of exposure data has been identified as critical in all 3 questions on exposure (Q11, Q12 and Q13).

13.2. Overall assessment / conclusions

- The three exposure questions (Q11, Q12 and Q13) are not mutually exclusive; there is substantial overlap and linkage between them. In the same way there is substantial overlap and linkage in the Exposure tasks in NANoREG.
- NANoREG has made an identification and prioritisation of "which scenarios could denote potential exposure" and has collected "what information we have on them" (figure 1 below).
- Life cycle use maps, identifying exposure scenarios along the life cycle, have been developed for the main applications for the core set of NANoREG NMs.
- 135 occupational inhalation scenario, 139 occupational dermal scenarios, and 153 environmental scenarios, plus additional contribution exposure scenarios have been ranked.
- A general life cycle map approach was developed and used, as well as a prioritisation algorithm, developed as part of a ranking tool for prioritisation of exposure scenarios. The prioritisation tool provides the basis for screening for other new scenarios.
- A main output in relation to this question is new data about determinants, emissions, exposures and risk management measures, which adds to the existing data collected and collated.
- Improved data on exposures supports the development of better exposure scenarios in REACH and better, more appropriate and proportionate risk assessment at both the regulatory and industrial level.
- Several SOPs have been reviewed or created and published by WP3.

13.3. Any other relevant issues?

- Some points will remain outstanding at the end of the project. The lack of consumer exposure data is acknowledged as are the difficulties in collecting new field data. The question also points to the further development on the Life Cycle Assessment (LCA, according to the ISO definition) are yet to be used in regulatory contexts. In NANoREG, the researchers have (deliberately) not started from an LCA methodology but rather adopted a life cycle approach to the development of value chains and the exposure scenarios which contribute to them. The question of whether there are any advantages in trying to build a process derived from a LCA methodology remains an open one at this point.

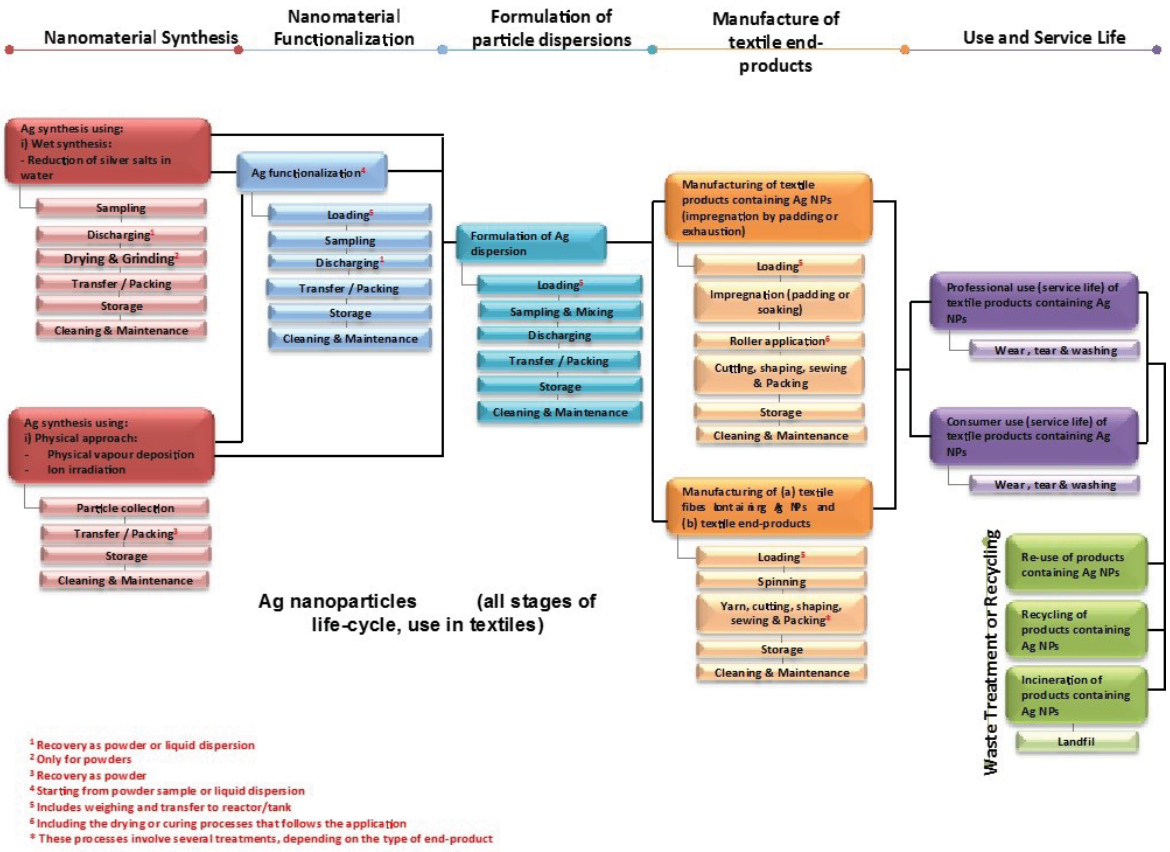


Figure 1: example scenario map

-oOo-

14. Question 14

NANoREG Question of Regulatory Relevance number 14
Risk assessment

T1.3 – Information-gathering master document

Question theme	*Risk assessment*
Keywords	*Risk assessment, dose-response assessment, effect assessment, no adverse effect dose, benchmark dose, long-term exposure, chronic toxicity*
Regulatory context:	*What are the no-adverse-effect or benchmark dose levels of long-term (low dose) exposures and can they be derived from short-term exposures (acute and sub-acute)? If not, what kind of information should be generated?*
Owner(s):	*Lead Contact Person(s): Paula Jantunen – Hugues Crutzen, JRC*

14.1. NANoREG (elements of) answer to the question

- Generation of chronic in vivo toxicity data; inhalation (with toxicokinetics; cerium dioxide), oral (dose-response; silicon dioxide). Several assays linked to RA were conducted in NANoREG:
    - A combined chronic/carcinogenicity whole-body inhalation study is currently performed to verify the hypothesis that based on quantifying particle mass concentration, particle agglomerate volume seems to be the best applicable metric to describe longer-term toxicity for a highly relevant category of nanomaterials. These nanomaterials are called poorly soluble, low toxicity particles (PSLT), poorly soluble particles of low cytotoxicity (PSP) or respirable granular biodurable particles without known significant specific toxicity (GBP).
    - Within this study, a satellite experiment was carried out to assess systemic genotoxicity induced in hematopoietic system of rats exposed by inhalation for 3 or 6 months to 0.1, 0.3, 1 or 3 mg/m3 CeO2 NM-212 or 50 mg/m3 BaSO4 NM-220. Results obtained in this study demonstrate that subchronic inhalation exposure to low doses of CeO2 or to a high dose of BaSO4 does not induce genotoxicity on the rat hematopoietic system at the DNA, gene or chromosomal levels. The negative data obtained after 6-month inhalation exposure were consistent with the negative outcome of the micronucleus test using RBCs upon 4-week inhalation exposure, suggesting that under the specific experimental conditions a short term exposure experiment could predict the outcome of a long term exposure. A quantitative comparison of the target doses obtained in these inhalation studies with those reached under oral exposure to the same nanomaterials – let alone with the effective dosages of the in vitro genotoxicity studies – may only be performed with a too great level of uncertainty to allow for a meaningful interpretation of the different outcomes.

This project is funded by the EU Framework 7 Programme, contract no 310584.

- Comparison of chronic (2 years) inhalation toxicity under NanoReg Project with sub-chronic (90 days) inhalation data in Wistar rats exposed to CeO_2 NPs in Project InhalT90 funded by German Federal Ministry of Education will be performed to find predictive and risk assessment relevant endpoints.
- 12 intratracheally instilled MWCNTs are studied in mice followed for one year of observation.
- A sub-chronic 90-day oral toxicity study in rats on the basis of the OECD TG 408 is performed including additional parameters as described in OECD TG 407, namely repro-endocrine-related endpoints, genotoxicity and immunotoxicity parameters. The test NM is relevant for food safety. The study represents the minimum requirement for RA of NM potentially present in food and feed according to the European Food Safety Authority approach.
- In WP 4 there were long term studies on nanomaterials including also HARN materials.

- Producing SOPs for aquatic ecotoxicity testing (for acute testing, as these are needed first, but with implications that may help with long-term methodology)
  - Short term ecotoxicity tests performed in the presence of natural organic matter (NOM) might provide useful insights for the development of long term assessment methods."
  - For the most soluble NMs, solubility in natural water may represent a useful indication for no-adverse-effect doses, as it relates with the threshold below which the NM is supposed to be dissolved and, thus, transformed into conventional chemicals (at least in long-term scenarios, as long as bio-uptake rates are slower than dissolution rates).
- Identification of data and testing (methods) that can be used for predicting long-term toxicity as a part of a risk assessment decision tree for NMs.
  - The objectives of WP5 (Advancement of Regulatory Risk Assessment and Testing) included developing *in vitro* screening methods for absorption/barrier crossing and a risk-assessment decision tree for nanomaterials which prioritizes information requirements, including those concerning chronic toxicity at low exposure doses or concentrations.
- Identification of parameters that need to be determined in order to predict long-term toxicity as a part of the Safe by Design approach, on basis of experiences from drug development.
  - D6.3 and D6.4 give some ideas about which type of information could be helpful per stage of innovation.
- Identification of screening methods suited for early assessment of potential EHS impacts of nanomaterials; studying the feasibility of using organ-on-a-chip in vitro methods for long-term toxicity testing.
  - The activity in T6.1 was linked to the “being prepared” theme. One part of the work concerned the early assessment of potential impacts on EHS of new technologies and applications that will likely reach the market. The dataset to be used in this activity was taken from literature, research reports, patents, and dialogue with experts, therefore the uncertainty (of the application reaching the market) is very high. However, the screening risk assessment that can be done in this exercise can provide some initial indications about critical MNM, and main targets for which long-term exposure is more likely. This information can be used to implement Risk Management or Safe by Design early on in the innovation process.
  - Task 6.1 aimed at raising awareness among regulators on (new) upcoming nanomaterials and gaining insight which kind of safety questions come along with it. Moreover, D6.2 came with an inventory in which stage of innovation which hurdles innovators come across to identify safety issues.

14.2. Overall assessment / conclusions

Work performed within the NANoREG project does not conclusively answer Question 14. The work done, however, contributes to an eventual understanding of the relationship of short-term and long-term toxicity of nanomaterials. It helps identify information and methodology that will facilitate prediction of exposure levels connected with long-term toxicity in practical contexts. Production of chronic ecotoxicological data, and/or standardized methodology for this purpose, remains a large gap in the context of this question.

14.3. Any other relevant issues?

At the workshop for NANoREG scientific task leaders in Bern (October 2015), the question of the persistence or reversibility of the effects of exposure to NMs in the long term was brought up. According to the participants in this discussion, there is some indication from 28/100-day toxicity studies that the timeline of NM clearance from tissues (after exposure has ceased) is longer than that for chemical substances in general, but whether the associated toxic effects are permanent or will go away with time is a relevant open question regarding various types of long-term toxicity.

-oOo-

.

15. Question 15

NANoREG Question of Regulatory Relevance 15
Risk *management*

T1.3 – Information-gathering master document

Question theme	*Risk Management: How can exposure to MNMs be minimized / eliminated? Are risk management measures (RMM), in particular existing personal protective equipment, effective and sufficient when hazards and/or risks are high, uncertain or unknown? Should the RMM be different from bulk powders? Are currently available control banding tools appropriate for NPs or will these need to be further evaluated, improved (related to exposure assessment, too)?*
Keywords	*PPE, control banding tools, 'zero' exposure*
Regulatory context:	*Human exposure to MNMs via the lung should be avoided (elimination, control, personal protective equipment (PPE)), particularly as long as their hazard is unknown. Therefore, the evaluation of exposure control measures, and especially PPE is important. Classification and labelling according to GHS/CLP are tools for risk management. The applicability of the classification criteria to MNM needs to be evaluated. Control banding tools can be used in Safe by Design approaches. They can support the assurance of sustainable MNM handling. Scientific knowledge is growing rapidly and existing control banding tools for MNM need regular updating.* ***Importance:*** *High* ***Time:*** *Now*
Owner(s):	*Christoph Studer (FOPH), Juergen Hoeck (TEMAS)*

15.1. NANoREG (elements of) answer to the question

Within task 3.5, the performance of different RMMs in the occupational environment was tested against NMs, either in real case scenarios or simulating the process under controlled conditions in an exposure chamber placed and also directly testing the efficiency of Personal Protective Equipment (PPE).

To this end, a complete review of the ISO standards protocols, ASTM, BSI, as well as guidelines published by relevant organizations (i.e. OECD WPMN, U.S. NIOSH, EU OSHA) has been

This project is funded by the EU Framework 7 Programme, contract no 310584.

conducted, and a set of protocols has been tested to proof the efficacy of RMMs against NMs. This has been done in three steps or tasks:

- Identification and compilation of the published harmonized standards for personal protective equipment and testing of engineered control measures.
- Development and description of protocols for RMM testing based on published standards.
- Evaluation of the adequacy and feasibility of the RMM testing methods for nanomaterials.

Table 1: General types of RMM regarding Engineering Controls and Personal Protective Equipment.

Category	General Type
Ventilation control	Local Exhaust Ventilation - (partial) enclosure
	Laminar Flow Booths & Laminar Flow Benches
	Local Exhaust Ventilation - captor hoods
	Local Exhaust Ventilation - receptor hoods
	Local Exhaust Ventilation - specialised applications
Personal Protective Equipment	Body protection
	Hand protection
	Respiratory protection

Regarding the efficiency of PPE under simulated conditions, different types of masks, coats and gloves were tested with different MNMs and concentrations.

For respiratory Protection Equipment (RPE), the Inward Leakage (IL), Total Inward leakage (TIL) and Filter Penetration (FP) were tested, either with the rubber head when testing nanopowders, which could be hazardous for the human health, or in tests subjects with NaCl to proof the fitting during movements.

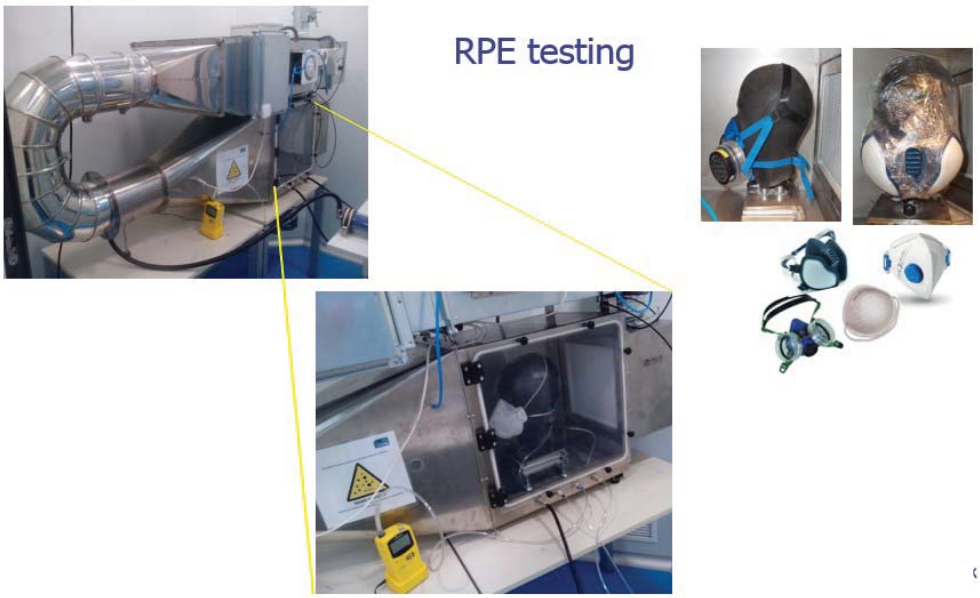


Figure 1: Testing for Respiratory Protection equipment within the simulation exposure chamber.

Experiments of IL and TIL were performed with three types of metal oxides (TiO_2, SiO_2, and ZnO) and NaCl at different sizes. Particle penetration through the masks was measured and in addition also blocked to measure the fitting properties (Fig. 1). Some representative results are shown in table 2.

Table 2: Efficiencies of different kinds of masks tested for several nanomaterials

Mask Type	TIL (%)	IL (%)	FP (%)	Nanomaterial
Half Mask – FFP3 filter	71,90 ±2,99	72,55 ±0,36	89,84 ±0,77	NaCl (Dpg = 35 nm)
Full Mask – FFP3 filter		99,58 ±0,74		SiO_2 (Dpg = 94-176 nm)
Half Mask – FFP2 filter	47,4		3,33	ZnO

To check the performance of the masks during occupational use, they have to be tested in test subjects performing movements like walking, talking, gesturing and moving the head, as shown in Fig. 2. In this case, only NaCl nanoparticles at different size distributions and concentrations can be tested, since they are the less hazardous for the human health.

Figure 2: Test Subject for RPE with NaCl on a treadmill.

Seven women and six men tried each a full and a half mask and two kinds of filters (FFP2 and FFP3). Results showed a high variability on the performance of the masks due to the fitting to the facial geometry, existing leaks where nanoparticles penetrate, especially in the case of women, and men with beards (see fig. 3 for a representative example).

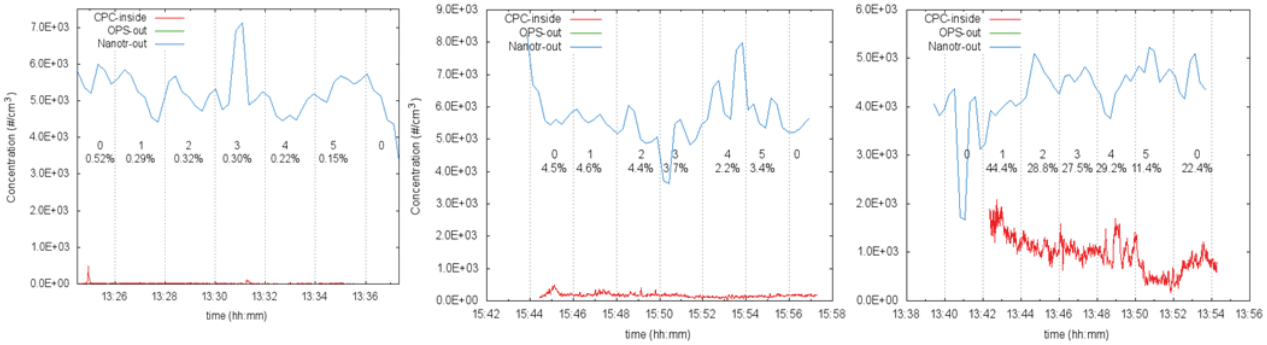


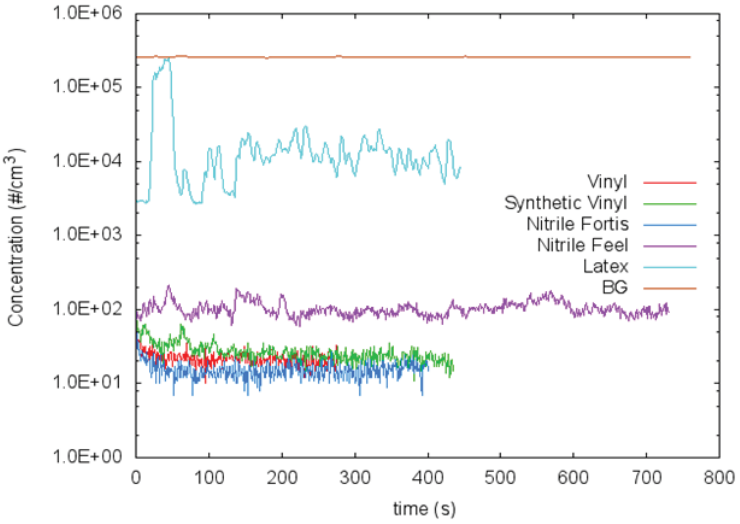
Figure 3: Three representative cases of masks fitting. Left: woman with Full Mask, centre: man with full mask, right: men with grown beard and half mask.

Dermal Protective Equipment (DPE) was tested against penetration of airborne nanoparticles and permeation of liquid dispersions with nanoparticles embed. In both cases, laboratory suits and protective gloves were tested.

In the case of resistance against penetration of airborne nanoparticles, the device shown in Fig. 4 is used. A circular sample of a glove material is placed in-between the path of a flow of nanoparticles and concentrations are measured at both sides of the glove and then compared.

Figure 4: Set up for gloves testing the penetration of airborne nanoparticles.

Concentrations of nanoparticles of NaCl with Dpg = 35 nm were measured for different glove materials, resulting the penetrations shown in Fig. 5.



Penetration (%)	
Vinyl	0,103
Latex	8,546
Nitrile Feel	0,040
Nitrile Fortis	0,006
Vinyl Synthetic	0,013

Figure 5: Concentration inside and outside the glove material.

In the case of resistance against penetration of nanoparticles of suits, tests can be performed with subjects when the aerosolized material is NaCl, to test the suits in movement, or with a mannequin, as shown in Fig. 6. Three points at the suit are selected to measure the concentration inside the suit, which is then compared with the concentration outside the suit.

A sheath flow of clean dry air has to be supplied inside the suit at the same flow rate as the measuring devices, which are suctioning in order to no create depression or a false result.

Figure 6: Tests of penetration of airborne nanoparticles in suits with a mannequin

For a typical Tyvek hooded cover suit, penetration concentrations are shown in Fig. 7. Particles, which lead to concentrations of 20,000 #/cm3 of NaCl of 35 nm of DpG, were nebulized at the height of the head of the mannequin. Penetration of airborne particles trough the suit decreased progressively with the height at which was measured, ranging from 6% at the knee up to 48% near the chest. Internal leaks are probably due to the seams and gaps between suit and skin.

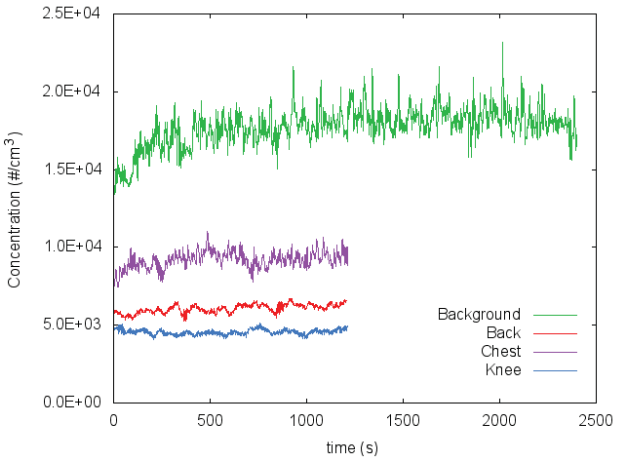


Figure 7: Penetration through theTyvek suit of airborne NaCl particles

The LEVs' efficiency is tested in real case studies, where a portable captor hood is driven to an actual working environment in which MNMs are handled or manufactured. In these cases, no previous ventilation system has been installed, thus the release of MNMs before and after the installation of the captor hood is compared (see Fig. 8).

Figure 8: Portable captor hood placed during the transfer of NM.

In Fig. 9 the decrease of concentration of MNMs released when a capturing system is placed near the source is shown for two examples. Graphene were the MNMs measured in this case, in the form of few layers for one case (left) and as nanoplatelets for the other (right).

The measuring devices were CPCs. The process of weighting the MNM and bagging were studied. Graphene is a highly volatile material, which spreads all along the room and which is difficult to capture by the aerosol devices since it may have only one dimension on the nanoscale, thus it has to coincide that precisely such dimension is detected by the measuring device.

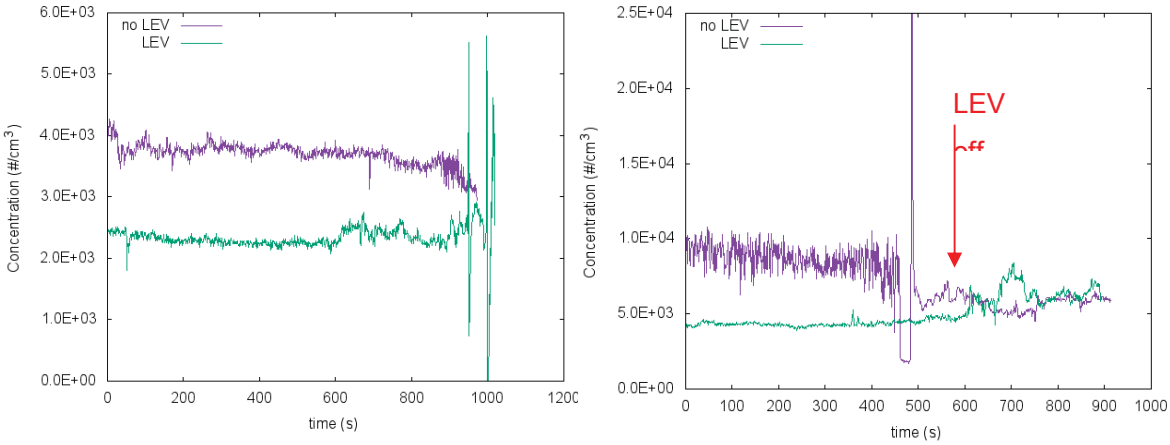


Fig. 9: Concentration measured during weight and bag of graphene in few layers (left) and nanoplatelets (right).

Simply with the use of a ventilation system, the release of nanoparticles can be reduced up to a 46% (Figure 9, left), or 36% (Figure 9, right). It can also be seen with the nanoplatelets that the concentration rapidly reaches the background levels when the exhaust system is switched off.

However, suction flow rates and locations of the LEVs must be carefully chosen, since a too high flow or a wrong position of the hood might cause the contrary effect, spreading the MNMs through the worker's exposure area. This occurred in Fig. 10, where the capture hood was placed at around 25 cm of the source and at 53 lpm.

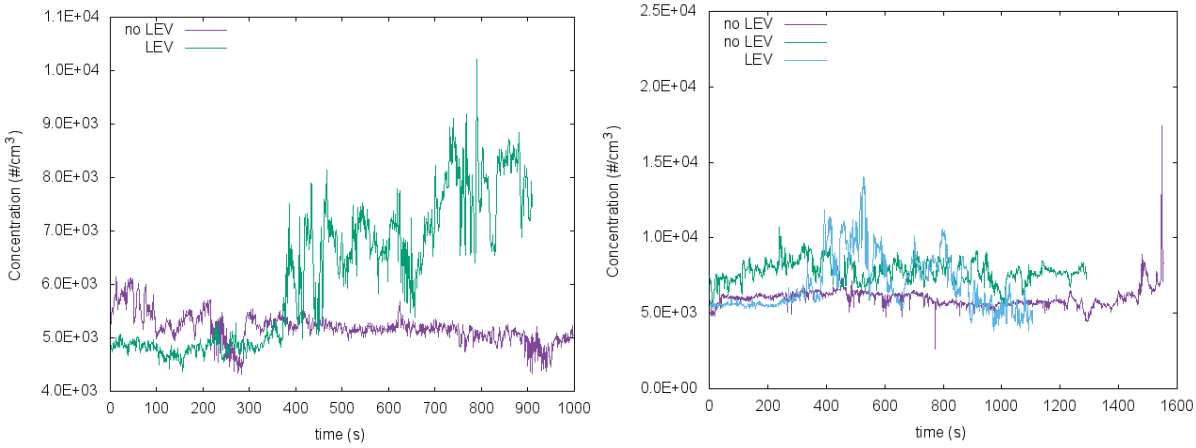


Figure 10: Concentration measured during weight and bag of graphene in few layers (left) and nanoplatelets (right) with LEV too close and too high suctioning flowrate.

It can be seen that the high volatility of the MNMs and the turbulence provoked cause that the MNMs are diffused and spread in a higher rate through the room than without any ventilation system. Thus, careful properties of the LEV must be set up for a proper protection against exposure.

15.2. Overall assessment / conclusions

- Valuable results for the regulator are the SOPs for the efficiency testing of individual RMMs.
- Deliverable D3.9 is now a very valuable source of information on reviewed RMMs and PPE for regulators and industry, and a notablee output of NANoREG.
- It includes for instance decision flow charts for respiratory protective equipment, gloves and protective clothing.
- RMMs for consumers and the environments as well as the improvement of control banding tools have to be developed or evaluated by other research programmes than NANoREG.

15.3. Any other relevant issues?

-oOo-

I. SVCCS1

NANoREG SVCCS number 1
GALANT – Glass surface coAting to reduce Leaching using NanoTiO$_2$

T1.3 – Information-gathering master document

***SVCCS* theme**	The aim of the SVCCS is to analyze possible leaching of titanium dioxide (TiO_2)-nanoparticles out of nano-coatings on the inner surface of glass containers that are used for storage of pharmaceutical solutions.
Regulatory Importance urgency and context	The project will clarify the stability of nanoTiO_2 coating of glass containers and whether this specific coating should be investigated regarding a possible effect on health and environment. Furthermore a more complete picture of a value chain will be produced, and the methods for SVCCS developed and refined.
Keywords	TiO_2 leakage, coating process, life cycle
Owner(s)	Lead Contact Person(s): Mats-Olof Mattsson, Andy Booth, Andreas Falk

I.1. NANoREG (elements of) answer to the question

Mapping of the value chain
Characterisation of TiO2-suspension
Analysis of TiO2 leaching
Analysis and compilation of the data and overall SVCCS analysis including needs for risk assessment

I.2. Impact / Implications for the stakeholders

Safety value chain case studies (SVCCS) are established to support and test the development of answers to the regulatory issues/questions. These case studies range from testing proposed risk reduction strategies to more detailed aspects of a risk/safety assessment. Depending on the available information and relevance, case studies either consider the entire value chain, from R&D and design over production/manufacturing, to use and disposal/recycling, or focus on specific parts of the value chain (e.g. GALANT).
The GALANT case study includes mapping of the value chain, experimental work on characterization and measurements, and overall SVCCS analysis and risk assessment.

This project is funded by the EU Framework 7 Programme, contract no 310584.

I.3. Overall assessment / conclusions

Results

- Big difference between 'denatured' and 'absolute' ethanol
  - 'denatured' more stable as a coating
- Significant difference between shaking and no shaking
- Shaking: higher Ti concentrations for 'denatured' ethanol
- Shaking: lower Ti concentrations for 'absolute' ethanol
- Most significant is the washing
- High dissociation of Ti from the bottle coating in 'absolute' ethanol
- No difference between washed and unwashed samples with 'denatured' ethanol
- In all samples, bulk of measurable Ti is in the particulate form.
  - 'Dissolved' Ti values may contain contribution from nano-sized TiO2

Conclusion:

There are significant differences in TiO2 leaching by using different approaches during the VC.

I.4. Any other relevant issues?

-oOo-