A common European approach to the regulatory testing of nanomaterials

NANoREG, a common European approach to the regulatory testing of nanomaterials

Final Report (part 1)

Grant Agreement number:	NMP-LA-2013-310584
Project acronym:	NANoREG
Project title:	"NANoREG, a common European approach to the regulatory testing of nanomaterials"
Funding Scheme:	
Date of latest version of Annex I:	November 2016 / updated 2017/02/21
Period covered:	1 March 2013 – 28 February 2017
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Version 20170518

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1 Introduction and Reading Guide

Aim of the NANoREG Final Report

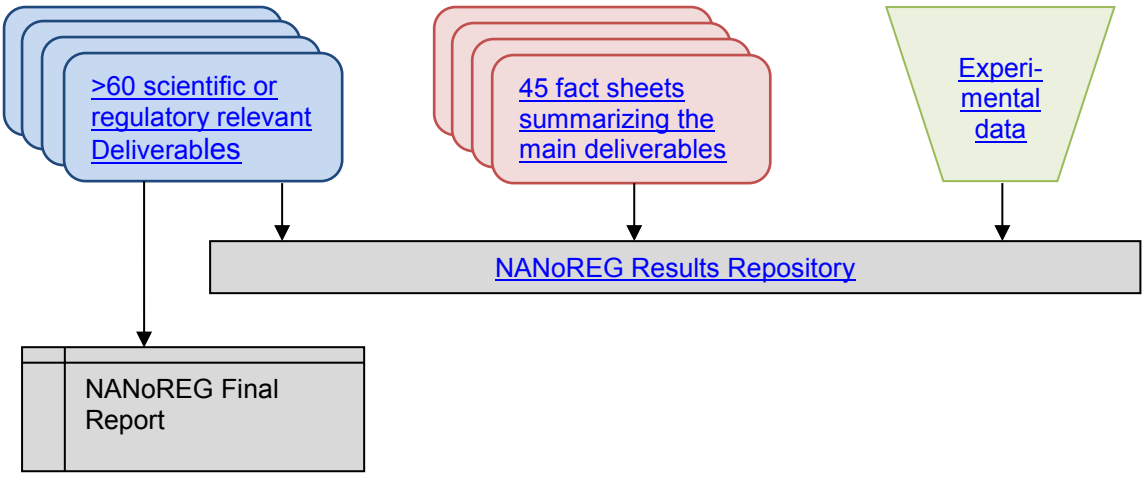
The NANoREG Progress Report informs interested parties on the results of the NANoREG project. At the same time it justifies the financial contribution to the NANoREG project by the EC and the involved member states. To this end we link the activities to the resources used and evaluate the implementation of the Description of Work. Finally yet importantly, we give an overview of our experiences regarding topics that also are of relevance for other FP7 and H2020 projects, such as hurdles for collaboration and data logging.

The two parts of this report

The NANoREG Final Report is split in two parts. Part 1 is a public document. It gives a complete overview of the scientific and regulatory relevant results of the project. The Final Report Part 2 reports on project management and dissemination including the use of resources. This part is for internal use only.

As probably one of the first –or possibly the first- FP7/H2020 projects, all NANoREG results – deliverables as well as experimental data- are publicly available as of the end of the project. So if you want more detailed information on topics reported in part 1 of the report, you can turn to the NANoREG Results Repository.

The graphic below gives an impression of the main elements of the NANoREG reporting structure.



Reading Guide and additional information

Chapter 2 of the Progress Report is a management summary. It sketches the implementation of the project and the achievements so far in rough strokes. Links are made to the more elaborated information in Chapter 3. This chapter “Project objectives, progress and achievements and project management” is the core of the report. It describes the activities, results and impact in more detail.

To keep the size of the report within reasonable limits, the Final Report refers to documents available in the NANoREG Results Repository by means of a hyperlink. By clicking on the hyperlink, the document referred to, is directly available.

2 Abstract

2.1 Project context and objectives

Nanotechnology is one of the six “Key Enabling Technologies” (KETs), the European Commission identified in its 2012 Communication on this topic. These technologies enable the development of new goods and services and the restructuring of industrial processes needed to modernize EU industry. They are of paramount importance for the transition to a knowledge-based and low carbon resource-efficient economy. KETs are regarded as crucial for ensuring the competitiveness of European industries in the knowledge economy.

A serious threat to the capitalization of the innovative and economic potential of Nanotechnology is the limited understanding of the environmental, health and safety (EHS) aspects of nanomaterials (NMs); often labelled nanoEHS. This limited understanding leads to uncertainty on how to judge the EHS aspects of these materials in a regulatory context. This has a negative impact on the investment climate and on societal appreciation of products containing NMs.

The NANoREG project aims to eliminate part of these uncertainties by:

1. Identifying what nanoEHS aspects are relevant from a regulatory point of view (“questions and needs of regulators”).
2. Identifying what the gaps in our knowledge are: what aspects are sufficiently covered by existing knowledge; what aspects need further research.
3. Carrying out the research to *fill in* the gaps.
4. Developing a NANoREG framework and toolbox for testing the EHS aspects and for the assessment and management of the risks. This includes proposed forward looking strategies such as safe-by-design to prioritize those nanomaterial applications that may lead to high exposure or high toxic potential and ultimately high risks for human health.
5. Creating support for the results of the project in order to contribute to a quick and broad implementation of the results.

2.2 Work performed and results

This section briefly summarizes the results of the project in terms of “products and findings”. For most topics, you will find a link to the more extensive section in the core of the report (chapter 3). In these sections you will also find hyperlinks to relevant documents such as deliverables, Standard Operation Procedures (SOPs), data sets, etc.

2.2.1 General overview

The R&D results of the project are impressive in terms of quantity and quality. Partners produced a large set of well-defined experimental nanoEHS data. This dataset is of great value in- and outside the project since the data on exposure and effects are linked to accurate physical-chemical data. These are required for meaningful QSAR approaches and in silico correlational studies regarding the toxicological mode of action (MoA) of nanomaterials.

Numerous Standard Operation Protocols (SOPs) have been developed and tested regarding their reliability, reproducibility and relevance. Their status varies from “proof of concept” to “validated” by inter laboratory comparison. New insights have been developed regarding the importance of a standardized way of preparing dispersions, the need to characterize test media before and during experiments, the applicability of *in vitro* tests, the use of high throughput screening (HTS) and the importance of harmonized data reporting formats (ISA-TAB based), just to name a few. The results and knowledge acquired has been condensed in overarching deliverables such as the NANoREG Framework and accompanying NANoREG Toolbox providing, among others, a risk assessment (RA) scheme that supports a more cost-efficient RA of nanomaterials. The results also have been translated into Answers on the Regulatory Questions that formed the demand side of the project.

Also “soft results” have been achieved. The NANoREG consortium has proven that it is feasible to come to a concerted action regarding the materials to be tested, test methods and cell lines to be applied, quality checks, etc. Such concerted action is an absolute must for generating meaningful

data. The project also has proven that the basic willingness of partners to collaborate can be used to come to an agreement (possibly for the first time) to make data and deliverables publically available. This makes it possible for other projects to build on the results of NANoREG. In this context, it can be noted that NanoReg^2 and caLIBRAte will further elaborate on the data generated in the project. It would be a major step forward if other nanosafety projects would copy the example NANoREG has set, with respect to opening up the results. All NANoREG results are available in the NANoREG Results Repository.

Those results are important building blocks for "the White Paper process" of the H2020 project ProSafe. This process aims at developing a White Paper with recommendations for regulators and innovators regarding cost efficient RA of nanomaterials now and in the future. In this context, a ProSafe Task Force of senior experts evaluated a great number of nanosafety projects, including NANoREG, concerning the regulatory relevance of their results and generated data sets. The results of this evaluation are condensed in the "the Joint Document". A draft of this document was discussed during a scientific conference that was organized by ProSafe together with the OECD from 29 November - 1 December 2016. This conference also was the final meeting of the NANoREG project. The final Joint Document together with the NANoREG Framework and Toolbox form the basis for the White Paper.

2.2.2 Basic conditions for the R&D work (WP 1&2):

At the start of the project, an inventory was carried out to identify the main questions and needs of regulatory relevance that should be addressed by NANoREG. The result is a list of 16 questions that formed the "demand side" of the NANoREG project (D1.01). The list was used during the project to keep focus on "doing what is needed". In the final stage of the project, most of the questions were (partly) answered on the basis of the results of the scientific work packages (D1.09). Further, a Gap Analysis of the knowledge needed in the area of regulatory toxicology and RA was made (D1.02).

To be able to combine and compare experimental data, a set of core nanomaterials was selected that all partners had to use. A web ordering system (NIWO) was set up. It provided partners with the core nanomaterials from known selected suppliers (D2.01).

For all NANoREG materials, a state-of-the-art physico-chemical characterization was done with the aim to cover as many of the key endpoints of the OECD WPMN sponsorship program as technically and practically possible (D2.02). Dispersion SOPs and minimum requirements for characterization for toxicological studies were established and laid down in a Guidance Document, thus supporting reliability and comparability of data. It was mandatory for the partners to perform their experiments in accordance with the Guidance Document. During the course of the project, those fundamental requirements were further refined.

A policy for NANoREG data management was established comprising a standardized way of data logging, mandatory uploading of data to the NANoREG data platform and opening up the data at the end of the project. This dataset is now available to- and exploitable by the nanosafety community.

2.2.3 NANoREG R&D work

Synthesis, supplying and characterization (WP2)

To support the implementation of the EC recommended definition of a nanomaterial, a SOP for measuring size distribution of nanomaterials by means of Transmission Electron Microscopy (TEM) was established (D2.10). The procedure for gas-adsorption BET analysis and the t-plot method for data analysis were improved to enable discrimination between external surface and porosity in NMs to feed into an alternative number-based VSSA approach developed to assess whether a powder was to be considered a NM or not. Identification of NMs by BET or VSSA may be applied for monitoring purposes, but the use of VSSA may be of limited use for identification of NM in a regulatory context (D2.11).

To identify and quantify selected types of inorganic- and organic chemical surface modifications, including surface functionalization and coatings, a Technical Guideline with several analytical

SOPs was developed. The Guideline also can be used to screen NMs for the presence of associated impurities (D2.04).

To address the need for a practical system to categorize NMs, a scheme for an advanced categorization was developed. The scheme takes into account REACH naming and identification guidance to fit the existing European chemicals regulation (D2.05).

Ten OECD Test Guidelines were evaluated regarding the applicability for NMs. It resulted in several proposals for modifications of existing Guidelines or proposals for new Test Guidelines (D2.03/2.09), which have been proposed as a starting point for revision of relevant OECD TGs as part of a new project within the OECD WPMN.

A set of protocols for dispersion of NMs for aquatic eco-toxicological testing was developed or approved followed by extensive documentation and validation through interlaboratory comparison (D2.06). To better interpret and compare the results of *in vitro* tests, procedures for accurate quantification of NM exposure concentrations and characteristics, including NM reactivity and dissolution (fate) in ecotoxicity and in vitro exposure media were also developed and demonstrated (D2.08). All these methods form a solid basis for the regulatory (eco)-toxicological testing. One of the methods for assessment of MNM reactivity and dissolution during *in vitro* testing is under development as a CEN technical specification as part of a CEN/TC352 project.

Based on a literature review and experimental work conducted on different aerosol generation devices and NMs, a strategy was defined to characterize test aerosols. The strategy contributes to a better comparability and interpretation of inhalation toxicology results and can be applied for future inhalation toxicology studies (D2.07).

The deliverable “Framework and procedures for characterization of NM for regulatory needs” integrates results of several WP2 deliverables. It comes forward with recommendations for the further harmonization and improvement of the materials identification and registration schemes and guidance in REACH (D2.12).

Exposure through life cycle analysis (WP3)

To identify the most critical exposure scenarios during the life cycle of a product (in terms of potential exposure and economic importance) a model was developed taking into account factors such as production volume, main applications and information on activities with NMs along the life cycle. Exposure scenarios have been rated and ranked (D3.01).

A testing strategy based on mesocosms was developed and applied to better mimic the effects and impact of exposure of ecosystems to nanomaterials at different stages of their life cycle (D3.05).

To quantify the dustiness of NMs, three test methods were evaluated and applied to CNTs. For two of these methods, the work performed within NANoREG contributed to the development of draft standards in the framework of the CEN / TC 137 (assessment of workplace exposure to chemical and biological agents) (D3.02). These drafts now circulate for comments.

Standardized methods were developed and tested to quantify the release of core NMs, for selected processes during their lifecycle (D.3.03). Several improved measurement instruments, tools and methods were developed to make the link between release and exposure (workers, consumers and environment) (D3.06). Some methods were applied during a campaign of field measurements aimed at filling in a part of the gap in exposure data needed for modelling exposure and for further assessing the risks associated with NMs (D3.07).

A series of comprehensively-monitored nanoparticle dispersion experiment were undertaken inside a large climate-controlled chamber (D3.04). The resulting data can be used to test quantitative aerosol dispersion models and enable an assessment of the accuracy and uncertainty of model-predicted concentrations. Such aerosol dispersion models form the foundation for human exposure assessments to MNM.

To cover the knowledge gap on the effectiveness of currently available Risk Management Measures (RMMs) during NMs production and handling processes, a reliable methodology to obtain quantitative data on the effectiveness of personal protective equipment (PPE) and engineering controls (ECs) was provided and validated (D3.09).

Four Control Banding Tools models have been evaluated with respect to their applicability domain, assumptions made, inputs required and outputs as well as performance. For five different tools an inter-user study has been performed. The I-Nano tool developed under the umbrella of NANoREG is described and the demonstrated (D3.08).

Biokinetics and toxicity testing in vivo (WP4)

A long term (two year) inhalation study with female rats was performed to identify effects of two well characterized granular nanomaterials to determine concentration-response relationships and to verify/falsify the assumed mode of threshold-like action for carcinogenicity. The further aim was to investigate lung carcinogenicity and putative systemic effects of low-dose exposures to biopersistent nanoparticles. After 12 months of inhalation exposure, CeO_2 exposure-related histopathological findings were exclusively observed in the respiratory tract but not systemically. Adverse effects in the lung included alveolar/interstitial inflammatory cell infiltration, granulomatous inflammation and interstitial fibrosis. Although statistically not significant, some adverse effects were already observed in the 0.1 mg/m³ low-dose CeO_2 exposure group. After 12 months of inhalation exposure neither neoplastic nor pre-neoplastic treatment-related findings were seen in the lungs of CeO_2-exposed animals. No macroscopically visible tumours were found after 24 and 30 months.

Also in concentrations below overload, the CeO_2 the lung burden increased in a linear manner with a factor of ~5-7 including the accumulation over exposure time. The CeO_2 burden of liver, kidney, spleen, brain, heart, lymph nodes, bone and olfactory bulb was generally very low. In brain, maximum CeO_2 levels were 0.005 µg/g tissue, which is a factor of 700.000 below the lung burden. There was no evidence for systemic toxicity in the interim section after 12 months including the lung-associated lymph nodes although the cerium levels were relatively high in this tissue (D4.01-D4.07).

Ten commercial short, non-rigid, high aspect ratio nanomaterials (HARN, average length < 5 µm) have been tested after deposition of three doses in the lungs. There was no evidence of genotoxic effects in livers and spleens, or acute phase response in plasma. There was no evidence of MWCNT fibrogenicity. Remarkably, nanofibrillated celluloses were rather inflammogenic and persistent in mouse lung. The inflammatory responses in mice and in rats were strongly correlated. Some HARN materials were more inflammogenic and genotoxic than others. A high specific surface area (BET) and a low diameter were identified as a predictor of increased pulmonary inflammation. In addition, length significantly predicted pulmonary inflammation, whereas surface oxidation (-OH and -COOH) was a predictor of lowered inflammation. BET surface area, and therefore diameter size, significantly predicted genotoxicity in bronchia alveolar lavage (BAL) fluid cells and lung tissue.

In inhalation experiments with two pristine MWCNT, “long and thick” NM-401 and “short and thin” NM-403, NM-403 was more inflammogenic than NM-401. Since NM-403 had a 10-fold higher specific surface area than NM-401, these results were in agreement with those obtained by pulmonary instillation. Despite the persistent presence of carbon nanotubes in lung tissues, no significant histopathological changes were observed. The results may be helpful for the development of safer HARN materials (D4.13).

A repeated-dose 90-day oral toxicity study in rat with amorphous silica (NM203) did not find marked and clearly dose-dependent effects after oral doses of maximally 50 mg/kg bw per day (D4.11).

In prenatal toxicity studies with cerium dioxide JRCNM02102a and multi-walled carbon nanotubes (JRCNM04001a) in mice, no overt toxicity in terms of miscarriage or malformations was found (D4.14).

An instillation study was performed in rats using different metallic oxide NMs (TiO_2, CeO_2). All NMs were detected in the tracheobronchial lymph nodes after 35 and 90 days. There was no significant systemic distribution in liver, kidneys and spleen. No marked effects were seen for all tested NMs regarding the production of oxidative stress. Considering overall pro-inflammatory effects, lung inflammation seemed somewhat more pronounced for TiO_2 NM-105, TiO_2 NM-101 and CeO_2 NM-212 than for TiO_2 NM-100. In animals exposed to NM-100 and NM-101, no significant histopatho-

logical changes were observed. Pulmonary instillation of all tested NMs did not induce the formation of micronuclei in blood polychromatic (immature) erythrocytes (D4.15).

PBPK models for inert NPs (polyacrylamide, gold, titanium dioxide) and a PBPK model for inhalation exposure to cerium dioxide NPs (self-generated material) were developed. Further development is needed; especially with respect to regulatory use (D4.17).

Existing OECD and ISO standard methods for ecotoxicity assessment have been adapted specifically for NM testing and developed into defined SOPs. They have been applied on three priority species representing different trophic levels. Silver NPs (JRCNM03000a) showed high toxic potency. A lower toxicity was generally observed in the test systems for MWCNTs (JRCNM04000a, JRCNM04001a, JRCNM04100a) and titanium, cerium, silica and zinc oxide NMs (JRCNM01000a, JRCNM01001a, JRCNM01003a, JRCNM02000a, JRCNM01100a, JRCNM02102a). Concrete guidance to design and conduct eco-toxicity experiments is given in the form of decision trees and hazard potency categories based on specific cut off values (D4.12).

Immunotoxic and genotoxic effects of biopersistent nanofibrillated celluloses differed among four materials studied. Effects were also seen with the bulk-sized cellulose studied. The outcome of the in vivo toxicity tests was not consistently predicted by in vitro toxicity studies performed with the same materials (D4.16).

A summary and evaluation of the results of work package 4 is presented in D4.18

Advancement of Regulatory Risk Assessment and Testing (WP5)

To address the need for more efficient ways to evaluate potential adverse effects of a NM, a system for the grouping and read-across of NMs was developed. It is based on expected biological, ecological and/or toxicological effects (D5.01).

Since solubility is a crucial factor when predicting the effects and risks of NMs, test procedures for application in regulatory testing were investigated. It was concluded that dissolution in a complex matrix is highly challenging. It was not possible to devise one universal, robust and rapid test method for regulatory testing that is applicable for all types of NMs in all types of matrices (D5.02).

The potential internalization and crossing of several NANoREG NMs through different *in vitro* barrier models and their impact on tissue integrity was evaluated. Results indicate that *in vitro* models have currently a limited suitability to allow reliable evaluation of NMs crossing barriers (D5.03).

Several *in vitro* techniques mimicking inhalation exposure were evaluated. Results were linked to the results of *in vivo* tests for the same materials. Classical monolayer culturing produced similar results to cells grown in an air/liquid interface (ALI) model. Results indicate that it is better to use co-culture cellular models, which were shown to be more sensitive under the conditions of the different studies. It was difficult to correlate between methodologies due to the low toxicity of the NM under study (D5.04, D5.05).

The suitability of *in vitro* assays in terms of reliability and predictive value was evaluated on the basis of a great number of experiments and a comparison with the result of *in vivo* experiments with the same materials. Due to very low toxicity of the NMs, it was difficult to correlate results with the *in vivo* situation. However it was found that *in vitro* methodologies were able to rank NMs according to their toxicological outcomes in a fashion similar to the results obtained *in vivo*. For ease of data extrapolation to the *in vivo* situation, doses are better provided as µg/cm^2 (D5.06).

Based on a literature review and own experiments, an overview was made of the high throughput screening methods (HTS) and high content analysis (HCA) that can be applied nowadays. Several methods were standardized and applied for NM testing. They are promising to be used as robust methods for hazard assessment. Results of a preliminary evaluation show that standard test methods and the HTS/HCA approaches give similar results. The analysis of the generated data and the comparison, within and between different HTS/HCA tests as well as with standard *in vitro* assays is ongoing (D5.07).

A strategy was developed to prioritize those nanomaterial applications that may lead to high exposure or high toxic potential and ultimately high risks for human health. These aspects are summarized in six elements, which play a key role in the strategy: exposure potential, dissolution, NM transformation, accumulation, genotoxicity and immunotoxicity. With this approach it is possible to

identify those situations where the use of nano-specific grouping, read-across and (Q)SAR tools is likely to become feasible in the future, and to point towards the generation of the type of data that is needed for scientific justification, which may lead to regulatory acceptance of nano-specific applications of these tools (D5.08).

Keeping pace with innovation (WP6)

A NANoREG Foresight system was developed aimed at monitoring innovation and evaluating the potential adverse impacts of NMs and their likely applications in a time horizon of 5 to 10 years. The Foresight system was applied to Graphene as a first test case (D6.01).

To get a better insight in the causes and remedies for the increasing gap between innovation and risk analysis regarding manufactured NMs, an analysis has been made of the social and technical issues currently inhibiting robust safety assessment of NMs and the key bottlenecks inhibiting the ability of researchers to deliver answers to regulatory questions (D6.02).

Taking into account lessons learnt from drug development testing, a NANoREG approach for Safe by Design has been elaborated building on the innovation Stage Gate Model. The concept of “risk potentials for nanomaterials” introduced in this deliverable, has been elaborated regarding relevance and availability of test methods for their applicability by innovators and for their relevance for later stages facing a regulatory context (D6.03, D6.04).

A data structure was developed as a basis for a database encompassing relevant physico-chemical characteristics in relation to toxicity endpoints. To that aim the database was filled with literature data screened for relevancy and quality (D6.05).

A literature review was performed aimed at identifying key physicochemical parameters of NMs that may influence the functionality in terms of cell uptake, optical properties, electronic properties and catalytic activity/biorecognition to physicochemical parameters (D6.06).

2.2.4 Integrating results (WP1)

Deliverable D1.09 “*NANoREG final report with (elements of) answers to selected issues/questions*” integrates the results of the R&D work packages. Several conclusions and elements of answers have been directly or indirectly produced by this FP7 project, as can be seen by reading through the "*Summary Of The Findings And Elements Of Answers*" (section A.3 of D1.09). In several cases, procedures (SOPs) to tackle part of an issue have been identified, developed and published, though for some SOPs the verification/validation process ('testing the tests') requires more time and resources than what the project had to offer. D1.09 findings fed directly into the development of the NANoREG Framework for the safety assessment of nanomaterials (D1.11) and the related NANoREG Toolbox (D1.12).

The NANoREG Framework provides a detailed overview of how the safety of NMs should be addressed / assessed in the context of the European REACH Regulation (Part I of the document). It also presents forward-looking strategies aiming at making safety assessment more practical and economically efficient (Part II) (D1.11). Its self-standing annex I – report on a harmonised terminology – is a rare effort in the nanoEHS community, which is attracting interest from parties in Europe and beyond.

The NANoREG Toolbox (D1.12) supports the implementation of the NANoREG Framework by providing an overview of test methods, datasets, models etc., applicable in a regulatory context. Just like the Framework, it will be a building block for the ProSafe White Paper mentioned above.

2.2.5 Expected results and their impact

The progress report describes the impact on different levels. This paragraph highlights the impact on a general level. The results and their impact on work package level are described in the “Results and impact table” on top of the section for each work package (section 3.1). Part 2 of the Final Report gives an evaluation and update of the Impact chapter of the NANoREG DoW.

Impact on general level

√ *Relevance and quality of data in a regulatory context*

Working in a regulatory context is, when it comes to science, no "business as usual". It means that reliability, comparability, exchangeability and relevance of nanoEHS data are crucial for the usefulness of data. Contributing to the awareness regarding this statement is probably one of the main "soft" impacts of the NANoREG project. Almost all partners have been confronted with and discussed the necessity of using the same well-characterized materials to make it possible to combine their results with the results of other partners and projects. The same applies for the mandatory use of selected SOPs, the use of benchmark data and the testing with a limited number of agreed cell lines. It is a soft impact that actually goes well beyond the NANoREG project and that -nowadays- gets more and more attention in the EU NanoSafety Cluster (NSC) and at global level (e.g. in the US and in the US-EU Communities of Research (CoR)).

√ *Accessibility of results*

In the same category as "data quality", the awareness regarding the accessibility of the information generated under the umbrella of the project should be mentioned as an impact of the NANoREG project. Important in this context was the decision by the NANoREG General Assembly in June 2016 to open up all deliverables and experimental nanoEHS data at the end of the project. The decision was based on the conviction that open access to results of nanosafety projects is key to the effectiveness and efficiency of nanosafety research. It is the only way to build on the results of previous projects.

To operationalize this decision, formal and practical hurdles had to be taken such as an amendment of the NANoREG Consortium Agreement, the development of data logging templates and data entry tool and creating a NANoREG Results Repository (including a database for the experimental data).

By making the NANoREG legacy available outside the project, an example has been set that hopefully will inspire other projects in the NSC and will lead to measures by EC and member states to make the opening up of project results common practice. Recommendations to this end are listed in section 4.1.

√ *Collaboration*

The NANoREG project has strongly contributed to the (science- and policy-oriented) dialogue regarding nanosafety at EU level and beyond. The National Coordinators have played -and will play- an important role in this field; acting as link between science, industry, regulators and funding agencies. The established collaboration with the Czech Republic, Greece, South Korea and Brazil also contributed to this dialogue. The start of the ProSafe project early 2015 gave an additional boost to collaboration in- and outside the EU.

√ *Impact of specific activities*

The Result-Impact tables in section 3.2 summarize the impact of specific products and activities for each work package. Part 2 of the NANoREG Final Report will mirror expectations regarding impact to the actual situation on the basis of the impact chapter of the NANoREG Description of Work (DoW).

√ *Impact via White Paper process*

The ProSafe White Paper Process (2.2.1) is crucial for achieving impact of the NANoREG project, since it will integrate main NANoREG outcome (Framework and Toolbox) and the ProSafe Joint Document into the Policy Recommendations of the White Paper. The Final NANoREG meeting/Scientific Conference held at the end of 2016 in Paris contributed to the credibility of this document. The consultation procedure for the draft White Paper and the workshop with policymakers and innovators foreseen for autumn 2017 will also contribute to the support for the recommendations.

3 Project achievements and management

3.1 Progress and achievements

This section gives an overview of the results of each WP. Information on the use of resources for each Work Package (in terms of personnel effort) will be reported in the Final Report part 2.

3.1.1 Work package 1

Results	*Impact*
Answers to questions of regulatory relevance	*NANoREG provided elements of answers to regulatory relevant questions defined by regulators and policy makers. Noteworthy advances were made in answering questions in PPC, dose metrics, kinetics and risk management.*
NANoREG Framework for the safety assessment of nanomaterials (D1.11) and NANoREG Harmonised Terminology	*Supports industry and regulators by providing guidance on the implementation of the REACH Regulation for NMs.* *Forward-looking strategies (nano-specific RA, SbD and LCA) are promising options for policy recommendations in the ProSafe White Paper.* *A unique review of key nanoEHS terminology at global level.*
NANoREG Toolbox (D1.12)	*SOPs, guidance documents, IT-based tools, datasets, etc. from NANoREG and much beyond categorized into a Toolbox to support regulatory testing.*
NANoREG templates for data logging	*First concrete step to implement a harmonized and user-friendly experimental data logging system for the nanoEHS community. Strong interest from the US counterparts has been expressed.*
nanoEHS data stored in standardized way and publicly available (D1.04)	*The huge set of high-quality nanoEHS data for well-characterized NMs is a good basis for further evaluation of the risks of NMs. Relevant for SbD. Strong collaborative work with eNanoMapper.* *Contributes to EU-US collaboration in the field of data management.*

Task 1.1: Refinement of problem identification and formulation of questions and requirements, including interaction with stakeholders (D1.01)

At the start of the project, a wide consultation of stakeholders was carried out to identify the main questions and needs of regulators that should be addressed by the project. This resulted in a list of 16 questions that formed the “demand side” of NANoREG. The list was used during the project to keep R&D work focused: “are we doing what is needed?” D1.09 provides the elements of answers to a substantial part of the questions.

Resulting documents		Input for
Deliverable 1.01: Virtual Workshop to identify, formulate and prioritize issues/questions		Demand side of the project
Factsheet 1.01		

Task 1.2: Gap analysis (D1.02)

A Gap analysis of the knowledge that is needed in the area of regulatory toxicology and RA made clear that most regulatory questions relate to the following three general knowledge gaps:

a) Characteristics that influence the risk of NMs in the environment and humans,
b) Standardized methods to determine these characteristics, and
c) Nano-specific RA strategies and approaches.

Regarding short-term research needs it was concluded that more insight is needed into implications of the implementation of the EC definition within the regulatory frameworks. Furthermore, there is a need to develop methods to test or predict the extent and rates of transformation of NMs by incineration, chemical reactions and other processes.
The needs for long-term research mainly concern:

- Further standardization and validation of methods for identification, quantification, characterization and transformation of NMs,
- Further identification and verification of the key characteristics that influence the release, exposure, behaviour (fate and kinetics), effects (hazards) and subsequent risks,
- Further development and verification of nano-specific RA approaches or strategies, including extrapolation, interpolation, read across, grouping and approaches for safe design,
- Implementation of these nano-specific RA strategies and approaches within regulatory frameworks.

There is also a lack in systematic sets of high quality data of well-characterized NMs on exposure, kinetics and toxicity to further develop, verify and validate nano-specific methods and approaches. Until then, the implementation of nano-specific RA strategies and approaches within the regulatory frameworks strongly depends on the willingness to accept a substantial amount of uncertainty in which the use of decision strategies and/or risk governance approaches seems essential.

Resulting documents		Input for
Deliverable 1.02: Results of GAP analysis		R&D WPs

Task 1.3: Interaction with WPs 2-6 on the scientific answers to the regulatory issues / questions (D1.09).

This task is a key outcome of NANoREG and was aimed at answering the prioritized questions and issues from task 1.1 (D1.01) and the gaps identified in task 1.2.

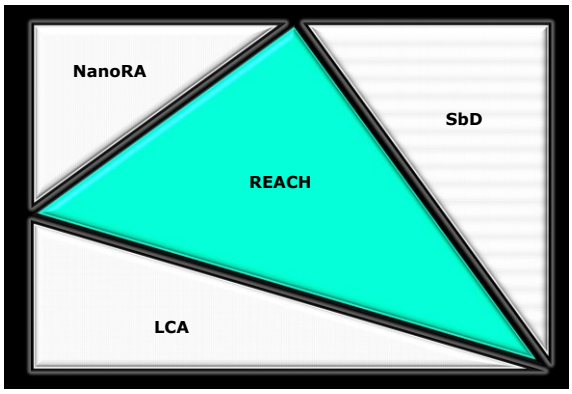
The NANoREG partners working on R&D aspects have contributed during the project lifetime to answering, at least partly, 15 of the 16 complex questions of regulatory nature identified during the first year of NANoREG. The consensually unaddressed one is no.16 on Health Surveillance.

The elements of answers collected and compiled by NANoREG T1.3 have been discussed with the National Advisors and Coordinators of several countries that co-funded this large initiative (Den Haag, 11-12 October 2016). Interesting conclusions have been put forward by NANoREG and appreciated by those stakeholders, among others in the areas of physicochemical characterization, dose metrics, kinetics and risk management measures.

Resulting documents		Input for
Deliverable 1.09: Final report with answers to selected issue/questions		ProSafe White Paper Process

Task 1.4: Framework development

The NANoREG Framework for the safety assessment of NMs is another major project output. The document has absorbed as far as possible the answers to the regulatory questions.



The four components of the NANoREG Framework, with REACH (green triangle) in Part I at its core. The forward-looking strategies are described in Part II: NanoRA = Nano-specific Risk Assessment; SbD = Safe-by-Design; LCA = Life Cycle Assessment

Split into two parts, it first provides a detailed over-

view of how the safety of NMs can be assessed in the context of the European REACH Regulation (Part I of the document). It then proposes in Part II three forward-looking strategies aiming at making safety assessment more practical and economically efficient (see figure).

Being a rather complete document covering physicochemical characterization, *in vivo* and *in vitro* aspects, RA and RMMs, and forward-looking strategies, it is rather large (210+ pages). Readers will find summarized information in the Executive Summary and, importantly, in section 7 *'Take-home messages and final considerations'*.

This document is expected to be useful for all stakeholders in the nanoEHS arena, in Europe and beyond and will be one of the cornerstones of the ProSafe White Paper; a policy-oriented document with recommendations regarding the assessment of NMs in a regulatory context.

Its self-standing annex I – report on a harmonised terminology – is a rare effort in the nanoEHS community, which is attracting interest from parties in Europe and beyond.

Task 1.7: NANoREG Instruments Toolbox for regulators and legislators.

The NANoREG Toolbox (D1.12) is another major project output that supports the implementation of the Framework by providing an overview of test methods, datasets, models, guidance documents, decision trees, etc., useful to regulators and other stakeholders. The tools are not only limited to NANoREG results, but are also collected and categorized from many other initiatives, at EU level, in European states and beyond. The tools are catalogued in relation to each section of the Framework document. This way, the user of the toolbox, or the reader of the Framework, can easily see the link between available tools and nano-specific hurdles to be addressed and reported in the various sections of the Framework.

Just like the other deliverables, the Toolbox is publicly available and is a building block for the H2020 ProSafe White Paper.

Resulting documents		Input for
Deliverable 1.11: Definitive Framework / Published Science for Policy Report		ProSafe White Paper
Deliverable 1.12: Toolbox		
Deliverable 1.12: NANoREG-ToolBox.xlsx		
Deliverable 1.12: NANoREG-Prospective tools.xlsx		
NANoREG harmonised terminology for environmental health and safety assessment of nanomaterials		
Factsheet D1.11 and D1.12		

Task 1.5: Data platform and data management

NANoREG produced a large set of well-defined and reliable data. To be able to fully exploit these data in- and outside the project, much effort has been made on data management. Also thanks to the fruitful collaboration with the FP7 project eNanoMapper and the willingness of all partners to open up their data at the end of the project, the following has been achieved:

- Partners uploaded all their nanoEHS data in accordance with the 'ISA-TAB inspired' templates to the NANoREG-TNO database or to CIRCABC.
- The templates for uploading data of assays in a standardized way have been initially released publicly in April 2017. The NANoREG templates set (January 2017) is now available as an open access JRC publication, which ensures long-term public accessibility and correct referencing to this NANoREG project by those who wish to exploit them and build newer, improved templates.
- (Almost) all of the NANoREG nanoEHS data have been transferred to the NANoREG - eNanoMapper database instance. They are available and exploitable for partners and other parties. The data base (and in future other databases) can be searched via this link
- By decision of the NANoREG General Assembly, all data are publicly available as of 1 March 2017 (end of NANoREG), thus setting an example and stimulating the NanoSafety community to do the same.

Endpoint - Assay	Number of entries
Characterization	**3012**
In vitro	**2682**
In vivo	**489**
Grand Total	**6183**
Number of data points	*23372*

Number of entries to the NANoREG Data Entry Tool. Entry defined as one value or an endpoint for one material and/or a series of values for different doses for one material and endpoint

Aside of the data collection and storage strategy outlined above, T1.5 has also implemented other key components of the critical project data management system:

- A content management system (CMS) to exchange all sorts of electronic files within the project. An Interest Group was created early on (2013) in CIRCABC by JRC and the management of access rights and security policies was setup by TEMAS before being transferred to the Project Office (2014).
- NIWO, the NM web ordering system, was conceived initially and designed in collaboration with T2.1 (NRCWE), and then fully implemented by NRCWE in T2.1.

Resulting documents		Input for
Deliverable 1.04: NANoREG data platform operational		Project and Nanosafety community
Templates for standardized reporting experimental data		
NANoREG – eNanoMapper database		Other nanosafety projects

Task 1.6: Working groups addressing Value Chain Case Studies (VCCS) and other R&D related activities.

The aim of the Value Chain Studies as defined in the DoW was to support and test the development of answers to the regulatory issues/questions ranging from testing proposed risk reduction strategies to more detailed aspects of a risk/safety assessment. Depending on the available information and relevance, case studies were to consider the entire value chain, from R&D and design over production/manufacturing, to use and disposal/recycling.

Due to several reasons, the original aim could not completely be achieved. It proved to be difficult to involve industry in this task. Furthermore, it was not possible to test and apply (draft) results of the NANoREG project due to their late availability. Also, it turned out to be quite difficult to address the whole value chain of a NM case at hand. The small Safety Value Chain Case Studies (SVCCS) projects focused on selected rings of a chain. However, several relevant results were achieved.

Deliverable 1.06 provides an analysis of the value chain concept, refocused on safety. It describes how SVCCSs are defined and performed within the framework of NANoREG. Furthermore, it presents examples of SVCCSs and an overview of the conclusions that can be drawn from those. D1.06 forms the basis for the SVCCS reported in deliverables D1.07 and D1.08:

- *A case study on possible leaching from TiO_2 coating of glass surfaces (acronym GALANT).* Results and conclusions from this study reported in this deliverable are compatible with expectations. Importantly, the obtained results were useful for the involved industrial partner.
- *A specific SVCCS focusing on carbon-based (CNT and graphene) NMs in the waste stream of electronic goods (mainly involving the handling of shredded battery material).* The results of the experimental work indicated that shredding, (downstream) and incineration of electronic goods poses a risk for release of NMs. Appropriate risk management measures need to be in place to prevent exposure to these NMs.
- A third SVCCS focusing on the use of nano-TiO2 from liquid suspensions applied to ceramic honeycomb components of air treatment equipment. Exposure scenarios have been assessed applying REACH and the outcome has proved beneficial both for the industrial partner and for the NANoREG partner.

The work performed has shown that the NANoREG approach to SVCCS is feasible, and that useful outcomes from the industry perspective can be obtained.

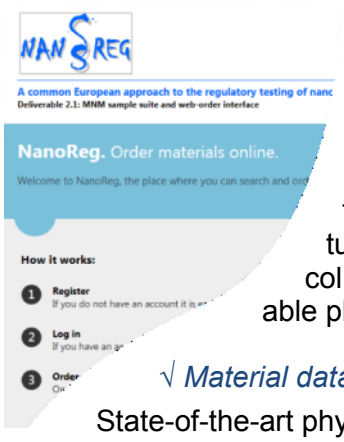
Resulting documents		Input for
Deliverable 1.06: NANoREG Assessment of value chain case studies		
Deliverable 1.07: Case studies of the different investigated materials and the different proposed answers		
Deliverable 1.08: Case study summary report including feasibility check of the proposed answers		

3.1.2 Work package 2

Results	*Impact*
Web ordering system with state-of-the-art Technical Datasheets from NANoREG	*All partners use the same well-characterized NMs thus fulfilling a basic condition for comparison and linking experimental data*
Protocol for determination of the minimum particle size distribution of nano-objects	*Essential condition for applying the EC definition for a NM*
Protocol for determination of porosity and outer volume-specific surface area of NM powders	*Key method for using VSSA as a NM monitoring method in industry and NM categorization for regulatory use*
Protocols for identification and quantification of surface-chemical modifications of NM	*Essential for identification of chemical modifications that may alter the toxicity and fate of NM and essential for proper categorization of NM in a regulatory context*
Scheme and nomenclature for proper material-specific categorization of NMs	*The scheme provides a generic regulatory categorization principle for NMs and their chemical derivatives of simple to complex structures. The associated proposed nomenclature provides a robust system for naming of NM that provides a minimum set of descriptors that enables one to understand the chemical and structural composition of a NM.*
Evaluation OECD Technical Guidelines and recommendations for modifications	*Basis for "formal" modification of OECD Technical Guidelines to make them applicable for NMs*
Protocols for preparing dispersions	*Basic condition to generate reliable and comparable data in both physico-chemical characterization and toxicological testing.*
Protocols for characterization and determination of particle fate of NMs in test media	*Provides essential information for interpreting experimental data of in vitro- and ecotoxicity testing and forms the basis for new characterization guidelines*
Assessment of particle generation methods for in vivo inhalation exposure	*The results provide evidence that careful considerations must be made to select the proper aerosolization method to produce relevant dusts for in vivo inhalation exposure studies.*
Framework for NM categorization for regulatory use	*A proposal for methods-supported framework and guidance for the identification and NM-specific categorization of NM*

Task 2.1: NM synthesis and procurement

√ *NANoREG core materials (D2.1)*



To be able to combine and compare experimental data, a set of core NMs was selected that all partners should use for the experimentation. A web ordering system was set up that enabled partners ordering of both NANoREG core and supplemental NM.

Eleven CNTs and one graphene material were added to the JRC Nanomaterials Repository. For use in WP2 and WP5 fluorescent and non-fluorescent silica nanoparticles where produced with the following features: 1) stable (in different media and under the NANOGENOTOX protocol conditions), monodisperse, 2) biocompatible, endotoxin free, with tuneable phys-chem properties (size, surface charge).

√ *Material data sheet (D2.02)*

State-of-the-art physico-chemical characterization was done for all NANoREG NM considering key characterization end-points in the OECD WPMN sponsorship programme. These data make it possible to link experimental data on the hazard of materials to their phys-chem properties. State-of-the-Art Technical Data Sheets were developed for all NANoREG core NMs and made available via the NIWO system. These TDS form the basis for development of future TDS considering all data in the final NANoREG data-base.

Resulting documents		Input for
Deliverable 2.01: NM benchmark sample suite. Establishment of primary NM sample suite and web-based resource for sample order (NIWO)		Project as a whole
Deliverable 2.02: Material data sheet on primary NM suite up-linked with internet resource		Project and Nanosafety community

Task 2.2: Identification of NM according to the EC regulatory definition

√ *Size distribution by TEM (D2.10)*

To support the implementation of the EC-definition of a nanomaterial, a SOP for measuring the number based size distribution of the minimal external dimension of nano-objects by means of Transmission Electron Microscopy (TEM) was established. The SOPs were validated on a series of near-spherical, granular and aggregated NMs, by successful implementation of the SOPs in nine laboratories and performing intra-laboratory and inter-laboratory comparisons to describe the uncertainty of the method.

The intra-laboratory validation demonstrated that the TEM size measurements were accurate considering uncertainties of 23 size and shape measurands of colloidal near-spherical (8.9-202 nm) as well as granular and fractal-like NMN aggregates.

Based on the work, the SOPs are ready for adoption into guidance documents for identification of near-spherical and agglomerated non-spherical NMs in various regulatory fields.

Resulting documents/data		Input for
Deliverable 2.10: Protocol(s) for size-distribution analysis of primary NNM particles in air, powders, and liquids for compliance with the EU definition		
Factsheet 2.10		
NANoREG D2.10 SOP 01 Preparation of EM-grids containing a representative sample of a dispersed NM		Toolbox
NANoREG D2.10 SOP 02 Transmission electron microscopic imaging of nanomaterials		
NANoREG D2.10 SOP 03 Qualitative description of a NM based on TEM micrographs		
NANoREG D2.10 SOP 04 Electron microscopic image analysis of nanomaterials		
NANoREG D2.10 SOP 05 Electron microscopic image analysis of primary particles in aggregated nanomaterials		

√ *Size distribution by VSSA (D2.11)*

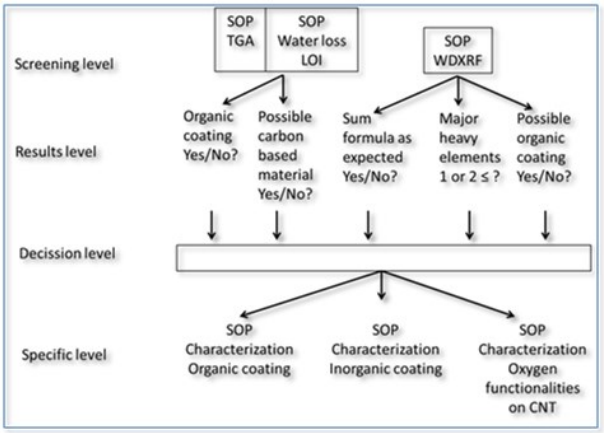
A procedure for analysing the BET gas-adsorption results by means of a T-plot was modified to discriminate between the specific surface area ascribed to porosity and the "outer" particle surface area. This procedure is developed as an alternative approach for identification of NM by electron microscopy information on size-distributions and morphology of the nano-objects. Combined with microscopic information on general morphology and modality, a correction factor is proposed as a solution to correct the conventional VSSA into a number-based VSSA (nVSSA), which could allow better identification of NM than VSSA alone.

Application of the nVSSA approach on a range of nano- and non-NMs demonstrates that the nVSSA approach may be an instrument for identification of near-monomodal and uncoated NM. The procedure differs from the VSSA proposed by SCENIHR and the EC-recommendation for VSSA as a supporting procedure.

Resulting documents		Input for
Deliverable 2.11: Protocol(s) for VSSA analysis of primary NM in air, powders, and liquids		
SOP for BET and t-plot analysis (annex to Deliverable)		Toolbox

√ *Quantitative analysis of organic and inorganic NM surface coatings (D2.04)*

To identify and quantify the presence of inorganic and organic surface-chemical functionalization and coatings, a Technical Guideline with several analytical SOPs was developed. The Guideline also can be used to screen NM for the presence of associated impurities.



The Technical Guideline consists of an initial screening procedure using relatively simple methods to first identify NM, which may have been chemically modified by doping and/or surface coating and/or functionalization, which is then followed by more advanced specific analysis using different proposed SOPs depending on the type(s) of possible modifications observed. The main focus, however, has been set on methods suitable for assessment of surface-chemical modifications.

The guideline and SOPs are intended as a future work item for the OECD Working Party on NM.

Resulting documents		Input for
Deliverable 2.04: Protocol for quantitative analysis of organic NM surface coatings including)		OECD WPNM
Fact sheet 2.04		
SOP for determination of organic content in MNMs by thermogravimetric analysis (TGA) (Appendix I to deliverable)		Toolbox; OECD WPNM
SOP for assessment of the contents of water, organic compounds, and degradable inorganics in MNMs using a drying oven and a laboratory furnace (Appendix II to deliverable)		
SOP for determination of inorganic content in MNMs by Wavelength dispersive X-ray fluorescence (WDXRF) (Appendix III to deliverable)		
SOP for characterization of non-covalent bound organic surface coating on MNM by extraction and mass spectrometry (Appendix IV to deliverable)		
SOP for determination of inorganic coating in MNMs by Transmission Electron Microscopy. (Appendix V to deliverable)		
SOP for determination of oxygen containing functional groups on CNT by decarbonylisation, decarboxylation, and dehydration in inert atmosphere using TGA-MS (Appendix VI to deliverable)		

√ *Characterization and categorization of NM in powders and liquid dispersions (2.05)*

To address the need for a practical system to define and categorize NMs, an advanced material-specific categorization scheme was developed, which is suitable for a range of NMs. The main

assumption of the scheme is that we deal with a nanomaterial according to a regulatory definition accepted by EU authorities, whatever this definition could be.

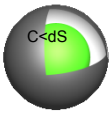
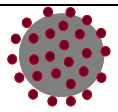
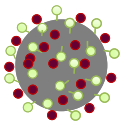
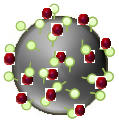
The scheme introduces relevant physico-chemical properties of NM by subgrouping them into:

- Structure/Chemical composition,
- Shape/Porosity,
- Specific Physico-chemical properties.

The applicability of the proposed scheme was assessed by applying it to specific NANoREG core-NMs and available data from the NANoREG Technical Data Sheets (TDS). The proposed classification scheme seems to fit well with the majority of the NMs, and after further testing it could be proposed as a system for substance identification and classification of NM. Unless otherwise stated, the terminologies used in the scheme are coherent with Regulation (EC) No 1907/2006 (REACH) and ISO standards.

Deliverable 2.5 also presents a system of nomenclature for NMs on the basis of physical and chemical properties. It provides a minimum set of descriptors to understand the chemical composition and structural relationships of a substance immediately. The approach for naming is generic, each material-group such as metals, metal oxides, carbon, organics etc., must be considered as individual cases.

The deliverable gives examples for metal oxides and carbon nanotubes, to define their naming and compatibility to the classification scheme.

Simple Nomenclature	Fully described Nomenclature	Simplified Image	Description
SiO_2 (Amorphous) – $1G_n$	SiO_2 (Amorphous) – $1G_{99}$ (1% Al_2O_3)		Above 99% pure amorphous Silica with 1% of acceptable impurities Al_2O_3
SiO_2(Amorphous) – $2GIC_n$	SiO_2(Amorphous) – $2GI_{(CdS)}C_5$		Second generation silica shell on a core of 5 wt% CdS (core-shell type)
SiO_2(Amorphous) – $2GIE_n$	SiO_2(Amorphous) – $2GI_{(Fe2O3)}$ E_5		Second generation silica with 5 wt% of Fe_2O_3 coated on silica.
SiO_2(Amorphous/ Maghemite) – $3GO_nI_n$	SiO_2(Amorphous/ Maghemite) – $3GO_{10}I_5$ (10%APES, 5% Fe_2O_3)		Third generation External surface of silica is functionalized with 10 wt% APES and further coated with 5 wt% Fe_2O_3.
SiO_2(Amorphous/ Maghemite) – $3GO_nI_n$ /O_nI_n	SiO_2(Amorphous/ Maghemite) – $3GO_{10}I_5$ /$O_{10}I_5$		Third generation, a mixture of silica containing 10 wt% of functional groups, APES and coated with 5 wt% Fe_2O_3.

Resulting documents		Input for
Deliverable 2.05: NANoREG Protocol for characterization and categorization		
Factsheet 2.05		

Task 2.3: NM characterization SOPs for regulatory purposes

√ *Evaluation of OECD Technical Guidelines (D2.03, 2.09)*

Due to the unique properties of NMs, not all OECD Technical Guidelines are applicable for the characterization and the assessment of NMs. Ten selected OECD TGs relevant in REACH context, were evaluated.

Delivery 2.3 concludes that the OECD guidelines **TG 106** and **TG 107/117/123** are for thermodynamic reasons not applicable to nanomaterials. In D2.9 the modification of the OECD guidelines proposed in D2.3 were experimentally developed and evaluated. Most of the modified technical guidelines are now ready for validation in inter-laboratory tests.

It was concluded that the **dispersibility** of nanoparticles is still not well defined, and no protocol exists for its determination. Based on existing protocols for dispersion of nanoparticles and size measurement, D2.09 describes a first draft protocol to determine the dispersibility of nanoparticles in water in a reproducible manner.

OECD Technical Guideline	End-point appropriate for NM?	Modified protocol established	Future work
TG 109 (relative density)	Partially	Yes; New protocol for relative (skeletal) and agglomerate density	Validation
TG 110 (granulometry)	Partially	Yes, including dispersion protocol (CLS, DLS and SEM (image analysis))	Validation
TG 106 (sorption-desorption)	NO	From a thermodynamic point of view not possible	
TG 105 (water solubility)	Partially	Yes; A revision of the TG to address dissolution is proposed, and two additional protocols were established and demonstrated.	Validation
TG 115 (surface tension of aqueous solutions	YES	No modification necessary; replacement of solution by solution/suspension	
TG 107/117/123 (*n*-Octanol-water partition coefficient)	NO.	Generally not applicable to solids. No testing or modification established in NANoREG	
TG 112 (dissociation constant in water)	NO	Protocols were developed for other more relevant endpoint such as isoelectric point.	Validation
TG 108 (complex formation in water)	Partially.	Guideline modification for focusing on the adsorption of trace metals on NMs.	Validation
Dispersibility	NO	New protocol developed	Validation

Additional proposals for TGs were developed for analysing the particle surface charge by zeta-potential and water dissolution rates. The biggest challenge in dissolution testing is separation of the solid phase where suitable filtration or better in situ ion measurements can be made. Further work is also needed to consider whether dissolution should be assessed in buffered or unbuffered water as well as in presence with a controlled atmosphere as test conditions may influence the dissolution and solubility of the test material considerably.

Resulting documents		Input for
Deliverable 2.03: NANoREG Experimental evaluation of OECD methods		OECD harmonization programme
Factsheet 2.03		
Deliverable 2.09: Revised OECD methods for determination of physico-chemical NM properties		
Factsheet 2.09		
NANoREG D2.09 SOP 01 Protocol for true density measurements		
NANoREG D2.09 SOP 02 Protocol for effective density measurements		
NANoREG D2.09 SOP 03 Protocol for the measurement of water solubility		
NANoREG D2.09 SOP 04 Protocol for the determination of the Dispersibility		
NANoREG D2.09 SOP 05 Protocol for IEP determination		

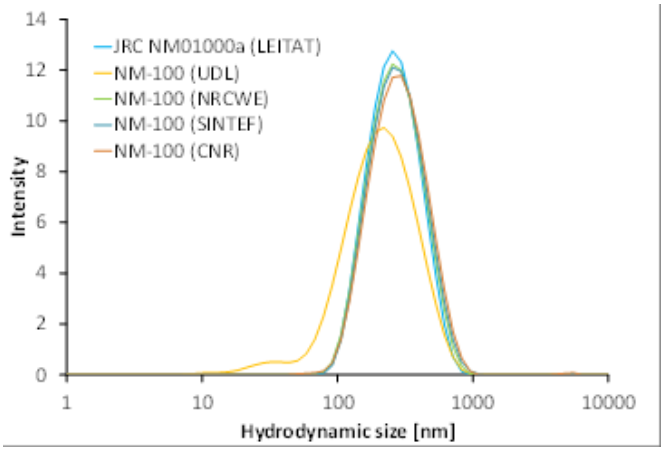
Task 2.4: Test item preparation, exposure, dose and fate for regulatory purposes and toxicology

√ *Dispersions protocols (D2.06)*

Task 2.4 was the core WP2 activity to establish the NANoREG Guidance Document for (eco)-toxicological testing to facilitate generation of reliability and comparability of data. For partners it was mandatory to perform their experiments in accordance with the Guidance Document. During the course of the project, some of the procedures proposed were further refined (without affecting the initial guideline) and documented.

Most assays for eco- and in vitro toxicological testing of NMs require dispersion of solid particles into liquid test mediums. The way this is done strongly affects the outcome of (eco-)toxicity tests. Standardization of the dispersion procedures is therefore the first indispensable step towards establishing reliable and comparable toxicological testing. It is a prerequisite for comparative analyses of results from different laboratories and different toxicological assays and for the regulatory testing of nanomaterials.

To ensure that for the NANoREG project such comparative analysis of results would be possible, a Guidance Document (July 2014) was established during the preparatory phase of the project. The document describes dispersion procedures and characterization requirements to be applied by all partners. During the course of the NANoREG project, validation of the dispersion procedures took place and benchmark values were established for all NANoREG core materials. The final result is a set of validated protocols that can be used as a solid basis for the regulatory (eco)-toxicological testing. All protocols are performance tested and validated using an interlaboratory comparison approach.



The results showed that all dispersion protocols used in NANoREG enable establishment of comparable dispersions for granular NM. However, some variations were observed for some granular NM, which is generally ascribed to sample variability.

Due to the method, DLS results are generally poor for high aspect ratio NMs such as carbon nanotubes and nanocellulose samples with comparability between laboratories being generally poor. Optical microscopy screening of dispersions made with the NANOGENOTOX dispersion protocol showed that only NM-411 (SWCNT) was poorly dispersed among all 19 NANoREG core test materials.

Resulting documents		Input for
Deliverable 2.06: Validated protocols for test item preparation for key in vitro and ecotoxicity studies		
Factsheet 2.06		
The validated probe sonicator calibration protocol for the NANOGENOTOX batch dispersion protocol (Deliverable Chapter 3)		Toolbox
The validated Standard Dispersion Protocol for ecotoxicological studies (Deliverable Chapter 4)		
The validated Enhanced Dispersion Protocol (NOM-water) for ecotoxicological studies (Deliverable Chapter 5)		
The validated generic NANOGENOTOX dispersion protocol for in vitro studies (Deliverable Chapter 6)		

√ *Test item preparation, exposure, dose and fate for regulatory purposes and toxicology (2.08)*

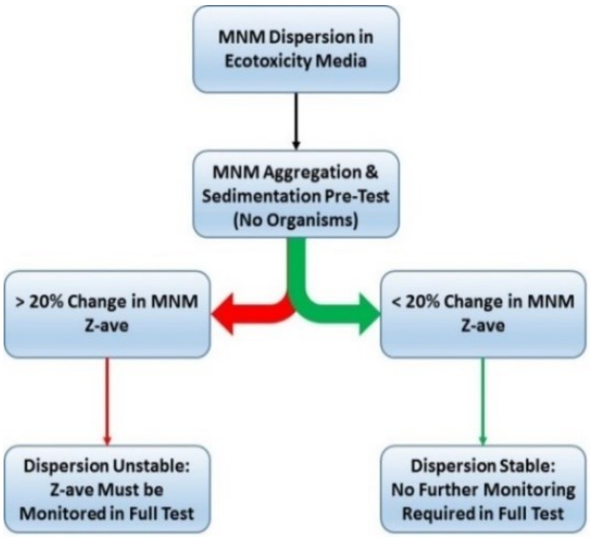
In ecotoxicology and –submerged- *in vitro* testing, exposure to NMs occurs via dispersion in a liquid medium containing both inorganic and organic compounds. For a meaningful interpretation and comparison of data generated in such tests, it is important to understand the variation in physico-chemical exposure-dose characteristics -“fate”- during the experiment. Relevant parameters include (i) the dispersion stability / sedimentation rate, (ii) particle agglomeration/size-fractionation in

the exposure medium and (iii) NM reactivity; including their interaction with medium constituents and dissolution as well acid-base and redox activity.

*Procedures for quantification of NM **exposure and fate** in dispersions for **ecotoxicological** studies*

The "NANoREG ECOTOX Dispersion Characterization Technical Guidance Document (TGD)" was developed as a framework requesting the following steps at both the start and end of the experiment:

- Characterization of the NMs.
- Determination of the total concentration mass and SSA of the NM (mass and SSA concentrations) in the water phase.
- The mass and SSA concentration of dissolved NM and particulate NM in the water phase (actual).
  As well as:
- Estimation of the amount of test NM (mass and SSA), which has either sedimented out of the water phase or adsorbed to the surfaces of the exposure system during the test.



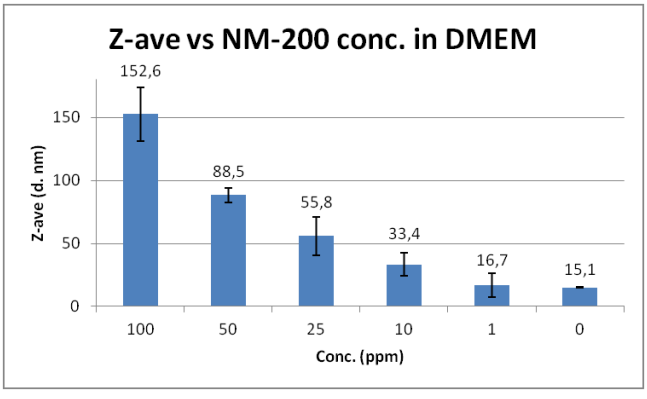
Pre-test for aggregation and sedimentation

Application of this TGD in NANoREG WP4 clearly highlights the need for conducting detailed physico-chemical characterization of NM dispersion exposures throughout the duration of an ecotoxicity test. It enables a reproducible decision-making process for exposure-fate characterization in aquatic ecotoxicity tests.

*Procedures for quantification of NM **exposure and fate** in dispersions for **in vitro** studies*

Procedures for providing the key physico-chemical NM exposure-dose characteristics requested in the NANoREG TGD for *in vitro* studies were developed and/or demonstrated. They include:

- A Centrifuge Liquid Sedimentation (CLS) and two Dynamic Light-Scattering (DLS) procedures to assess dispersion stability (plus zeta-potential by DLS) and size-evolutions during testing in cell culture mediums.
- A Proton Induced X-ray Emission (PIXE) procedure for quantification of deposited (elemental) dose to cells.
- An ELISA-procedure for quantifying the NM-biomolecule interaction (LDH, IL-6 and IL-8) in cell culture mediums.
- A Sensor-Dish Reader (SDR) procedure for real-time measurement of the pH- and O_2-reactivity and offline determination of particle dissolution rate during in vitro testing.
- A method for measuring the dissolved fraction of NM using Ultrafiltration Inductive Coupled Plasma Atomic Emission Spectrometry (UF/ICP-AES).



Z-ave sizes of dispersions of different concentrations of NM-200 in DMEM measured with DLS

Even-though CLS has a higher size resolution than DLS, the DLS measurements following the NANoREG TGD minimum characterization requests are still robust and useful. Any measurement should also be verified at least by qualitative imaging.

Continuous DLS monitoring enables assessment of the temporal dispersion behaviour. Assessed particle sedimentation rates from DLS showed that 50% of NM-110 (0.256 mg/mL RPMI with 0.2% glutamax) were deposited after 2.7 hours. For NM-400, only 25% were deposited after 8.4 hours.

Quantification of deposited dose by PIXE is a more precise chemical dose determination for most metal compounds. The effective PIXE dose can differ significantly from the nominal dose. A PIXE dose of only ~50% nominal dose was observed in a 72-hour test with NM-110.

Assessment of NM reactivity and dissolution during incubation was demonstrated using the Sensor Dish Reader method and showed a serious increase in pH-activity of ZnO (NM-110 and NM-111) dissolution in HAMs F12 cell medium. Incubation of Ag (NM-300K) was associated with important changes in the O_2 concentrations.

Deposition times assessed from relative intensity of the derived count rate measured by DLS

Deposited fraction	**Deposition time NM-110 [hour]**	**Deposition time NM-400 [hour]**
25%	1.0	8.4
50%	2.7	16.4[€]
75%	4.5	24.4[€]
99%	6.2[£]	32.2[€]

[£]The suspension near the bottom may start to agglomerate and accumulate at the base of the vial. [€]Apparent deposition time projected from initial slope. The NM appears to form a suspended accumulation layer in the lower volume of test vial.

No reactivity was observed for silica (NM-200), which however undergoes extensive dissolution. The test procedure established for assessment of biomolecule interactions, showed important LDH and interleukin interactions with the NANoREG core NM under *in vitro* test conditions. In conclusion, exposure-fate characterization is essential to understand NM toxicological test results.

*Procedures for characterization of MN **hydrochemical reactivity** in **synthetic biological fluids***

In this task, two different example studies demonstrated the use of an:

- Atmosphere-Temperature-pH-controlled stirred batch reactor (ATempH-SBR) with monitoring of redox potential (E_h) to assess the NM reactivity and dissolution in a simulant phagolysomal fluid (PSF; pH 4.5).
- Acellular *in vitro* test procedure to assess the NM behaviour in the gastro-intestinal different fluids.

Results obtained using the ATempH-SBR demonstrated that NM-110 showed rapid dissolution and a short-term destabilization of pH and reduction on E_h. Dissolution of NM-300K was slower and resulted in a continuous reduction in E_h during extensive buffering with NaOH to maintain pH.

Investigations of NM state, exposure-fate and reactivity in GI-fluids clearly showed differences in particle charge with test mediums, which was also reflected in UV-vis and DLS size-measurements. TEM was needed to show morphological changes in the different fluids. The NM dissolution also varied in the different mediums and with NM type. Overall, it can be concluded that NM are generally reactive and do not maintain their primary characteristics in liquid mediums. The changes affect the exposure characteristics which should influence on cellular permeability and uptake (for *in vitro* studies) and ADME parameters (in *in vivo* studies).

Zeta-pot of three NM in synthetic human digestive compartments. CTRL refers to the Zeta-pot of NMs pre-dispersed in 0.05% (v/v) BSA-water

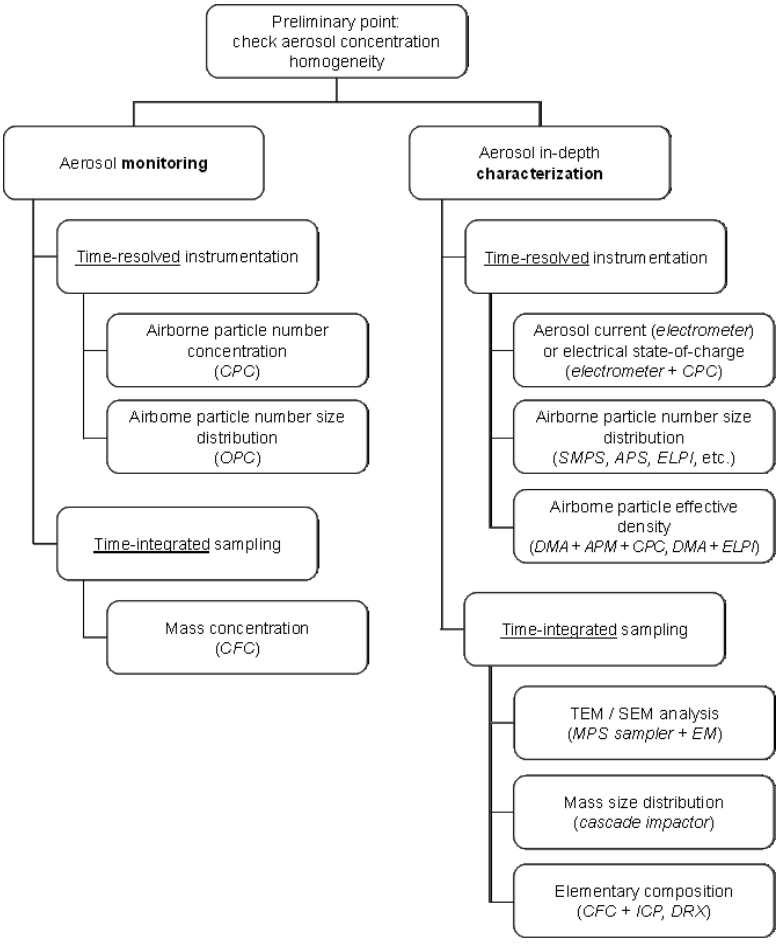
	Nominal Charge (mV)	**CTRL charge (mV)**	**Saliva (mV)**	**Stomach (mV)**	**Intestine (mV)**
NM-110	-24.3	-20.6	-36.0	-9.0	-35.0
NM-200	-47.5	-28.0	-30.0	-10.0	-39.0
NM-300K	-11.0	-24.4	-35.0	-3.0	-32.0

Resulting documents		**Input for**
Deliverable 2.08: Test item preparation, exposure, dose and fate for regulatory purposes and toxicology		
Factsheet 2.08		
NANoREG D2.08 SOP 01 Methodology for sedimented dose determination with CLS and PIXE techniques		Toolbox
NANoREG D2.08 SOP 02 For measurement of hydrodynamic Size-Distribution and Dispersion Stability by DLS		
NANoREG D2.08 SOP 03 For particle size determination of a given MNM by the CLS technique		

NANoREG D2.08 SOP 04 To quantify the concentration of a given MNM in cell culture media with the PIXE technique		
NANoREG D2.08 SOP 05 For test item preparation and comparability of results during in vitro testing		
NANoREG D2.08 SOP 06 For characterizing MNM fate in biological media and digestive fluids by multi-technique based method		
NANoREG D2.08 SOP 07 For determination of LDH, IL-6, IL-8 adsorption onto MNM		
NANoREG D2.08 SOP 08 For hydrochemical reactivity and biodurability testing using an Atmosphere-Temperature-pH-controlled SBR		
NANoREG D2.08 SOP 09 For SDR analyses of MNM hydrochemical reactivity and dissolution in in vitro medium		
NANoREG D2.08 TG Document for Environmental Exposure Characterisation		

√ *Characteristics of test aerosols using different generation methods for inhalation studies [D 2.07]*

An essential element of inhalation studies is dispersion of NMs in air in a strongly conditioned way. Until now, various methods have been developed and used to produce aerosols with NMs for inhalation studies:



As a first step a literature review has been carried out to develop an aerosol measurement strategy. As a follow up, two dry based methods (*Vibrating air velocity jet generator* and *Rotating brush generator)* and a direct synthesis method (*Spark discharge generator and evaporator/condensation in a high temperature furnace*) have been tested applying the developed measurement strategy.

In the set-up with the vibrating air velocity jet generator, the stability and reproducibility of the generated aerosol was tested for one core nanomaterial (NM-105). The use of this device leads to unstable aerosols. After optimising the settings, better aerosol stability is observed with acceptable deviations between three repeated experiments.

The results of a comparison of aerosol generation with nine different NANoREG core materials in a Rotating Brush Generator (PALAS RGB 1000) are summarised in the figure below.

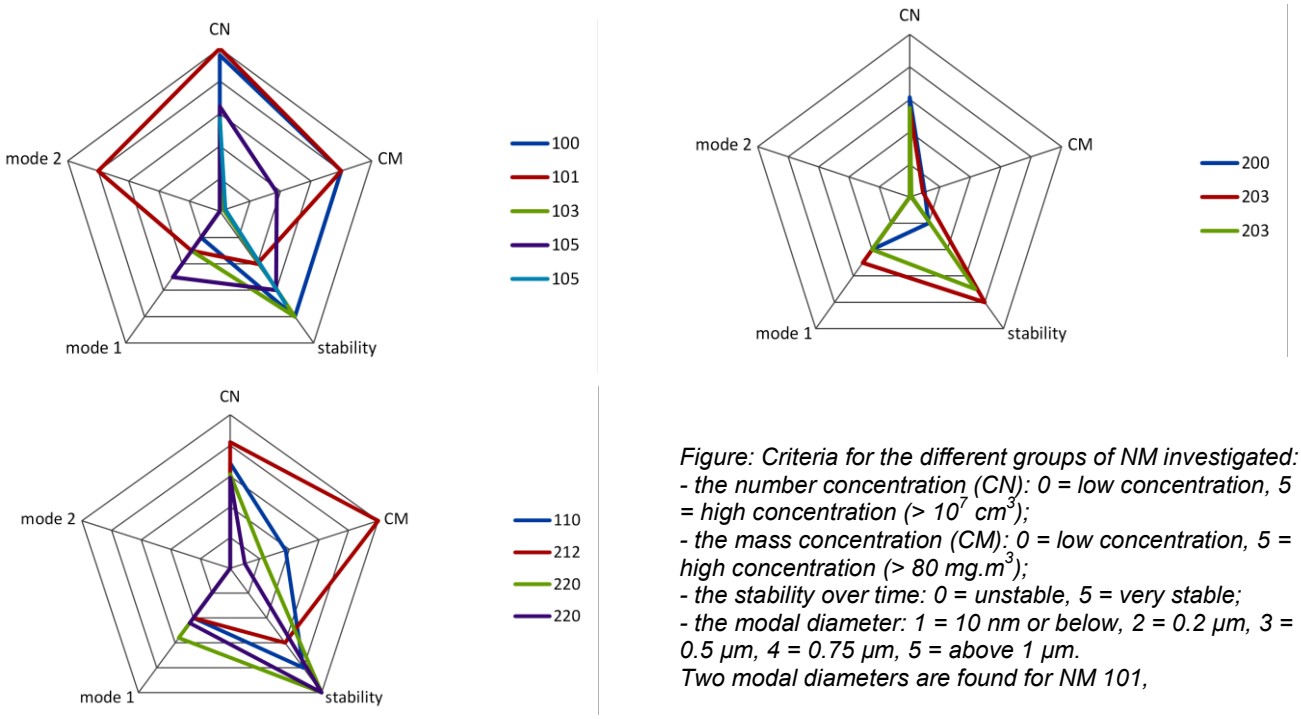


Figure: Criteria for the different groups of NM investigated:
- the number concentration (CN): 0 = low concentration, 5 = high concentration (> 10^7 cm^3);
- the mass concentration (CM): 0 = low concentration, 5 = high concentration (> 80 $mg.m^3$);
- the stability over time: 0 = unstable, 5 = very stable;
- the modal diameter: 1 = 10 nm or below, 2 = 0.2 µm, 3 = 0.5 µm, 4 = 0.75 µm, 5 = above 1 µm.
Two modal diameters are found for NM 101,

The two spark discharge generators and a high temperature evaporation furnace produced a stable output of nanostructured metal agglomerate aerosol particles in the size range 30 to 300 nm, but at different mass concentrations ~5 to 50 mg/m3. The deliverable also presents the results of the application of the measurement strategy for two operational inhalation systems.

For the dry based aerosol generations system the following conclusions are drawn:

- The applied methods cover the particle size range from about a few tens of nanometers up to several micrometers, the particles and are mainly composed of aggregates and/or agglomerates.
- The conditions required for inhalation studies (stability, repeatability, level of concentration) are sometimes difficult to obtain, but possible.
- It is necessary to carry out preliminary tests to ascertain the performances of the chosen devices and their suitability for the inhalation facility.
- Specialised expertise is needed for in-depths characterization.

The applied direct-synthesis methods cover particle size range from about a few nanometers up to several hundred of nanometers. With this method, higher concentrations in number can be obtained. Stability and repeatability is -in general- very good.

The work carried out shows that generation and characterization of test aerosols for inhalation studies is a complex but essential element of inhalation studies. The application of the developed measurement strategy during the NANoREG project has proven to be feasible.

The results reported in the deliverable has been used to comment the current revision of the OECD guidance document on inhalation toxicity testing.

Resulting documents		Input for
Deliverable 2.07: Characteristics of test aerosols using different generation methods for inhalation studies		
Factsheet 2.07		

Framework and procedures for characterization and reporting of manufactured nanomaterials for regulatory use (Deliverable 2.12)

The REACH regulations oblige companies operating on the European Market to identify and manage risks linked to the substances they manufacture and market in the EU. They have to register

their substances and in this context, carry out a chemical safety assessment aimed at defining and describing the conditions under which the risks of the substance are controlled. To facilitate this registration process ECHA (The European Chemicals Agency) provides guidance on what information should be provided and what methods can be applied for generating the data on properties of substances, for predicting exposure levels and for RA.

Deliverable 2.12 provides recommendations for revision of these guidance documents which address NMs. The recommendations are related to schemes for substance identification and methods to generate the physico-chemical end-points required for the substance identification, grouping, QSAR and read-across schemes for NMs in REACH. The recommendations stem from an over-arching activity bridging several tasks of work package 2 of the NANoREG project.

Recommendations regarding characterization and identification of nanomaterials

The analysis of the current (draft) guidance documents and Q&As on substance identification reveals that the guidance with respect to substance identification in the different documents is not consistent. The deliverable comes forward with several concrete proposals for modification, such as reporting of particle size distribution and adjustment of the scheme for morphological categorization to be used as basis for grouping and read across purposes. In this context already developed schemes for shape type classification by ISO are mentioned as a more solid base.

Reporting on surface-chemistry is considered as an important improvement. However, surface-chemistry is not the only chemical modification that should be included in the information requirements for NMs. Nanomaterials may be modified in many different ways in modern material design including atomic substitution, doping, porosity-filling, physical coating and chemical functionalization. They all should be reported since all these modifications potentially change the properties, reactivity, fate and hazard of the NM.

Recommendations regarding substance identification scheme and material categorisation

Following up on Deliverable 2.05, a proposal is presented for a modified categorization scheme. It is suggested that the final identification and reporting should include characterization of physico-chemical properties according to the (nano-) materials by subgrouping them into 1) Structure/Chemical composition, 2) Shape/Porosity, and 3) Specific Physico-chemical properties. The number of characterization end-points under “Specific physico-chemical properties” could vary from limited to rather extensive depending on the material type and information needs.

Recommendations regarding methods to support data generation

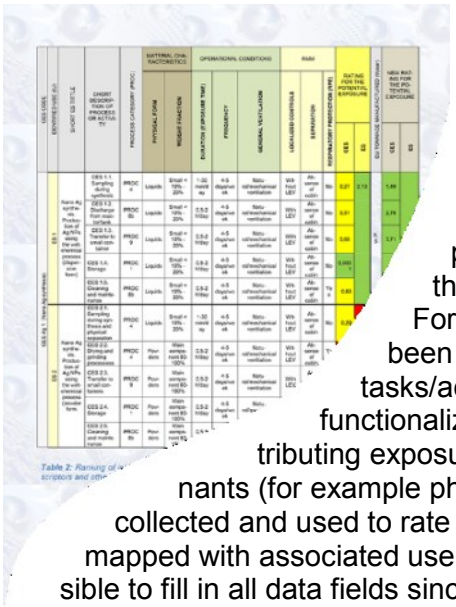
In the ECHA Guidance Documents information on a minimum of 13 physico-chemical characterization end-points is requested. Recommendations on how to generate the data also are sometimes given, however it is mentioned that the methods mentioned are rarely fully applicable for characterisation of NMs.

To support regulatory characterisation, WP2 developed and demonstrated SOPs for identification of MNM by sizing, using electron microscopy and BET gas-adsorption and de-sorption profiles. Procedures were developed for several other end-points of regulatory relevance, including identification and quantification of surface chemical modifications, dissolution testing, and reactivity. Revisions of several of the OECD TGs were proposed or proposed to be replaced with alternative or new methods and presented in NANoREG D2.09.

3.1.3 Work package 3

Results	*Impact*
Model for identifying critical exposure scenarios	*Contributes to an efficient assessment of risks of a NM by identifying activities during the life cycle of a NM with the highest (potential) exposure*
Development of Mesocosms	*Better mimic the effects and impact of exposure of ecosystems to nanomaterials. Determine differences in effects between pristine material, formulation products and released material.*
SOP for measuring releases (aging, sanding, dustiness, …) and exposure	*Assures a better reliability and reproducibility of experimental data on the fields mentioned*
Additional data on exposure (field measurements, measurements in a large exposure chamber and simulated approaches)	*Fills the gap in data needed for modelling of exposure and further assessing the risks associated with NMs*
SOP to test the effectiveness of protective measures	*Diagram of decision for choosing the most appropriate Personal protection Equipment contributes to labour safety.*
Evaluation to tools for first tier risk assessment of occupational exposure	*Insight in factors causing possible miscalculation of risk classification.*
Development and demonstration of the I-Nano tool (quantative model for predicting inhalation exposure)	*Better (and quantitative) prediction of inhalation exposure to nanomaterials*

Task 3.1: Identification and elaboration of exposure scenarios (D3.01):



To identify the most critical exposure scenarios during the life cycle of a product (in terms of potential exposure and economic importance) a model was developed.

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 European production, main market applications of NM used in nano-enabled products and existing exposure data. Based on this information, the main applications for the core set of NMs have been selected. For these main applications a method for “Mapping of Uses” has been developed. The maps give an overview of all relevant tasks/activities along the life cycle of a nanomaterial, i.e., synthesis, functionalization, formulation, use and end of life. For all processes (“contributing exposure scenarios”; CES), use descriptors and exposure determinants (for example physical form, duration and frequency of the activity) have been collected and used to rate and rank the exposure scenarios. In total, 29 applications were mapped with associated use descriptors and exposure determinants. It was not always feasible to fill in all data fields since the information needed to build exposure scenarios is rarely included in the peer-reviewed literature.

The scenarios with the highest exposure along the life cycle of nano products are distinguished in four general source domains:

- NM synthesis, examples of production processes such as chemical vapour condensation, arc-vapour, laser ablation, thermal decomposition or flame pyrolysis
- Handling and transfer of powdered NM (e.g. weighing, bagging or packaging of powders)
- Manufacturing of intermediate materials containing NM
- Cleaning and maintenance processes

Resulting documents		Input for
Deliverable 3.01: Gap analysis report, identifying the critical exposure scenarios with-in the key value chains (will be updated at end of project)		Toolbox section 3.5 REACH exposure assessment.
Factsheet 3.01		

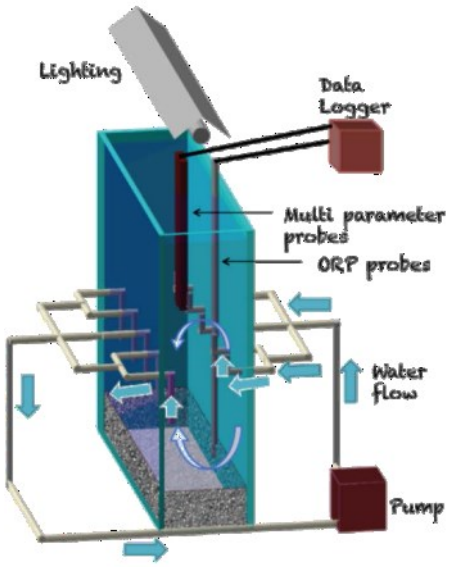
Task 3.2: Release of NM

√ *Development of an aquatic Mesocosms Platform (D3.05)*

Current strategies for assessing the environmental safety of NMs are largely based on the exposure of a single species or model organism to such materials. Such strategies separate the organism from the environment and community of organisms (i.e. the ecosystem) in which the environmental risks of NMs ultimately need to be understood. To better mimic the effects and impact of exposure of ecosystems to NMs a testing strategy based on mesocosm (*experimental system that simulates real-life conditions as closely as possible, while allowing the manipulation of environmental factors)* was developed and applied with satisfactory results.

Three different types of mesocosms have been tested: Freshwater mesocosms of 3 Litres and 60 Litres and a marine water mesocosm of 60 Litres. To take into account the various stages of the life cycle, the mesocosms were exposed to different pristine NMs (CeO_2, TiO_2, Ag and ZnO) as well as to formulation products and released materials from nano-enabled products (diesel additive, Cement and PET and PP film.

The developed experimental design, provides more realistic conditions of exposure by taking into account transformation of NM; an aspect that has proven to be critical in assessing the potential and is missing many studies. It was successfully shown that mesocosms can be used to simulate a NMs runoff rain or vent loading (e.g. single pulse) versus a continuous point source discharge such as wastewater treatment plant or industrial discharge (e.g. multiple dosing). A good reproducibility could be achieved across the 9-mesocosm located at one site; reproducibility was also found between two sites.



Such versatility and reproducibility allows for contrasting different environmentally relevant exposure scenarios. By creating representative conditions for environmental transformation and ecosystem exposure, such platform facilitates the integration of the ecotoxicological data into an environmental RA model related to nanotechnologies based on reliable exposure and impact data.

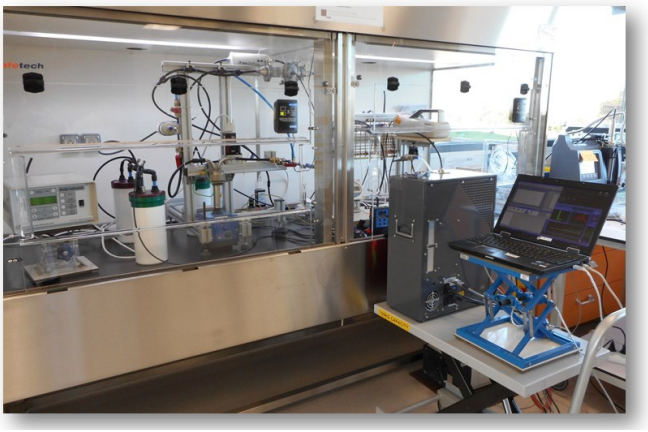
The complexity of food web and the species diversity in mesocosms is smaller than in real ecosystems. This exacerbates the exposure and impact in mesocosms compared to real natural environments. Consequently, mesocosms will provide the upper limit of the risks expected in the mimicked ecosystems.

Resulting documents		Input for
Deliverable 3.05: Development of an aquatic mesocosms platform allowing the evaluation of kinetics of aggregation		
Factsheet 3.05		
D3.5 SOP Characterization of the exposure to NMs in mesocosms_2016.11.16		Toolbox
Compendium of Protocols for testing the release of nanomaterials (see section on D3.03)		

√ *Comparison of existing dustiness methods (D3.02)*

Dustiness is a term addressing the ability of a material (e.g., loose, granulated, or pelletized powder) to generate an aerosol (airborne particles) during agitation. It is an important consideration for

worker exposure. In the EN15051 standard, two methods for dustiness testing are proposed: Rotating Drum (RD) and the continuous drop (CD) method. The methods intend to simulate different handling scenarios: dropping and mixing for the RD method and loading of trucks by a conveyor belt for the CD method. The methods also differ with respect to the intensity and the duration of treatment of the powdered material.



New dustiness approaches have been developed and tested over the last ten years in order to enable testing of smaller amounts in smaller and easy to handle setups mimicking potentially different scenarios in a workplace. Three of these methods have been tested for their applicability with NMs:

- the Vortex Shaker (VS) method,
- the Small rotating Drum (SD) method
- the Vibro Fluidization (VF) method.

The testing of the VS and SD method is part of the evaluation and standardization of dustiness testing in the framework of the CEN / TC 137. The tests were carried out with a variety of carbon nanotubes (CNT) since so far no CNT's had been tested within the CEN/TC137 activity. The testing of the VF method, a specific method developed by BAuA (no part of the current standardization work) was aimed at comparing VS and the SD which represent different "exposure scenario". However this second aim was not achieved.

Overall, results show good reproducibility for each method. However, dustiness indices and size distributions vary significantly between in the different methods. The number-based and mass-based dustiness indices of the investigated CNTs present a large span over several orders of magnitude suggesting that the levels of exposure could differ significantly when handling these carbon nanotubes.

Each method is supposed to simulate a given handling or release scenario. The available information however does not justify a conclusion regarding the scenario best mimicked by the different methods. It can be hypothesized that SRD simulates the dropping and mixing of the powder and that the VS simulates a worst case exposure scenario that may take place during the (non-recommended) use of compressed air blow guns to clean contaminated worker coveralls and dry work surfaces. These hypotheses have not been systematically studied.

Resulting documents		Input for
Deliverable 3.02: Comparison of existing dustiness methods		
Factsheet 3.02		

√ *Methods for the use of simulation approaches (D3.03)*

To quantify the release of NMs during their lifecycle, standardized methods were developed to quantify the release for selected processes and NMs (so called exposure scenarios). It resulted in a compendium of protocols for applying the test methods

The table below gives an overview of the methods for which protocols have been described in the deliverable including information on the field of application.

Method	To be applied on
Nanoparticle release from textiles to the water compartment during washing cycles	Textiles impregnated with: - Mixture of TiO_2 nanoparticles and AgCl, - Two different sized Ag nanoparticles.
Nanoparticle release from polymers to the water compartment during accelerated aging.	Polypropylene, polyamide, poly urethane doped with nanosilica and clays (Montmorillonite)
Nanoparticle release during sanding processes.	Nano enabled paints, coatings and nanocomposites with Nano fillers such as SiO_2, TiO_2, ZnO
Nanoparticle release during environmental aging.	Nano enabled paints, coatings and nanocomposites with Nano fillers such as SiO_2, TiO_2, ZnO
Release rate for processes placed under fume cupboards.	Processes placed in fume cupboards (or equivalent) emitting ultrafine or nano-sized particles (Chemical vapour deposition reactors, flame spray pyrolysis, weathering processes, lab grinders, spray drying, weighing or transferring of powders, cleaning…)
Controlled generation of aerosols from NMs (carbon nanotubes and fibres) using the Shaker-Method.	Carbon nano tubes and fibres in a test chamber to determine dustiness behaviour or to compare measurement instruments. This Standard Operating Procedure (SOP) is not valid for granular biopersistent particle (GBP).
Controlled generation of aerosols from NMs (granular biopersistent particles) using the Shaker-Method.	Dry/powder NMs, more specific GBP – granular biopersistent particles, to determine dustiness behaviour or to compare measurement instruments. This Standard Operating Procedure (SOP) is not valid for fibrous NMs.
Agitation method to test dustiness: small rotating drum (SD) method.	The method is used to characterize nanomaterial powders in order to determine their dustiness indexes. The small rotating drum was designed as a downscaled version of the EN 15051 rotating drum while maintaining important test parameters. This enabled testing of smaller material amounts (~6g).
Agitation method to test dustiness: vortex shaker (VS) method.	The vortex shaker method is used to characterize nanomaterial powders in order to determine their dustiness indexes with supposedly higher energy than SD which participates to de-agglomeration processes. This method enabled testing of very small material amounts (~0.5 cm^3).

Resulting documents		Input for
Deliverable 3.03: Methods for the use of simulation approaches		
Factsheet 3.03		
Compendium of Protocols for testing the release of NMs:		Toolbox
SOP 1_Method for the evaluation of nanoparticle release from textiles to the water compartment during washing cycles (Deliverable section 2.3.1)		
SOP2_Method for the evaluation of nanoparticle release from polymers to the water compartment during accelerated aging (Deliverable section 2.3.2)		
SOP3_Method for the evaluation of nanoparticle release during sanding processes (Deliverable section 2.3.3)		
SOP4_Method for the evaluation of nanoparticle release during environmental aging (Deliverable section 2.3.4)		
SOP5_Method for the evaluation of nanoparticle release rates for processes placed under fume cupboards and hoods (Deliverable section 2.3.5)		
SOP6_Method to generate controlled aerosol from nanomaterials using the shaker method (Deliverable section 2.3.6)		
SOP7_Method to generate controlled aerosol from nanomaterials using the shaker method (Deliverable section 2.3.7)		
SOP8_Small rotating drum (SD) method (Deliverable section 2.3.8)		
SOP9_Vortex Shaker(VS) method (Deliverable section 2.3.9)		

Task 3.3: Measurement of exposure

To assess and manage the risks related to the occupational-, consumer- and environmental exposure to nanomaterials (NMs), information is needed respectively on the actual exposure of workers, consumers and the environment to such materials. Work package 3 provides such information by evaluating methods to determine the release of nano materials from products containing such

materials (Deliverable 3.03), by evaluating methods to measure actual exposure to nanomaterials (Deliverable 3.06) and by applying such methods in real life situations (Deliverable 3.07

√ *Improved measurement instruments, tools and methods (D3.06)*

Fourteen instruments, tools and methods to measure actual exposure have been selected, evaluated and tested. They covered various route of exposure: inhalation, dermal contact and ingestion. It follows up on (and is complementary to) previous projects like NanoGEM and nanoIndEx. Both projects investigated the accuracy, comparability and field applicability of granulometers, counters and personal devices to characterize airborne NMs and produced several Standard Operation Procedures for commercial instruments.

Figure: The NANOBADGE worn as personal sampler during field measurements

The (additional) tools selected by task 3.3. of the NANoREG project were evaluated and compared to reference instruments. Improved sampling strategies were developed in cooperation with Task 3.2 (D3.03 and D3.07) to make the link between release and exposure. Some approaches towards conversion between metrics were evaluated. Attempts to discriminate background from actual (environmental) exposure were evaluated based on chemical composition (XRF, LIBS) and on isotope analysis.

Personal devices for exposure measurement

Mini Particle Sampler	Samples the aerosol particles onto a TEM grid to allow further characterization of the NMs.
NANOBADGE	Provides information on the elemental composition, size distribution and morphology of material collected in the personal breathing zone of workers thanks to physical-chemical characterization by XRF and SEM-EDS.
Surface swab method; tape stripping technique	Complementary to air sampling; determines surface contamination by NMs at workplaces that may be released by cleaning activities, movement of the workers etc. Together, these methods (air and surface sampling) provide an overview of the hygienic situation in workplaces where nanomaterials are handled.

Used in a coherent strategy, those easy-to-use personal devices are able to provide a good picture of the potential release sources and emissions of NMs in occupational settings. Those devices could deliver mass concentration shift average with the advantage to enable the identification of specific morphological and chemical features. A limitation of these methods is the limit of detection that could require a longer sampling time than actual exposure duration. In such situations one might prefer personal monitoring using for instance commercial devices such as DiSCmini, NanoTracer or Partector. Moreover, depending on the analytical technique associated to the sampling method, information on the particle identity might be partial (shape, morphology, chemical. composition …).

Biomonitoring tools

Nasal paper flag and the Exhaled Breath Condensate method	Methods to measure biomarkers of exposure and biomarkers of effects in order to assess the actual personal exposure to occupational and environmental toxicants. They can be used for occupational health surveillance and human biomonitoring.

More sensitive, reliable and versatile instruments that require highly qualified personnel were also investigated since they could provide valuable information on potential exposure.

More sensitive, reliable and versatile instruments

Small Angle X-rays Scattering	Provides information on size, shape, aggregation state and structure of aggregates. This technique could be applied to samples with various physical states (i.e. solids, liquid suspensions, aerosols…). It has however a limited applicability for very heterogeneous samples in size and chemical composition.
X-ray computed tomography	Technique that provides semi-quantitative 3D chemical mapping for solids or frozen liquids (100 ppm concentration). Its main limitation is due to the long data acquisition period during which the sample has to remain stable (e.g. drying, deforming that can cause modifications).
Cryogenic – Transmission	An *in situ* analytical technical that allows imaging materials and biological samples in a

Electron Microscopy	frozen state, directly within the TEM. Samples and processes that previously required deposition on substrates (and presented aggregation), or could not be imaged in their native environment, could now be studied and observed in an amorphous frozen liquid and with high resolution.
Laser Induced Breakdown Spectroscopy	Another candidate for nanoparticle *detection in situ* in real time or semi real time. Applied in air or on substrate, this technique does not require sampling preparation and allows measuring to the entire list of atomic species of the periodic table.
Asymmetric-Flow Field Flow Fractionation	Separation technique that allow physical-chemical characterization of nanoparticles in suspension in complex matrices. An example is given on the detection, the characterization and the quantification of silver nanoparticles in an aqueous matrix.
Isotopic labelling approaches	Radioisotope labelling can be used as an aid to the detection and localisation of nanoparticle in environmental media, as well as kinetic studies of stability and bioavailability. It allows the detection of nanoparticle selectively from natural nanoparticles and the large variety of amorphous materials present in environmental media.

Resulting documents		Input for
Deliverable 3.06: Improved measurement instruments, tools and methods [draft]		
Factsheet 3.06		
Compendium of protocols for using the instruments, tools and methods (annex to deliverable) SOP_01_MPS SOP_02_LIBS SOP_03_NANOBADGE SOP_04_ELPI SOP_05_Nasal paper flag SOP_06_Exhaled Breath Condensate SOP_07_FFF-ICPMS SOP_08_Cryo-TEM SOP_09_X-ray computed tomography SOP_10_SAXS SOP_11_Surface swab SOP_12_Tape stripping method SOP_13_SMPS GRIMM		Toolbox

√ *Simulation approaches and field measurements (D3.07)*

Simulation approaches

By applying the methods described in Deliverable 3.03, the release of nanomaterials has been simulated for the following products/processes:

- Aging and sanding of photocatalytic paints (w TiO_2)
- Sanding of nanocomposites (w MWCNT)
- Shredding of electrodes from Li-ion batteries (w/wo C-based NMs)
- Incineration studies (several materials)
- Drilling cement (w TiO_2)
- Cement alteration in climatic chamber (w TiO_2)
- Preservative wood stain coating alteration in climatic chamber (w CeO_2)

The experiments showed they were effective in terms of simulating processes that occurs in complex environments (background issues, low release rates, parameter control …). They allow getting insight on potential release, time and space dependent transformation, fate and behaviour of nano-enabled product along their whole life cycle. Those approaches are particularly useful since they allow through laboratory experiments to simulate processes that would take place over long period of time outdoor, in accelerated and controlled conditions indoor. Those approaches save time and resources compared to field measurements and consumer surveys. Experimental work showed that simulated approaches were able to address four classes according to the ENM state such as:

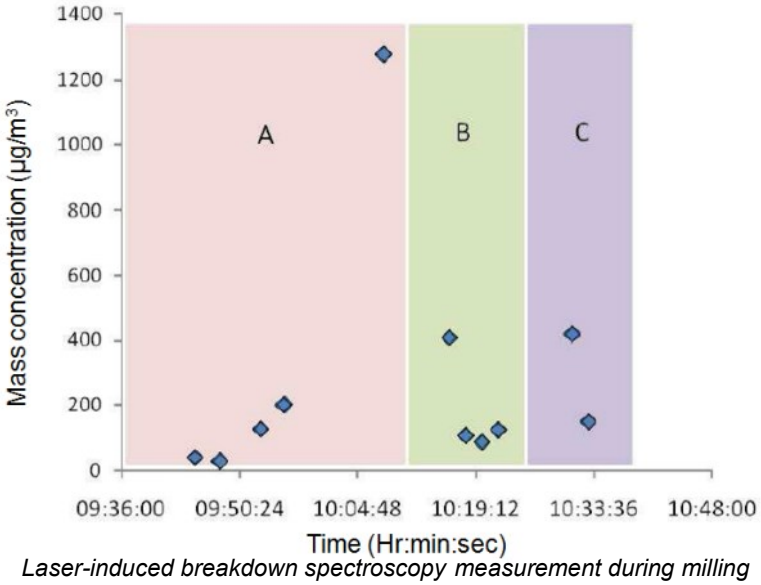
- Powders (e.g. dustiness testing)
- Suspensions (e.g. washing, sonication, spraying)
- Coatings (e.g. mild abrasion process, sanding, weathering …)
- Composites (e.g. cutting, sawing, grinding, drilling, milling, shredding …)

Field measurements

The methods mentioned above in D3.3 and D3.6 have been applied during a campaign of field measurements aimed at filling in a part of the gap in exposure data identified in the previous mentioned deliverable 3.01

It proved to be difficult to engage with companies which manufactured, handled or used NMs, but a limited number of companies volunteered to take part in the project. Studies include collection of comprehensive contextual information, information about determinants as well as quantitative data from direct reading instruments and samplers. Around twenty field studies were performed covering the most-produced NMs on different life cycle stages (production, use and end-of-life) and in different environments (academic labs, pilot lines and industrial plants).

The gathered data will be used for future activities for modelling exposure and to further assess the risks associated with NMs.



Laser-induced breakdown spectroscopy measurement during milling process which consisted of incorporating Silicon Carbide nanoparticles into Aluminium particles under extraction hood

Temporal evolution of mass concentration of aluminum particles
- A. when the hood is aspirating
- B. when the hood is turned off
- C. when the hood is aspirating at the end of the operations.

Resulting documents/data		Input for
Deliverable 3.07: Improved (exposure) data on specific scenarios (levels, physico-chemical characteristics, etc.)		
Factsheet 3.07		
Data set		Toolbox

Task 3.4: Exposure modelling

√ *Improved data for the modelling of the exposure to NMs (D3.04)*

Measuring of exposure levels of airborne NMs in industrial settings is complicated and costly. For this reason, there is a great need for models that can adequately predict aerosol exposure levels. Calibration and validation of such models requires data on the exposure in real-world situations. To provide such data, a series of comprehensively-monitored nanoparticle exposure experiment were undertaken inside a large climate-controlled chamber.

Besides preparatory experiments, *"source variation experiments*" were carried out to measure the influence of source position on particle concentrations throughout the room. "*Spike experiments*" were carried out to mimic emission scenarios where the nanoparticles are released intermittently (e.g. pouring nanomaterial from bags) and to study the emission spike broadening according to ventilation rate and aerosol monitor location. "*Coagulation experiments*" studied effects of injecting SiO_2 nanoparticles into an existing background aerosol, focusing on interactions between

engineered and natural particle emissions. Also nanoparticle wall losses were studied using SiO_2 and fluorescein particles and compared to theoretical predictions.

The resulting data will be used to test quantitative exposure models and enable an assessment of the accuracy and uncertainty of model-predicted concentrations. The resulting guidance document will identify the applicability domain of each tool/model, what information is essential to obtain a sensible estimate of exposure, and how reliable these estimates are likely to be. In addition, recommendations will be provided for model developers regarding potential issues with their model.

The dataset will be made available with many potential uses for those dealing with measuring and modelling of occupational exposures to NM.

Resulting documents/data		Input for
Deliverable 3.04: Improved data for the modelling of the exposure to NMs		
Factsheet 3.04		
Guidance document for quantitative exposure modelling		Toolbox
Dataset		Toolbox

√ Improved and validated occupational exposure models of release, exposure, dispersion and transfer (D3.08)

First tier RA of manufactured nanomaterial often uses control banding tools like the CB NanoTool Nanosafer, Stoffenmanager-NANO for occupational exposures and ConsExpo-nano for consumer exposure. The input for such tools is information provided by the user on the hazard of the nanomaterials, and the exposure conditions (emission potential, duration and frequency of exposure). Based on this input these tools make an estimation of hazard and exposure level (usually a category) and combine them into a risk score. A number of these tools also recommend control measures -if necessary- for the scenario being considered. These tools do not provide quantitative estimates of exposure.

Four models have been evaluated with respect to their applicability domain, assumptions made, inputs required and outputs as well as performance. For two of the most common used tools, the Advance Reach Tool (ART) and Stoffenmanager-NANO, a comparison was made between the outputs and measurement data. For five different tools an inter-user study has been performed to evaluate the potential variability in the answers obtained from different users of the tools.

Furthermore this task developed and demonstrated a quantitative inhalation exposure model.

Category	Spearman correlation	P-value	Spearman correlation	P-value
	ART		**Stoffenmanager - NANO**	
Transfer	0.464	**0.017**	0.195	0.340
Coating	0.515	**0.000**	0.590	**0.000**
Spraying	-0.596	0.001	-0.177	0.377
Welding	0.075	0.680	-0.148	0.413
hot process	-0.473	0.088	0.244	0.401
Impaction	0.476	**0.016**	0.651	**0.000**

Table: Correlations between particle number concentrations and scores

The comparison between 24 field measurements provided by the Swiss Accident Insurance Fund and the results of Stoffenmanager-NANO and ART (not specifically designed for nanomaterials) show little correlation with the particle number concentration. In the case of ART, the tool is not calibrated for MNMs, so this is perhaps not unexpected but in the case of Stoffenmanager-NANO a better correlation was expected.

The potential variability in outcome of exposure assessment tools when applied by different users was investigated for CB NanoTool, Stoffenmanager-NANO, Nanosafer, ConsExpo-nano and ART tool; although, the latter has not been calibrated for MNMs. Only the exposure assessment module of the tool was studied -and not the hazard assessment.

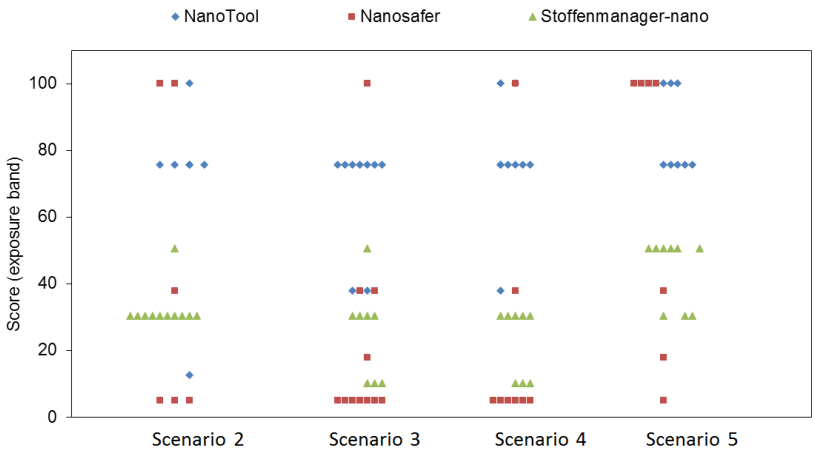


Figure Tools' scores for each scenario; normalised to a scale of 1-100

Five exposure scenarios were applied by 28 people with different areas of expertise. For the same tool and the same scenario all data points should be aligned along the same exposure score. However for all the tools a high inter-user variability was observed. Nanosafer showed the largest variability; Stoffenmanager-NANO resulted in less inter-user variability. The scores obtained by the different tools also show large variability.

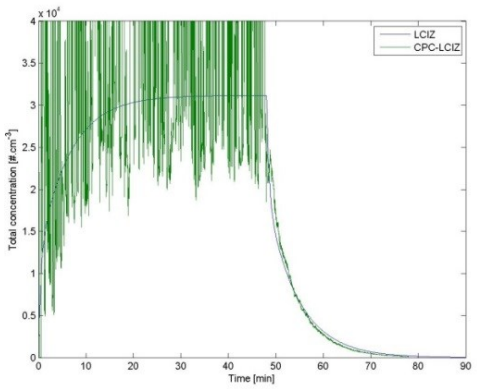
The results make clear that user variation in interpretation of exposure scenario information and converting these into the input variables is an important source of uncertainty. It can lead to completely different conclusions based on exactly the same set of information. This uncertainty needs to be taken into account during the development of the tools and its supporting guidance documentation and also by users of the tools; for use of more advanced tools, proper introduction and training is essential to reduce variability.

Generally speaking, the conclusion is that that the tools can lead to misclassification of the risks.

Quantitative models

Apart from the inter-user variability study of ART and ConsExpo-nano and the comparison of ART outputs with measurement data, the deliverable also presents the I-Nano tool developed under the umbrella of NANoREG and the demonstration of this model with the results of data measured in a large exposure chamber reported in deliverable 3.04.

The predictions from the I-Nano tool compared well with the measured data collected in the 'purpose build' simulation study. Concentrations in some locations were overestimated for some scenarios but overall the model appeared to be reliable at least within this experimental setup.



Unfortunately the concentrations achieved where not sufficiently high to cause coagulation between the released nanoparticles. Therefore, the model could not be tested for its sensitivity to pick up changes in the particle size distribution over time.

Resulting documents/data		Input for
Deliverable 3.08: Improved and validated occupational exposure models of release, exposure, dispersion and transfer		
Factsheet 3.08		
I-Nano two box model		Toolbox

Task 3.5: Effectiveness of risk management measures (D3.9):

There is a severe knowledge gap on the effectiveness of currently available Risk Management Measures (RMMs) for nanomaterial production and handling processes. To help bridge this, reliable methodologies to obtain quantitative data on the effectiveness of personal protective equip-

ment (PPE) and engineering controls (ECs) has been developed. For NMs in dry form as well as NMs in liquids.

Eight standard operating procedures (SOPs) to test the effectiveness of relevant RMMs have been developed, including 2 for respiratory protection (masks, filters), 2 for chemical protective gloves, 2 for protective clothing (suits), and 2 for engineering controls (LEVs). In order to confirm the reproducibility and robustness of the SOPs, a number of validation studies were specifically designed and conducted under controlled conditions to support a quantitative evaluation of the potential differences in the performance factors in order to ensure the transferability of the information provided in each SOP.

For all the Risk Management Measures mentioned above, the deliverable presents an extensive overview of the experimental results in terms of protection efficiency in different situations. Respiratory protection equipment for example has been tested by as half mask and full mask, new and aged, with different human subjects, shaven and unshaven walking, talking etc.

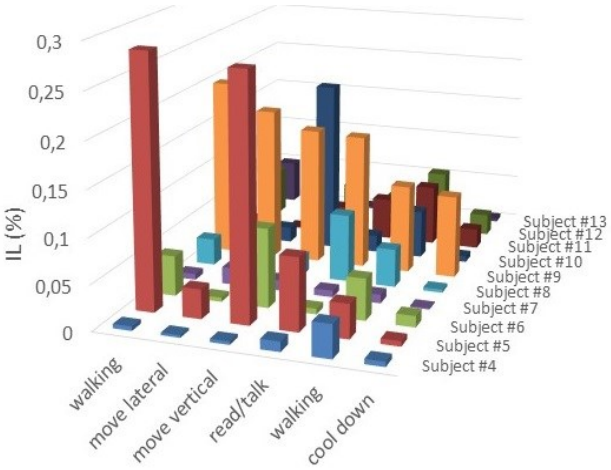
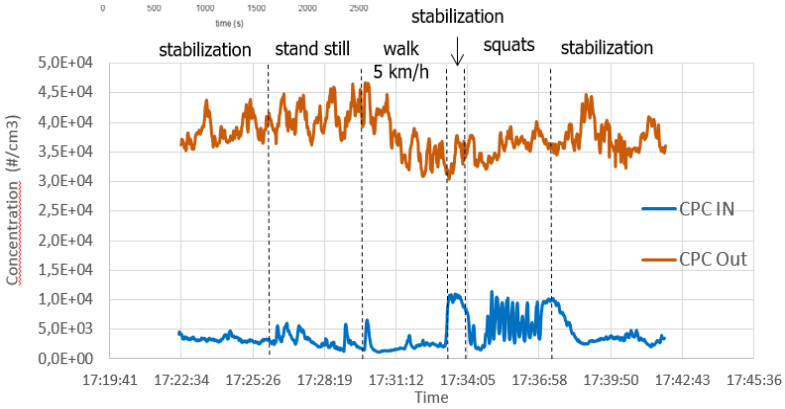


Figure: Percentage of Inward Leakage (IL) of Hall mask respirators measured on human subjects



The experimental studies within NANoREG confirmed the findings of other studies that face seal leakleakage, and not filter penetration, is a key parameter to be considered.

Figure: Particle number concentrations inside the suit and in the chamber along the exercises during a generic test of a suit.

In general terms, the results of the study conducted revealed that an adequate protection of the human health and the environment against NMs can be achieved by means of the combination of administrative controls, engineering controls and personal protective equipment. Notwithstanding, a proper risk evaluation by expertise staff should be conducted to evaluate the risk in the workplace.

The experimental studies within NANoREG confirmed the findings of other studies that face seal leakage, and not filter penetration, is a key parameter to be considered.

Left Static test (Clothes). Right Dynamic test with volunteers

Based on the results of testing the PPEs and considering data from relevant guidelines and scientific publications, recommendations can be defined with respect to PPE for inhalation as well protective gloves and clothes.

The results of the testing activities are compiled in a library of nano-specific Risk Management Measures (RMM) and designed to provide small and medium sized enterprises (SMEs), large companies, and other relevant stakeholders with an easy to use tool to select proper measures to achieve a high level of protection of the human health and the environment. The library follows the structure of the RMM library developed under the scope of the REACH implementation project 3.2, which provides insight into the efficacy of technical control measures in the workplace.

The data generated on the effectiveness of common RMMs for different exposure situations will allow regulators and industry to make appropriate choices in risk management decisions and measurements for specific exposure situations. Moreover, the development of standard procedures for RMM effectiveness testing will allow the regulatory bodies to define new performance requirement to ensure a high level of protection for the human health and the environment.

The performance factors defined per each RMM category will be transferred to existing tools oriented to support risk characterization and risk management, including the Nano Exposure & Contextual Information Database (NECID) hosted by PEROSH , the ECEL library hosted by TNO, and the exposure estimation models NanoSafer and the Advanced REACH Tool (ART).

Resulting documents/data		Input for
Deliverable 3.09: NANoREG Improved measurement instruments, tools and methods		Toolbox section 4.0
Factsheet 3.09		
Library on nanospecific Risk Management Measures		
SOP1 Respiratory protective devices. Determination of inward leakage (IL) and total inward leakage (TIL) of nanoparticles (annex 1 to deliverable)		
SOP2 Respiratory protective devices. Determination of particle filter penetration (annex 2 to deliverable)		
SOP3 Chemical protective gloves. Determination of the penetration resistance to nanoparticles (annex 3 to deliverable)		
SOP4 Chemical protective gloves. Determination of the permeation resistance to nanoparticles (annex 4 to deliverable)		
SOP5 Protective clothing. Determination of the inward leakage of aerosols of nanoparticles into suits (annex 5 to deliverable)		
SOP6 Protective clothing. Determination of resistance to penetration by a spray of liquid (spray test) (annex 6 to deliverable)		
SOP7 Local Exhaust Ventilation. Determination of the nanoparticle capture efficiency of fume hoods (annex 7 to deliverable)		
SOP8. Local Exhaust Ventilation. Determination of the nanoparticle capture efficiency of Movable LEVs (annex 8 to deliverable)		

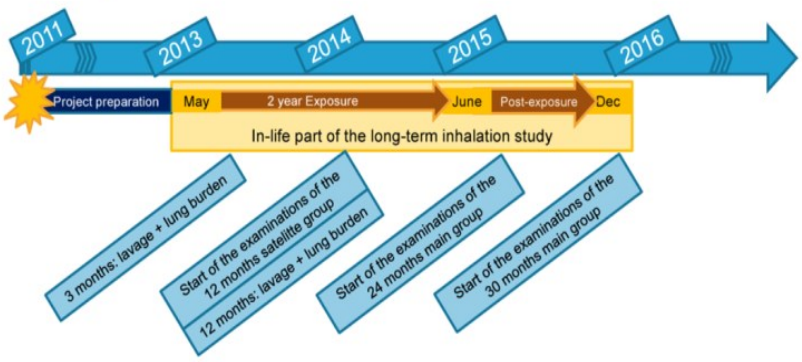
3.1.4 Work package 4

Results	*Impact*
So far no evidence for nano-specific toxicity/ new toxicity endpoints in *in vivo* testing	*Contributions to adequate regulatory assessment of granular and fibrous nanomaterials*
Systemic low level accumulation of granular CeO_2 nanomaterial did not lead to systemic toxicity so far	*Contributions to adequate regulatory assessment of granular nanomaterials*
No macroscopically visible lung tumours were found after 24 and 30 months with low dose exposures of nano-CeO_2 and $BaSO_4$	*Contributions to adequate regulatory assessment of granular nanomaterials*
Studies with fibrous nanomaterials found that physical and chemical properties (surface area, surface oxidation, length and diameter) influenced toxicity	*Contributions to safer by design approaches for fibrous nanomaterials*
Biopersistent nano- and bulk nanofibrillated celluloses materials showed a relative high toxicity and biopersistence.	*Contributions to adequate regulatory assessment of biopersistent nano- and bulk nanofibrillated celluloses*
Cellulose materials: the outcome of the *in vivo* toxicity tests was not consistently predicted by *in vitro* toxicity studies	*Contributions to adequateness of in vivo/in vitro test comparison*
No marked and clearly dose-dependent effects after low oral doses of amorphous silicon dioxide	*Contributions to adequate regulatory assessment of amorphous silicon dioxide*
No prenatal toxicity in studies with cerium dioxide, amorphous silicon dioxide and multi-walled carbon nanotubes	*Contributions to adequate regulatory assessment of these material*
Methodological limitations in the standardisation of manufactured nanomaterials (NMs) ecotoxicity testing addressed	*Basis for more valid result outcome in future regulatory testing*
Silver nanoparticles showed high ecotoxic potency. A lower ecotoxicity was generally observed for MWCNTs and titanium, cerium, silicon and zinc oxide NMs.	*Contributions to adequate regulatory ecotoxicity assessment of these materials*

Task 4.1, 4.2, 4.3, 4.4: Long-term inhalation study (D4.01, D4.02, D4.03, D4.04)

Long-term studies are considered as the golden standard for RA. A long-term (two-year) inhalation study on Granular Biodurable Particles (GBP), was performed with two well characterized nanomaterials. The aim of the study is to identify chronic and carcinogenic effects of these materials, to determine concentration-response relationships and to verify/falsify the assumed mode of threshold-like action for carcinogenicity.

√ Performing the inhalation study



A chronic inhalation study was carried out according to OECD TG 453 under GLP (Good Laboratory Practice) with OECD depository material. It started with 100 rats per dose group. 50 animals per dose group were sacrificed after 24 months. The remaining animals were kept exposure-free till natural death or till month 30. Animals of each group which died during the exposure or post-exposure period were examined as well (see figure study timeline).

Based on a 28 day range-finding study with nanoscaled CeO_2 (NM212) and $BaSO_4$ (NM220), the following concentrations were selected: 3 mg/m³ as high concentration with expected toxic effects, 1 mg/m³ as mid concentration, 0.3 mg/m³ as a second low concentration, and 0.1 mg/m³ as low concentration. For $BaSO_4$ a high concentration of 50 mg/m³ was selected.

Study design

	CeO_2					$BaSO_4$
Test group Test concentration	0 control	1 0.1 mg/m³	2 0.3 mg/m³	3 1 mg/m³	4 3 mg/m³	5 50 mg/m³
Main group :	100	100	100	100	100	100
Main group OECD453 24 months	50	50	50	50	50	50
Main group 30 months	50	50	50	50	50	50
Satellite groups:						
Chronic OECD453 ***12 months***	10	10	10	10	10	10
Lavage and Lung Burden ***3 months***	5	5	5	5	5	5
Lavage and Lung Burden ***12 months***	5	5	5	5	5	5

Total: 840 animals

Satellite groups were sacrificed after 12 months (chronic group with 10 animals per dose for histopathology) and after 3 months, 12 months, and 24 months (for kinetic/organ burden evaluations) (see figure study design). The results have been reported in deliverables.

The mortality rates were in an acceptable range. Macroscopically evident tumours were not detected after 24 months of exposure.

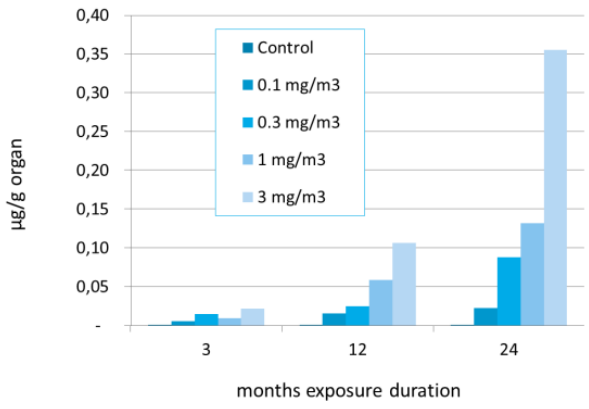
Resulting documents/data		Input for
Deliverable 4.01: Initiate and perform in-life long-term inhalation study		
Factsheet 4.01		
Deliverable 4.02: Provide histological samples from chronic study		
Deliverable 4.03: Lung burden and particle detection and quantification in olfactory bulbs, blood,- subacute exposure		
Factsheet 4.03		
Deliverable 4.04: Organ burden and particle detection pattern in other organs after subacute exposure		
Factsheet 4.04		

√ *Organ burden of CeO_2 and nanoparticle organ distribution (D4.05, D4.06)*

The CeO_2 (NM-212) burdens in peripheral organs and faeces out of the 2 year chronic inhalation study was investigated. The lung burdens were maximally 3500 µg/g lung at the highest exposure concentration of 3 mg/m³. Independent on the exposure concentration the CeO_2 the organ burden increased from 3 to 24 months in a linear manner with a factor of ~5-7 including the concentrations below overload despite accumulation over exposure time (see figure).

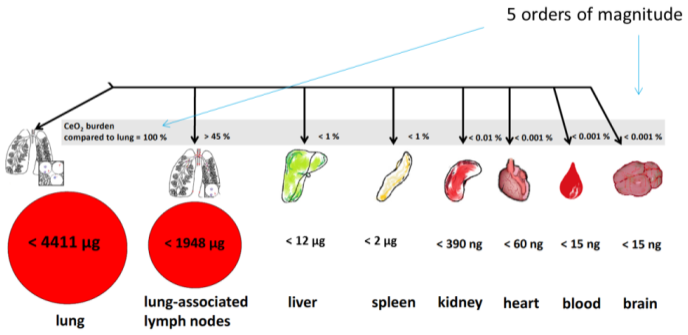
The CeO_2 burden of liver, kidney, spleen, brain, heart, bone, and olfactory bulb was generally very low (far less than 1 µg/g organ) (see figure).

Example: cerium burden in kidney



The highest CeO_2 burdens in organs remote to the exposed organ lung was bone, liver and spleen with maximally 1.9, 1.4, and 1 µg/g tissue. In brain, maximum CeO_2 levels were 0.005 µg/g tissue which is a factor of 700000 below the lung burden. Only in tracheobronchial and mediastinal lymph nodes, high levels of CeO_2 were found with maximally 2.5 mg/g total organ. This may be due to the fact that these lymph nodes drain the lung. CeO_2 concentrations in blood were on a very low level of < 0.1 ng/ml.

Systemic CeO_2 distribution low



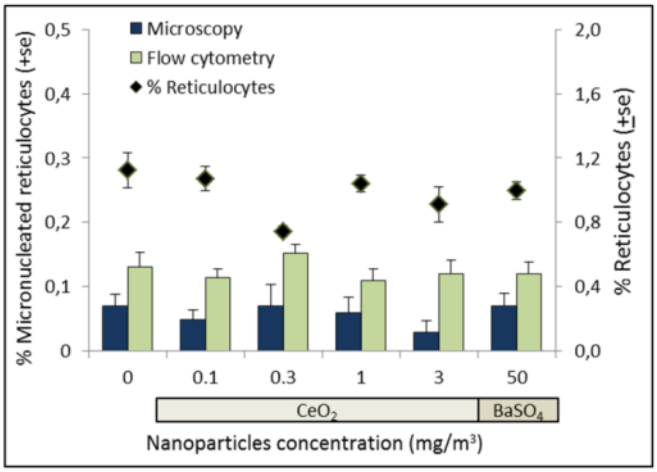
Lung tissue of Wistar rats showed an inhomogeneous distribution of the nanoparticles following 2 years of chronic CeO_2 inhalation. The CeO_2 particles were found mainly in macrophages and the alveolar septum. Nanoparticles were also detected in the endothelium of blood vessels and in close vicinity of the nucleus in pneumocytes. Some nanoparticles were able to cross epithelial and endothelial barriers of alveoli and showed translocation into other organs.

Resulting documents/data		Input for
Deliverable 4.05: Lung burden and particle detection and quantification in olfactory bulbs - chronic exposure		
Factsheet 4.05		
Deliverable 4.06: Organ burden, faeces analyses and particle detection pattern in other organs after chronic exposure		
Factsheet 4.06		

√ Long term effects of nanomaterials: systemic toxicity and histopathological evaluation (D4.07)

The histological examinations are ongoing until the end of 2017. Results are available from the 12-months interim section. There was some evidence that an inflammation-mediated secondary local genotoxicity in the lung could not be excluded at the higher exposure concentrations. On the other hand, CeO_2 inhalation exposure did not induce any significant effect on the analysed systemic genotoxicity endpoints, irrespective of dose and time (see figure).

No systemic genotoxicity after 3 and 6 months



CeO_2 exposure-related histopathological findings were exclusively observed in the respiratory tract but not systemically. In the nasal cavity, the incidence of age-related intra-epithelial eosinophilic globules was increased in the 3 mg/m³ high-dose CeO_2 exposure group as compared to the control group and associated with minimal inflammatory cell infiltration. Adverse effects in the lung included dose-dependent alveolar/interstitial inflammatory cell infiltration, alveolar/interstitial granulomatous inflammation and interstitial fibrosis. Alveolar lipoproteinosis was observed in the 3 mg/m³ high-dose CeO_2 exposure group only and cholesterol granulomas occurred in a single female each CeO_2 the 1 and 3 mg/m³ CeO_2 exposure groups. After 12 month of inhalation exposure neither neoplastic nor pre-neoplastic treatment-related findings were seen in the lungs of CeO_2 -exposed animals. No macroscopically visible tumours were detected at the 24 months and 30 months sacrifices. Although statistically not significant, some adverse effects such as alveolar/interstitial inflammatory cell infiltration, alveolar/interstitial granulomatous inflammation, and interstitial fibrosis have already been observed in the 0.1 mg/m³ low-dose CeO_2 exposure group (see table). Thus, a NOAEL (no observed adverse

effect level) could not be established for the lung after 12 months of exposure to the present CeO_2 nanoparticle concentrations.

There was no evidence for systemic toxicity in the interim section after 12 months including the lung-associated lymph nodes although the cerium levels were relatively high in this tissue.

The final results will be available end of 2017. The main result will deal with the question whether tumours will have been induced in the lower concentrations below the overload threshold postulated by Morrow. Also the results on a putative systemic toxicity, i.e. whether adverse effects were induced in organs remote to the exposed organ lung after chronic exposure, will be available.

Resulting documents/data		Input for
Deliverable 4.07: Histopathological evaluation (all organs) Immunohistochemical detection of local and systemic genotoxicity		
Factsheet 4.07		

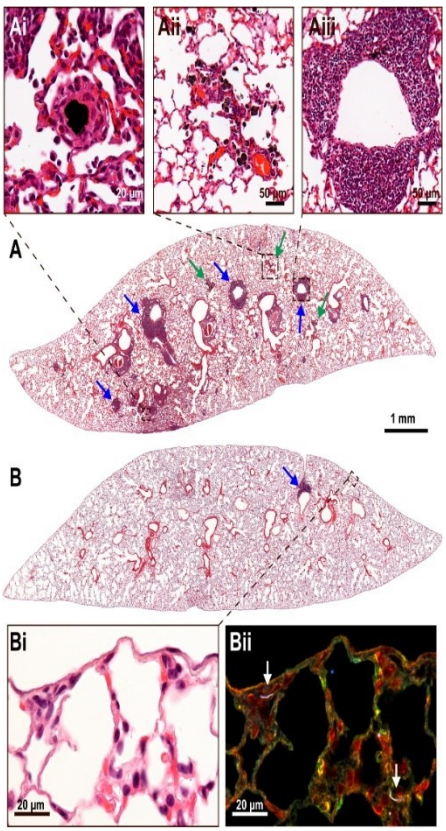
The deliverables mentioned above report the outcome of the different sub studies. A summary and overall evaluation of the results of the long term inhalation study will be presented in Deliverable 4.18.

Task 4.5: Other biokinetic and oral, dermal, inhalation toxicity studies in vivo

√ *Mode of toxic action of high aspect ratio nanomaterials (D4.13)*

To get more insight on the physico-chemical properties of MWCNT that cause toxicity, ten commercial carbon nanotubes in three groups of different dimensions with one pristine and two/three surface modified in each group were tested in mice by **intratracheal installation**.

Pulmonary inflammation and genotoxicity were determined on day 1, 28 or 92. Histopathology of the lungs was performed on day 28 and 92. The specific surface area (BET) was identified as a positive predictor of pulmonary inflammation on all post-exposure days. In addition, length significantly predicted pulmonary inflammation, whereas surface oxidation (–OH and –COOH) was predictor of attenuated inflammation on day 28. BET surface area, and therefore diameter, significantly predicted genotoxicity in BAL fluid cells and lung tissue such that lower BET surface area or correspondingly larger diameter was associated with increased genotoxicity



Effects of the ten MWCNTs and MWNT XNRI-7 were also evaluated after one year at the single dose 54 µg/mouse (Lung histopathology and genotoxicity in the secondary organs liver and spleen). MWNT XNRI-7 was selected since it was recently classified as possibly carcinogenic to humans.

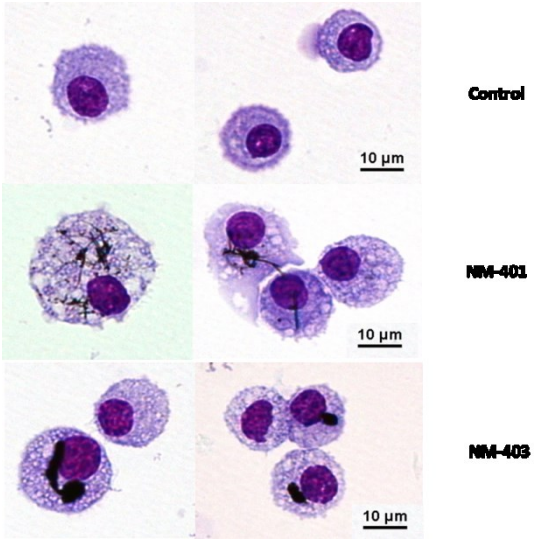
There were no treatment related neoplasms in pleura or lung. Examination of visceral pleura from the diaphragm and the chest revealed no tumours or other significant histopathological changes. In the lungs a few tumours were observed but the numbers of cases were too low to allow statistical evaluation. The study was not designed for evaluation for tumour formation, as the group size was too small and follow up time was too short. Only few fibrotic lesions were observed across the MWCNT exposed groups. The numbers of fibrotic lesions were also relatively low and the fibrotic lesions were rather focal. Lymphocytic aggregates were consistently observed

Figure: Representative overviews of H&E stained paraffin-embedded lung tissue from mice exposed to NM-403 (Fig 1A) and NRCWE-006 (Fig 1B) one year after exposure. MWCNT were generally observed as black aggregates in macrophages or granulomas (Fig 1Ai-ii and Fig S1-2) for all MWCNT except NM-401 and NRCWE-006. Only a few NRCWE-006 aggregates were found in lobes of a few exposed mice. With enhanced dark field microscopy, NRCWE-006 (Fig 1Bi-iiA) and NM-401 were found to be distributed as single fibres spread throughout the lung

signifying chronic inflammation. There was great variation in the induction of histopathological changes after one year with the 11 MWCNT with different physico-chemical properties. The pulmonary toxicological properties of two carbon nanotubes (NM 401 and NM 403; also tested in the previous mentioned tests) was examined in rats by **nose only inhalation**.

Inhalation of the highest concentration of NM-401 (1.5 mg/m3) led to a massive influx of granulocytic neutrophils 3 days after the end of the exposure an effect that abated over time but was still statistically significant after 180 days. There were more lymphocytes 3 days post-exposure and the number decreased over time. The sub-acute exposure to the lowest concentration of NM-401 (0.5 mg/m3) led to a small but statistically significant reduction of the number of macrophages in the BALF 3 and 30 days post-exposure; there were no changes in numbers of neutrophils or lymphocytes.
Inhalation of NM-403 increased the number of neutrophils for both concentrations 3 days after exposure and to a lesser extent at 30 days post-exposure. At day 90 day post-exposure there was no increase in neutrophils in animals that had inhaled 0.5 mg/m3 of NM-403, but it was not statistically significantly greater after inhalation of 1.5 mg/m3. NM-401 only induced significant DNA damages in lung cells at the 30 days post-exposure time, whereas the dose of 0.5 mg/m3 induced statistically significant DNA strand breaks at 30 days only with Fpg and at 180 days. (The Fpg enzyme detects oxidative damage).

Figure: *Representative optical microscope images of BALF macrophages from controls and animals exposed to NM-401 or NM-403 aerosol. BALF cells were cytospun on glass slides and stained with the May-Gunwald Giemsa method.*

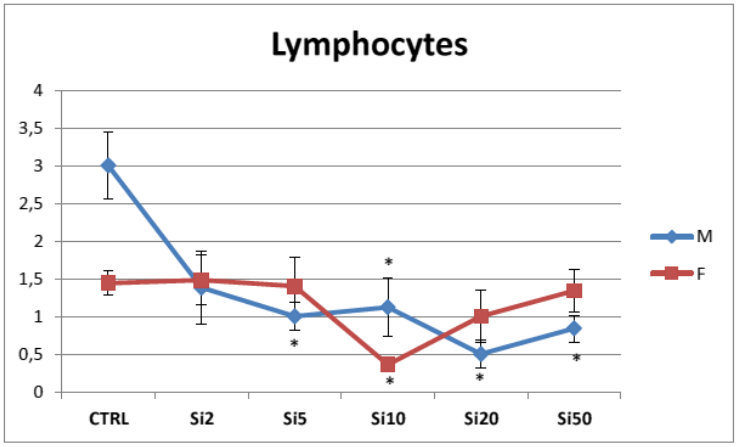


Evaluation of the testing of NRWCE-64 (carboxylated MWCNT) and NM 401 by **whole body inhalation** aimed at comparison of the nose-only results, is not completed yet. Preliminary results for NRWCE-64 (Carboxylated MWCNT) at 1.5 mg/m3 indicate no significant change to total BALF cell numbers at 3 and 30 days post-exposure.

Resulting documents/data		Input for
Deliverable 4.13: Mode(s) of toxic action of high aspect ratio nanomaterials – HARN		
Factsheet 4.13		

√ *Repeated-dose 90-day oral toxicity study (D4.11)*

A repeated-dose 90-day oral toxicity study in rat has been carried out on the basis of the OECD TG 408 with additional parameters (endocrine, immune, reproductive, genotoxic endpoints). This protocol is considered by EFSA (EFSA, 2011) as the minimum requirement to identify hazards and obtain dose-response data of nanomaterials relevant for food safety. The test material chosen for the study is amorphous silica dioxide (SiO_2 NM-203, JRC repository) - the food additive E551 - which has been selected as nanomaterial model in the Nanogenotox Joint Action - (http://www.nanogenotox.eu/) for oral testing due to its relevance in food safety and successfully tested in short-



term oral studies in rodents.

The administered doses were 2, 5 10, 20 and 50 mg/kg bw per day. Characterization of NM203 dispersion was carried out by DLS and FF-UV-MALS-ICP-MS on the basis of Nanogenotox dispersion protocol.

No effects have been recorded concerning general toxicity in both male and female rats during the treatment period at all doses tested. No reproductive or genotoxic effects have been reported after the 90-day treatment.

Although the dose-response was not linear for most parameters (see picture) and no NOAEL can be derived, it was possible to calculate the BMDs, although influenced by high confidence limits: 0.468 mg/kg body weight per day for decreased lymphocytes in blood count in male rats and for female rats the BMD of 0,079 mg/kg body weight per day for intralobular lymphoid infiltration in liver.

Resulting documents/data		Input for
Deliverable 4.11: Identification of hazards and NOAELs for amorphous silica after subchronic oral exposure		
Factsheet 4.11		

√ *Prenatal toxicity study (D4.14)*

To get insight in the effects of nanoparticles on pregnant women, prenatal toxicity studies were carried out. Pregnant mice were exposed to different concentrations of Cerium dioxide (CeO_2 JRCNM02102a 5 and 20 mg/kg) and multiwalled carbon nanotubes (MWCNT JRCNM04001a 200 and 800 mg/kg oral gavage) through two different routes: via the oral route for carbon nanotubes and, and via the pulmonary route for cerium dioxide. Maternal effects, characterized by a lower weight gain during gestation were observed in mice exposed to multi-walled carbon nanotubes at the highest dose. No overt toxicity in terms of miscarriage or malformations was found. The data suggest a lack of relevant embryo toxicity.

From the perspective of regulators and policy makers, the data imply that unintended oral exposure to CNTs and pulmonary exposure to cerium oxide nanoparticles at doses which can be realistically expected in occupational and environmental settings, should not pose a peculiar risk to pregnant women.

Resulting documents/data		Input for
Deliverable 4.14: Prenatal toxicity study with cerium oxide and SWCNT carbon nanotubes		
Factsheet 4.14		

√ *Acute and repeated nose-only inhalation toxicity study (4.15)*

The original aim of NANoREG task 4.5.7 was to define protocols for acute and repeated in-vivo inhalation exposure studies relevant to oxidative-stress, inflammation and genotoxicity, to give reference data on different nanomaterials (NMs) and to compare *in vivo* test results to *in vitro* results. Due to technical issues, inhalation exposure studies had to be replaced by an instillation study at doses similar to those expected for the acute and repeated inhalation studies.

All NMs were detected in the tracheobronchial lymph nodes after 35 and 90 d. There was no significant systemic distribution in liver, kidneys and spleen.

The table below summarizes the cytotoxicity, oxidative and pro-inflammatory potential of the 4 NM tested 3h, 24h and 5d after a single intratracheal instillation using the mass as dose metric. Genotoxicity was determined in the blood of the rats of the 5-day group, 2 days after instillation.

		Cytotoxicity			Oxidative stress			Inflammation			Genotoxicity
Exposure duration after a single IT		**3h**	**24h**	**5d**	**3h**	**24h**	**5d**	**3h**	**24h**	**5d**	**2d**
TiO_2	NM105	> 500 µg			> 500 µg			**ND**	500 µg	500 µg	> 500 µg
	NM101	> 500 µg			> 500 µg	> 500 µg	500 µg	> 500 µg	500 µg	> 500 µg	> 500 µg
	NM100	> 500 µg			> 500 µg			> 500 µg			> 500 µg
CeO_2	NM212	**500 µg**	> 500 µg		> 500 µg	ND	>500 µg	> 500 µg	500 µg	500 µg	> 500 µg
	significant effects allowing the determination of a LOAEL							IT	intratracheal instillation;		
	significant effects but no dose-related							ND	not determined		
	no significant observed effects										

Table: Lowest observed adverse effect levels (LOAEL in µg/rat). To assess the cytotoxicity, the LDH levels in BALF were assessed. For the oxidative stress, the levels of intracellular ROS were measured in BALF cells. For the inflammation, BALF cytology and total proteins, IL-1β, IL-6, Kc-Gro, TNF-α were assessed. Genotoxicity was evaluated by the micro-nucleus assay on blood erythrocytes.

The histopathological examination (for NM100 and 101) showed no treatment-related finding.

Using cytotoxicity and ROS measurements, it was not possible to discriminate between the different TiO_2 tested.

Considering the overall short-term toxicity in the used instillation protocol, the relative ranking of the NMs seemed to be best described by NM105~NM101~NM212 > NM100 when using the mass as dose metric. When taking into account the surface area of each NM, the NMs were ranked differently in term of toxicity.

Resulting documents/data		Input for
Deliverable 4.15: Protocol for inhalation exposure and choice of biological relevant endpoints		
Factsheet 4.15		

√ *Development of PBPK models (D4.17)*

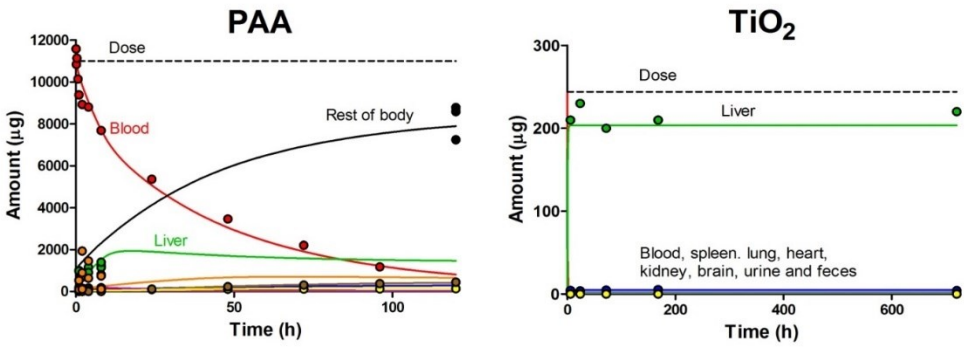
Physiologically based pharmacokinetic (PBPK) models have the potential to describe and predict the biokinetics of nanomaterials (NMs) in organisms on the basis of physico-chemical or other characteristics of a NM, and may strongly contribute to the efficiency of RA and the implementation of Safe by Design. They have the potential to reduce costs of- and the use of animals for assessing risks of NMs substantially.

A conceptual nanospecific physiologically-based pharmacokinetic (PBPK) model for intravenous administration to rats was developed and applied on different types of inert nanoparticles. As data generated in the NANoREG project only became available in a late stage of the project, the development and calibration of the model is based on datasets from other projects and with other materials than NANoREG core materials.

A conceptual model was constructed based on findings from literature review and organs analysed in study on PAA-PEG injected intravenously into rats. Next, the model was refined and optimized to data sets on PAA, gold and titanium dioxide. The model consists of 10 compartments; arterial blood, venous blood, liver, spleen, lung, kidney, heart, brain, bone marrow and carcass. Each compartment is divided into three sub compartments: capillary blood, tissue and phagocytic cells.

To evaluate if the PAA-PEG model can be used as a general model the model was tested on three additional types of nanoparticles; PAA, gold and titanium dioxide. The model adequately describes the biokinetic behaviour of all four NP types only by adjusting nanoparticle related parameters (R^2 on the log scale ranging from 0.88 to 0.96).

The PBPK model expanded with inhalation, describes the biodistribution of inhaled nanoceria well and is able to reproduce the different experimentally observed trends (R^2 on the log scale ranging from 0.68 to 0.95).



Simulated and experimentally observed amounts of polyacrylamide (PAA) and titanium dioxide (TiO2) nanoparticles in different tissues and organs of the rat after various time-points. Simulated (solid lines) and observed (symbols) time-courses

The conceptual model developed described in Deliverable 4.17 is the first one to include a separate compartment for saturable phagocytic cells. This structure has subsequently been adapted and modified in other published models, which supports its importance in nanospecific PBPK models.

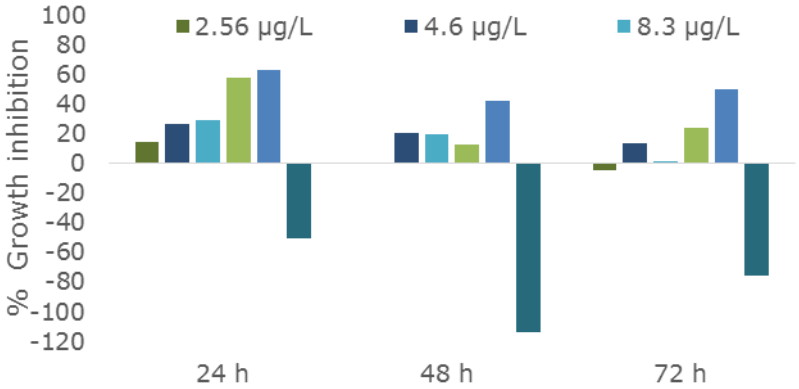
In agreement with the results from experimental biodistribution studies, the modelling exercises demonstrate that kinetics depends on both nanoparticles properties and exposure conditions.

Despite some major achievements, these nano-PBPK models are still in their infancy and cannot yet be readily used in the regulatory arena. On the other hand, PBPK modelling provides valuable information about uptake and distribution but need to be further refined before they can be successfully used as regulatory tools. The deliverable gives recommendations for further improving the nano-PBPK models.

Resulting documents/data		Input for
Deliverable 4.17: Development of nano specific physiologically based pharmacokinetic models		
Factsheet 4.17		

Task 4.6: Biokinetics and toxicity in aquatic organisms (D4.12).

Among the multiple pathways of NMs into the ecosystems, aquatic organisms constitute one of the most important for their entrance and transfer throughout the food web. Accumulation potential and toxicity of manufactured NMs at low concentrations in the aquatic environment however are largely unknown. To fill this gap in knowledge, a large scale toxicity test programme has been executed with a selection of the NANoREG core materials. Prior to that, Standard Operating Procedures (SOPs) have been developed to overcome the limitations of the existing procedures for ecotoxicity testing, mainly related to the lack of stability and reproducibility of NM dispersions. The SOPs include the optional addition of environmentally representative concentrations (10 mg/L) of Suwannee river natural organic matter (SR-NOM) to the culturing media where NM dispersions are unstable. An increase in the dispersion stability of several NMs (MWCNTs, CeO_2 NPs and TiO_2 NPs) has been observed after this addition of SR-NOM, leading to a corresponding improvement in reliability and reproducibility of the ecotoxicity test results.



Growth inhibition in P. subcapitata after exposure to Ag

Following the above-mentioned SOPs, reproducible dispersions for most of the 14 core NMs could be achieved at both the intra- and inter-laboratory levels. However, results for certain NMs such as the high aspect ratio nanomaterial (HARN) carbon nanotubes (JRCNM04000a, JRCNM04001a, JRCNM04100a) were limited.

Three priority species representing different trophic levels have been selected for testing with NANoREG core NMs: the unicellular green algae *Pseudokirchneriella subcapitata*, the crustacean *Daphnia magna* and the soil nematode *Caenorhabditis elegans*. Existing OECD and ISO standard methods for ecotoxicity assessment have been adapted specifically for NM testing and developed into defined SOPs.

To get insight in the biokinetics, particle accumulation in *Daphnia magna* has been analysed. Furthermore, the uptake, accumulation and hepatic gene expression in Brown trout (*Salmo trutta*) were analysed through water and diet.

Silver nanoparticles showed high ecotoxic potency, and lower ecotoxicity was generally observed in the test systems for MWCNTs and titanium, cerium, silica and zinc oxide NMs.

Ecotoxicity assay	Toxicity (decreasing)
Daphnia magna	Ag > ZnO > SiO_2 > CeO_2 > MWCNTs >TiO_2
P. subcapitata	Ag > ZnO > CeO_2 ≈ TiO_2
C. elegans	Ag > ZnO > SiO_2 > TiO_2 > CeO_2 > MWCNTs > Ag nanorods
Danio rerio	Adverse effects only for Ag NPs (JRCNM03000a)
Chironomus riparius	Adverse effects only for Ag NPs (JRCNM03000a); ZnO produced also an effect to some extent, but it was considerably alleviated in the presence of SR-NOM.

Regarding biokinetics, the tests conducted showed differences in uptake and accumulation in organs of brown trout exposed to silver ions and to nanoparticles both within and between exposure routs.

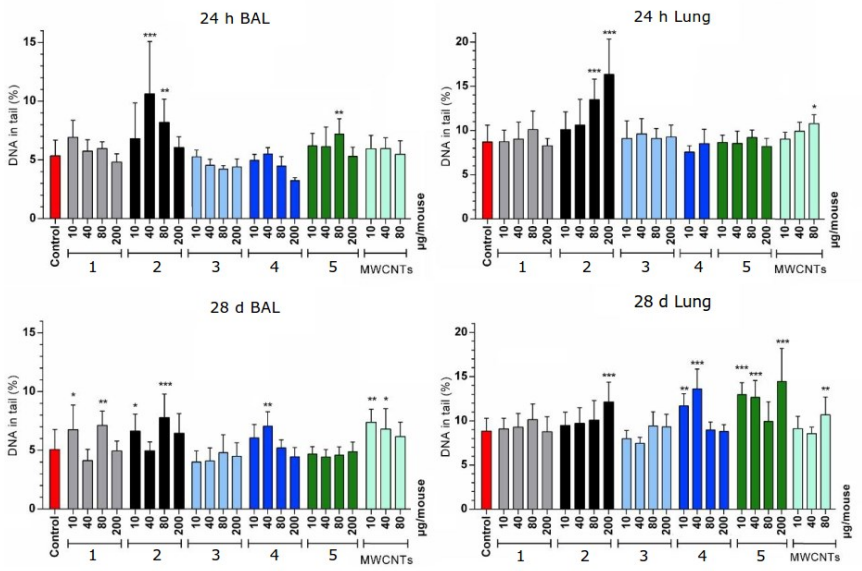
Finally, a regulatory approach to the results and conclusions has been provided in the form of decision trees and hazard potency categorization in order to make them clear and understandable for policymakers.

Resulting documents/data		Input for
Deliverable 4.12: Accumulation potential and aquatic toxicity of relevant groups of nanomaterials and products formula		
Factsheet 4.12		
SOP 01 Toxicity Test with *Daphnia magna* for NANoREG core nanomaterials (Annex 1 to Deliverable 4.12)		Toolbox section 3.6 Risk characterization
SOP 02 Toxicity Test with *Microalgae Pseudokirchneriella subcapitata* (Annex 2 to Deliverable 4.12)		
SOP 03 Toxicity test with the nematode *Caenorhabditis elegans* (Annex 3 to Deliverable 4.12)		
SOP 04 Fish, Acute Toxicity Test (Annex 4 to Deliverable 4.12)		
SOP for probe sonicator calibration of delivered acoustic power and de-agglomeration efficiency for ecotoxicological testing		
Protocol for producing reproducible dispersions of manufactured nanomaterials in environmental exposure media (see deliverable 2.06)		
Decision trees for the ecotoxicological assessment of MNMs (Section 2.5.3 Deliverable 4.12)		Toolbox section 4

Task 4.7: Acute immunotoxic and genotoxic effects of fibrous nanomaterials (D4.16).

Cellulose is a naturally occurring fibrous material that is generally considered safe. Cellulose pulp is exempted from the REACH regulation because it is considered to cause minimum risk, due to its intrinsic properties. Nanocellulose is one of the most promising innovations in the forest industry. The term nanocellulose generally refers to cellulosic materials having at least one dimension in the nanometre range. Nanocelluloses possess unique properties compared with the bulk material. Nanofibrillated cellulose (NFC) is a fibrous material with a higher surface area to volume ratio than bulk cellulose fibres. This raises some concern that NFC might act similarly to asbestos which is known to cause lung fibrosis and cancer. Toxicity studies on nanocellulose, especially the fibrillate form, are still very scarce.

Four nanofibrillated cellulose (NFC) materials have been tested. Also a sample of bulk-sized pulp and multiwalled carbon nanotubes (MWCNTs) was included in the study, to obtain comparative data on traditional cellulose and on another type of fibrous material. In vivo studies in mice were used to investigate both acute and subacute responses.



*In vivo comet assay (percentage of DNA in comet tail; mean ± SD). 1-4 = NFC materials, 5 = bulk-sized pulp. (***p<0.001, **p<0.01, *p<0.05; one-way ANOVA).*

All NFC materials, except one, caused DNA damage in lung or BAL cells, as determined by the comet assay. For one NFC, the effect was dose-dependent in lung cells both 24 h and 28 days after the exposure. The comparative materials, bulk-sized pulp and MWCNTs, were also able to induce DNA damage after 24 h and 28 days. None of the NFCs was shown to possess systemic genotoxic properties as measured by the micronucleus assay in bone marrow.

28 days after the exposure, no significant increases in the numbers of neutrophils or eosinophils were seen in BAL, indicating that the inflammation had resolved.

Histopathological assessment revealed that most of the NFC materials induced a moderate neutrophilia 24 h after the treatment. The neutrophilic response was accompanied by a low number of eosinophils. The bulk-sized pulp did not induce notable influx of inflammatory cells into lung tissue. Although the celluloses appeared to be biopersistent in the lungs during the follow-up time of 28 days after the treatment, no considerable lung tissue reactivity and no pathological changes were observed in response to the celluloses. MWCNTs induced neutrophil and eosinophil recruitment as well as aggregates surrounding the material in the 24-h sampling, and granuloma formation and PAS positivity were observed 28 days later.

In conclusion, the results indicate that, although the tested NFC materials were able to induce DNA damage and inflammatory responses at 24 h, the mice exposed to the NFCs showed signs of recovery of the inflammation at 28 d. On the contrary, the mice treated with MWCNTs exhibited characteristics of longer term adverse health effects. However, the observation that the NFCs, and the bulk-sized pulp, were biopersistent in the lungs for at least 28 days raises some concern, because some increase in the level of primary DNA damage was still observed at this time point. A longer follow-up would be required to better define the fate of the NFC material in the lungs and the duration of the increased level of DNA damage.

Resulting documents/data		Input for
Deliverable 4.16: immunotoxic and genotoxic effects after short-term inhalation of fibrous nanomaterials		
Factsheet 4.16		

Overall evaluation of results biokinetics and toxicity testing in vivo (Deliverable 4.18)

Deliverable 4.18 evaluates and summarises the findings of the work done by NANoREG work package 4. The main conclusions are presented below.

Relevant commercial forms of nanosized material like carbon black, titanium dioxide or cerium dioxide may be subsumed under the category nanoscaled granular biodurable particles without known specific toxicity. There is evidence that such particles induce lung tumours in rats, possibly also at low exposure doses. Concerns were expressed on a putative long-term accumulation of these particles in the body also leading to toxic effects in other organs. A long-term inhalation study was carried out to study these issues. The currently available first set of results showed that cerium dioxide particles accumulate at a rather low level in the body. After one year of exposure, no resulting damage in organs aside from the lung was found. In the lung, inflammation was detected but no macroscopically visible tumours were found after 24 and 30 months. Further histological evaluation of the available study material will show whether lung tumours were induced also at the lower dust exposures used in the study.

Studies with fibrous nanomaterials found evidence that physical and chemical properties (surface area, surface oxidation, length and diameter) influenced toxicity. Nano- and bulk cellulose materials showed a relatively high toxicity and biopersistence.

A 90 day oral study with amorphous silicon dioxide emphasized low dose toxicity testing. Numerous endpoints were covered but no signs of overt toxicity were found.

Some first PBPK models for nanomaterials were developed which need further development to be usable in regulatory practice.

The performed ecotoxicity studies addressed methodological limitations in the standardisation of manufactured nanomaterials (MNMs) ecotoxicity testing and performed round robin-style testing. Within NANoREG, the existing OECD and ISO standard methods for ecotoxicity assessment using three priority species representing different trophic levels have been adapted specifically for MNM testing and developed into defined Standard Operational Procedures (SOPs). Silver nanoparticles showed high ecotoxic potency. A lower ecotoxicity was generally observed in the test systems for MWCNTs and titanium, cerium, silicon and zinc oxide MNMs.

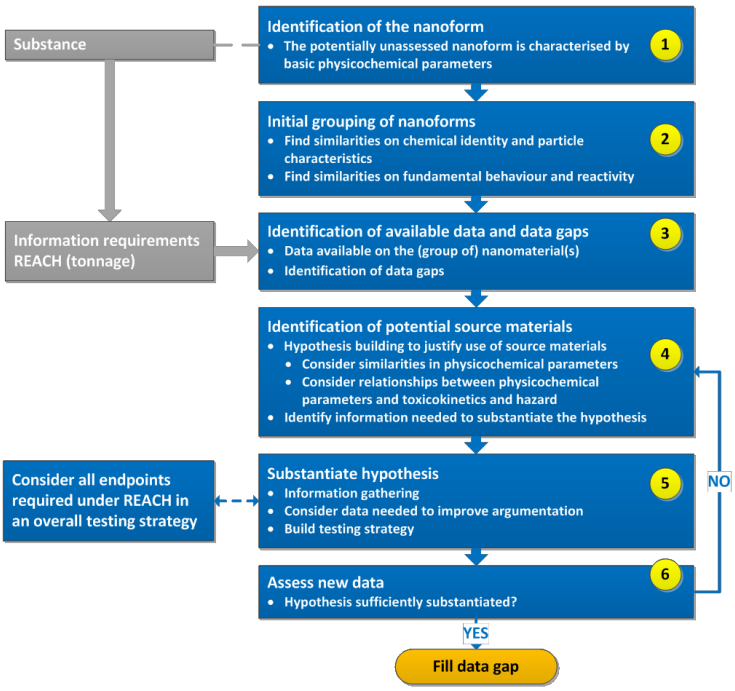
Resulting documents/data		Input for
Deliverable 4.18: Overall evaluation of data, publication, and transfer to OECD sponsorship programme		White Paper

3.1.5 Work package 5

Results	*Impact*
Criteria for categorisation, read-across and extra- and interpolation	*Contributes to a more cost-efficient testing of nano materials*
Methodology for testing solubility	*Determination of solubility is an important step in RA of NMs. The outcome strongly influences the testing strategy to be followed*
Methodology for *in vitro* testing inhalation toxicity	*Inhalation is the main route of entry of NM into the body. At present inhalation toxicology implies mainly in vivo experimentation since in vitro alternatives are mainly performed with submerged cultures. The contribution of this task is to look into the future and propose novel in vitro methodologies and exposure systems which better simulate in vivo situations without the ethical and economic implications of animal experimentation*
Methodology for in vitro testing in a regulatory context	*Identification of the most relevant methodologies to assess toxicity of NM in vitro mainly based on accepted guidelines.*
Insight in applicability of High Throughput screening and High Content Analysis for nanomaterials	*Contributes to the application of such techniques resulting in a higher testing volume and more cost-efficient testing of nano materials*
Flow chart for risk assessment	*Contributes to targeted RA: identification of elements that play a key role in human health RA, and how they can be used to prioritise nanomaterials that may lead to high exposure and/or high toxic potential.*

Task 5.1: Develop criteria for categorization, read-across, and extra-/interpolation (D5.01)

With the increasing number and variety of NMs, the need to develop more efficient ways to evaluate potential adverse effects of a nanomaterial is becoming more important. To address this need, a system for the grouping and read-across of NMs was developed, based on expected biological, ecological and/or toxicological effects. The recommendations for grouping, read-across, extrapolation and interpolation have been used in the development of a flowchart for RA that is part of the NANoREG regulatory framework/toolbox.



Two main goals of grouping are identified. The first is initial grouping for screening purposes and the second is grouping for the purpose of read-across to fill data gaps. In a scientific reference paper of ECHA, RIVM and JRC on read-across between nanoforms (ECHA et al., 2015), a stepwise procedure was proposed for using data between (nano)forms. For grouping for screening purposes, the first two steps of this procedure may already suffice.

From the examples of grouping and read across, it becomes clear that initial grouping for screening purposes is already possible for some limited NMs, route of exposures and endpoints. For nanoforms, examples of read-across used within regulatory RA are only available for specific narrow groups of NMs for a specific route of exposure and/or endpoint. Examples are the use of read across between high-aspect-ratio CNTs and asbestos (which is currently done in many control banding tools), the SCCS evaluation of TiO_2 in sunscreens and the NIOSH RELs for CNTs and TiO_2). Applying a stepwise approach to two hypothetical NMs showed that detailed information on the physicochemical characteristics is needed to identify and characterize the nanoform, and to support and justify read-across for specific exposure routes and/or endpoints.

Resulting documents/data		Input for
Deliverable 5.01: Report on identification and setting of categorization, read-across, and extra / intrapolation criteria		Toolbox section 3.3
Factsheet 5.01		

Task 5.2: Develop solubility testing procedures (D5.02)

Information on solubility of a nanomaterial is of paramount importance to assess or predict the effects and risks for human health and the environment. A solubility testing procedure was developed to be applied in rapid NM screening as part of the flowchart for RA.

An extensive review has been carried out on the “state of the art” concerning dissolution measurement methods. All possible methods for dissolution measurement were identified and evaluated. The review process was followed by experimental work. To improve comparability of the experimental work, and thus the methods investigated, all partners used in vitro digestion juices or cell culture media as complex media model systems. Several of the NANoREG core materials were selected for testing. Three ICP-MS/AES based methods have been tested. One was based on single particle (SP) ICP-MS measurement, and the other two consisted of a combination with ultrafiltration (UF) or ultracentrifugation (UC).

Besides ICP-MS/AES based methods also colorimetry methods were tested. They have the advantage to be cheaper and they work well with certain materials/metals. However, the choice of materials that work well with colorimetry is limited and complex matrices can interfere with the technique.

Finally, theoretical modelling of dissolution was evaluated, which was very helpful to provide insight in the dissolution behaviour of NMs.

Taken together, measuring dissolution in a complex matrix is highly challenging. At present it is not possible to devise one universal robust, rapid test method for regulatory testing that is applicable for all types of NMs in all types of matrices. However, several methods have been identified for future use (e.g. UF-ICP-MS/AES, UC-ICP-MS/AES, SP-ICP-MS, and colorimetry). A relative easy and highly robust method that can be used for a rather broad range of NMs appeared to be UF-ICP-MS/AES, given that no nanomaterial-matrix interactions take place. If these interactions do take place SP-ICP-MS would be a good choice for measurement, given that the material is not below the size detection limits. Nevertheless, to select the best suitable method, knowledge of physical-chemical properties of the NMs is crucial and it is recommended to use a combination of techniques.

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 standardize these procedures. When reporting dissolution analysis, the used protocol must be reported in detail, including all experimental conditions in which the data were collected. Finally, methods should be validated by conducting appropriate round robin studies and suitable reference materials should be available in order to obtain good quality results.

Resulting documents/data		Input for
Deliverable 5.02: Development of solubility testing procedures		Toolbox section 3.2
Factsheet 5.02		

Task 5.3: The relevance of barriers (D5.03)

A comprehensive understanding of the bio-distribution of NMs is essential to predict their effects and to evaluate their risks after exposure. The absorption of NMs through different biological barriers is the first step that determines internal exposure and systemic target tissue doses. Due to ethical and economical constrains, the development and use of relevant and alternative *in vitro* barrier models to predict the absorption of NMs should be of high priority.

Four *in vitro* barrier models (*i.e.* intestinal epithelium, lung, oral mucosa and blood-brain barrier) were either adopted from established protocols or developed and used to evaluate the potential internalization and crossing of several NANoREG NMs and their impact on cell model integrity. The suitability of several qualitative (TEM/SEM, confocal microscopy, CytoViva ultra high resolution microscope, flow cytometry) and quantitative (ICP-MS, PIXE, ICP-OES) techniques were considered in this task.

Before starting the crossing assays, the sub-toxic concentrations of the applied NMs have been determined using the MTS viability assay SOP developed in the NanoValid project. Core NM used in this task included TiO2 (NM100, NM101, NM103 and NM104), ZnO (NM110), SiO2 (NM200, NM203), CeO2 (NM212), BaSO4 (NM220), Ag (NM300K, NM302), and MWCNT (NM401). As a tracer fluorescent control, negatively and positively charged 50 nm SiO2 NMs were used.

The paracellular marker Lucifer yellow (LY) assay was used to evaluate the impact of NMs on the integrity of *in vitro* barrier models. This assay was shown to be suitable to evaluate the effect of NMs on intestinal epithelium, lung and BBB model integrity. No interference of NMs with this assay was observed. However, LY assay and multiple dye assay were found not suitable for the 3D oral mucosa model and thus the impact of NMs on the barrier integrity of this model could not be determined.

Limited or no crossing of selected NMs was observed through intestinal epithelium model or pulmonary and BBB models respectively. However, due to several analytical technique limitations and interferences, *in vitro* models have currently a limited suitability to allow reliable evaluation of NMs crossing. These include interference of insert membrane, limited sensibility of some quantitative methods (*e.g.* PIXE, ICP-MS for some NMs such as SiO_2 NMs), important volume of basolateral medium and presence of organic matter in culture media rendering difficult the reliable evaluation of NMs crossing.

In case of oral mucosa model, interference of membrane does not arise as the NMs were considered to successfully cross the oral mucosa barrier if they penetrated through the epithelium. So, oral mucosa model appears to be suitable for evaluation of NMs crossing but not for screening (medium or high throughput) as it constitutes a time- and resource (money and manpower) consuming model.

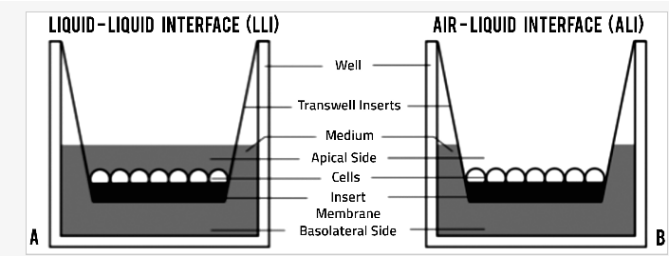
Given the relevance that absorption data through epithelial barriers have in RA of NMs, task 5.3 crossing results have to be considered with care to take into account the critical issues raised above. In particular, a combination of complementary analytical techniques (both qualitative and quantitative) is required to avoid under- or overestimation of crossing events. Altogether these data indicate that improvement of *in vitro* barrier models set-up is necessary and suggest that results obtained with these kind of *in vitro* models up to now have to be considered with caution taking into account limitations underlined in this project.

Resulting documents/data		Input for
Deliverable 5.03: In vitro screening methodology for absorption or crossing of other barriers [draft]		Toolbox section 3.4
Factsheet 5.03		
SOP 01 Protocol for organotypic cell culture modified for exposure to nanomaterials (Annex 1 to Deliverable 5.03)		Toolbox section 3.4
SOP 02 Caco-2 cell culture and differentiation (Annex 2 to Deliverable 5.03)		
SOP 03 Evaluation of NMs impact on Caco-2 cell barrier model (Annex 3 to Deliverable 5.03)		

Task 5.4: Inhalation toxicity modelling/*in vitro*

Inhalation represents the main route of exposure to NMs. Assessing the risks of this exposure route by performing *in vivo* tests is costly, time consuming and requires the use of animals. *In vitro* tests don't have these disadvantages; however, the predictive value of such tests is not yet known.

√ In vitro screening methodology to evaluate toxicity by inhalation (Deliverable 5.04)



Six NANoREG partners evaluated *in vitro* techniques mimicking inhalation exposure by a direct exposure of cells to NMs (air-liquid interface; ALI). Two of them also evaluated the more classical submerged technique alongside the ALI exposure experiments (for more information on submerged cultures the reader is referred to D5.06). Theoretically, the ALI - approach should mimic more closely the typical exposure route of cells in the respiratory tract, namely via air. Partners used the same pulmonary cell models (A549 monolayer, A549 co-culture with THP1 and 3D airway epithelia) and the same four NANoREG core NMs (NM100, NM101, NM212, NM220). Equipment however was not the same.

The six different experimental systems set up to expose cells at the ALI to aerosols of NMs achieved maximum doses of exposure in the order of few µg/cm². Some significant differences were nevertheless noted between the different systems, including the timing of the dose delivery. Except for one partner, the doses deposited were enough to observe significant biological responses (see tables below).

Expression of interleukins IL1β, IL6, IL8. IL10, MCP-1 and TNF-α was studied as biomarkers of inflammation. In general, the low toxicity and few data points collected did not allow for a robust correlation analysis among the different technologies used. Results obtained also highlight the importance of the test system, for example A549 co-cultured with THP-1 being more sensitive than A549 grown in ALI or as monocultures.

	Cytotoxicity						
	A549				A549+THP-1		Lung epithelia
Partner	INERIS	GAIKER	BAuA	TCD	INERIS	KI	GAIKER
NM105	>3				1		
NM101	>3			>0.68	>3		
NM100	>3			>0.68	>3		
NM212	>3	>1.5		>2.72	>3	5	>8
NM220		>1.5	>0.01	0.68			8*

	Inflammation				
	A549			A549+THP-1	
Partner	INERIS	BAuA	TCD	INERIS	KI
NM105	>3			1	
NM101	>3		0.68	1	
NM100	>3		0.68	1 to >3	
NM212	>3		2.72	1 to >3	>5
NM220		>0.01	>1.36		

Table: Lowest observed adverse effect levels (LOAEL in µg/cm² for 24 h exposure) for cytotoxicity in cells cultivated at the ALI and exposed to NM aerosols.

Lowest observed adverse effect levels (LOAEL in µg/cm² for 24 h exposure) for general pro-inflammatory effects in cells cultivated at the ALI and exposed to NM aerosols.

*Toxicity trends observed after 20 days exposure; "no effects" are mentioned in green, "significant effects" in red; Blank cells indicate experiments not carried out by the partners.

Regarding the co-culture and considering pro-inflammation results, comparison between ALI and submerged exposures showed that the ALI exposure was a more sensitive model than the submerged one. NM105, 101 and 212 appeared more toxic than NM100 both at the ALI and in submerged conditions

In general, it could be concluded that:

- It was difficult to correlate between ALI methodologies.
- The ALI exposure seems to be a more sensitive model than the submerge one. However, both methodologies provided similar relative ranking of NMs considering their potential toxicity
- It is better to use co-culture cellular models, which proved to be more sensitive under the conditions of the different studies.

Resulting documents/data		Input for
Deliverable 5.04: *In vitro*-screening methodology to evaluate toxicity by inhalation		Toolbox section 3.4
Factsheet 5.04		

√ *In vitro-in vivo correlation studies for inhalation toxicity (D5.05)*

For four sets of data a comparison has been made between the results of in vitro and in vivo assays related to pulmonary toxicity for the same materials. In all the comparisons, mass/cm^2 has been chosen as dose metrics. Unfortunately, the long term inhalation study with NM212 could not be included in this deliverable since full set of results will only become available after 2016.

Topic	In vivo	In vitro
Pulmonary toxicity of TiO_2 (NM100 and NM101) and CeO_2 (NM212)	Task 4.5.7. Acute and repeated nose-only inhalation toxicity study (D4.15)	Task 5.4. Inhalation toxicity modelling/*in vitro*
Uptake of CeO2	Task 4.4. Pattern of particle distribution in organs	Task 5.6. Develop a rapid high throughput screening methodology
Pulmonary toxicity of nanocellulose	Task 4.7. Acute immunotoxic and genotoxic effects of fibrous nanomaterials	Task 5.5. *In vitro* toxicity assays connected to regulatory questions
immunotoxicity of SiO2 (NM203)	Task 4.5.5. Repeated dose 90 days oral toxicity study	Task 5.5 In vitro toxicity assays connected to regulatory questions

From sets of experiments described above, the following conclusions can be drawn:

- *In vivo* approach appears to be the most sensitive and exhaustive one to assess absolute pulmonary toxicity of poorly soluble NM.
- *In vitro* approach may provide valuable information regarding relative ranking of NM, provided that the cell model is sensitive enough. In this context it is concluded that monocultures of pulmonary epithelial cells (A549 or BEAS-2B) are poorly sensitive models.
- ALI approach is more sensitive than submerge exposure.
- The development of advanced *in vitro* models, mimicking closer lung physiology and the reality of environmental exposure to assess pulmonary toxicity of low soluble NM should be a priority for the upcoming years.

To improve the *in vitro* predictivity after acute exposure to poorly soluble NMs it is important:

- To assess the real mass of NM deposited on the cell surface in vitro is fundamental
- To use compatible and relevant dose metrics between the *in vivo* and the *in vitro* is critical
- To use more realistic cell models (macrophages ++) and exposure methods

Resulting documents/data		Input for
Deliverable 5.05: Report on cell type and *in vitro-in vivo* correlation studies for inhalation toxicity		Toolbox section 3.4
Factsheet 5.05		

Task 5.5: *In vitro*-toxicity assays connected to regulatory questions (D5.06)

√ *Identification and optimization of the most suitable in vitro methodology*

The use of *in vitro* methods as part of a RA strategy for nanomaterials has considerable potential with regards to reducing costs and lead-time. *In vitro* methods can (1) reduce the numbers of animals used in both research and risk/hazard assessments, (2) Mimic more reliably aerosol exposure, (3) investigate susceptible effects in disease models and (4) highly reduce experimental cost.

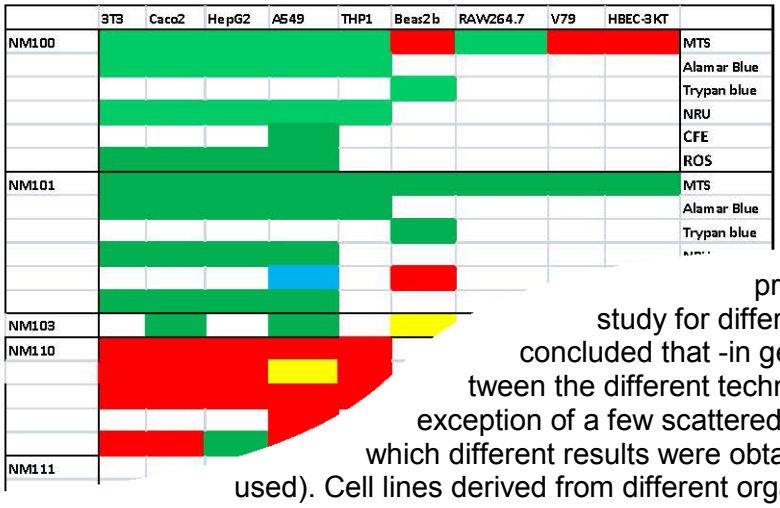
However to be used in a regulatory context *in vitro* methods have to be validated in order to ensure they are predictive and reliable.

Task 5.5 of the NANoREG project evaluated the suitability of *in vitro* assays in connection to the *in vivo* experiments performed in this project. All core NMs were evaluated in this task. To detect potential contamination of the nanomaterials with endotoxin (a toxic substance that strongly can influence the results of *in vitro* assays), all nanomaterials used in this task have been tested for this substance. Characterisation and dispersion of NM was carried out following the NANoREG Guidance Document.

For cytotoxicity 6 different assays have been performed addressing cellular viability (MTS, Alamar blue, Neutral Red Uptake (NRU), Lactate Dehydrogenase (LDH), Colony forming efficacy (CFE) and Reactive Oxygen Species (ROS). For MTS a round-robin exercise has been performed.

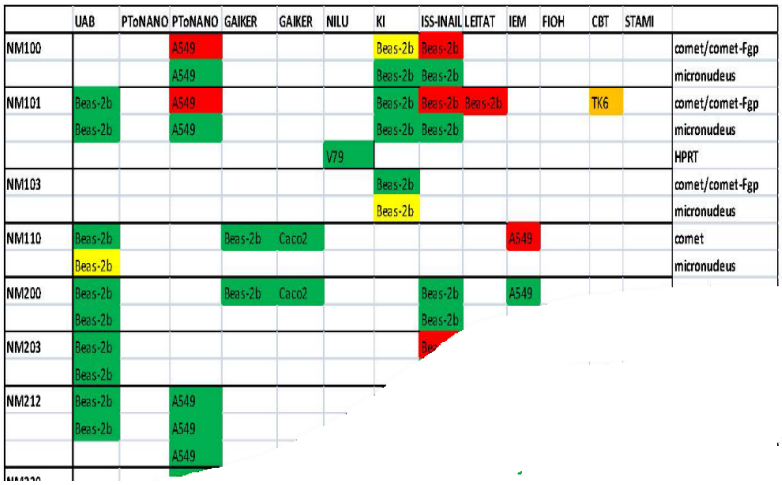
Potential genotoxic effects have been addressed by 4 assays (Comet assay, Mouse lymphoma assay (OECD490), micronucleus assay (OECD 487) and cell transformation assay).

Immunotoxicity has been tested by a study on macrophage and monocyte cell lines (RAW 264.7 mouse cell line and THP-1 human cell line), evaluating NO production and pro-inflammatory cytokine secretion.



A comparison study for the 8 different basic toxicity methodologies. Red: IC50 <50 µg/mL, yellow: IC50 >50-100 µg/mL and green: no toxicity observed. Blue represents inconsistencies between partner results. White: not tested.

Among many other topics, D5.06 presents the results of a comparison study for different toxicity methods (see figure). It is concluded that -in general- there was good correlation between the different techniques for all NMs under study (with the exception of a few scattered cases and the NM400 series, for which different results were obtained depending on the technique used). Cell lines derived from different organs show different sensitivity towards NM exposure. It is therefore important, while designing experiments, to select the cell line which best represents the intended exposure route.

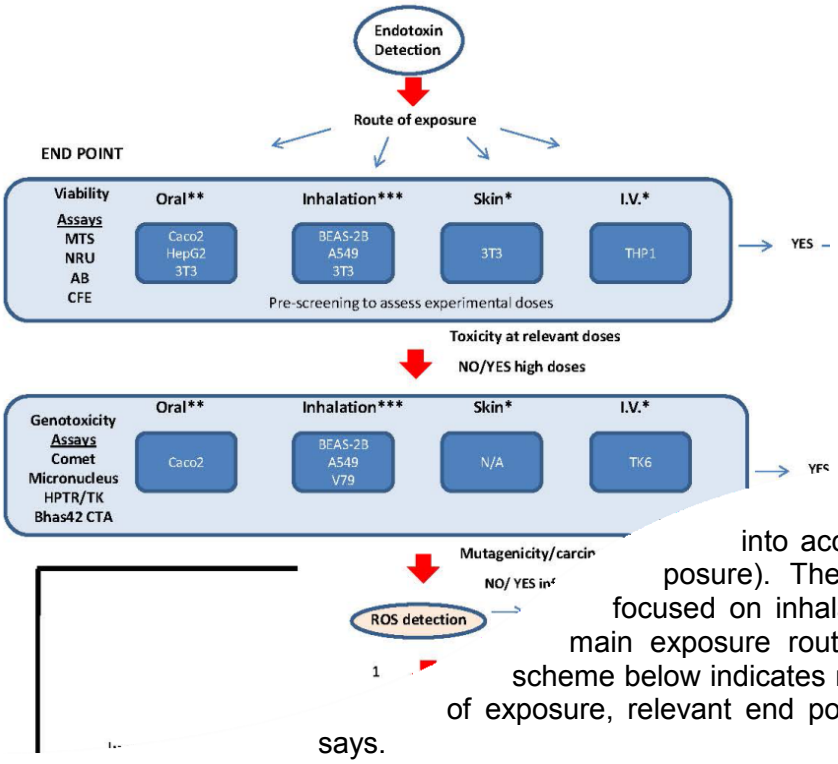


The effect of different dispersion methods on the size distribution and toxicity of a number of NMs also has been detailed. A limited effect of the dispersion procedure was observed for some of the tested NMs: Sonicated SiO_2 NM200 NMs and ZnO NM110 NMs induced a slightly higher toxic effect than stirred NPs. However, no significant impact of sonication was observed on the toxicity of Ag NM300K and NM302 NMs

The results of the genotoxicity tests reveal that most of the NMs are not genotoxic under the conditions of the study (consensus among different assays), except NM401 and NM402 that show genotoxicity in some of the cell types. In spite that comet and micronucleus assays are able to detect different types of genetic lesions, a good correlation between results obtained using these two methods was found. The exception was TiO_2 (NM100 and NM101) for which comet produced a

positive effect not observed by the micronucleus technique in 2 out of 3 partners. Overall the comet assay may represent a complementary test to the micronucleus assay. The comet assay allows for high-throughput adaptations (D5.07) and could potentially be included in the battery of in vitro tests for genotoxicity assessment of NMs.

The deliverable also presents results for inflammatory effects and immunotoxicity.



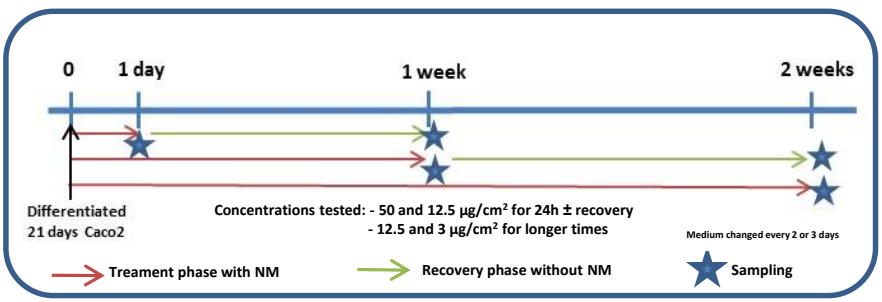
Based on the results of the *in vitro* experiments performed by NANoREG Task 5.5 and reported in deliverables 5.4, 5.5, 5.6 and 5.7 Deliverable 5.6 comes forward with a scheme providing a first line strategy to collect hazard information at early stages of product development (see figure). Information collected following the proposed scheme may assist product developers in collecting relevant safety information on their potential products, taking into account their final use (route of exposure). The NANoREG WP5 strategy has focused on inhalation and the oral routes as the main exposure routes of entry into the body. The scheme below indicates recommended cell lines per route of exposure, relevant end points and their corresponding assays.

The experiments that have been carried out have resulted in a huge set of well-defined and reliable nanoEHS data that will be of great value in and outside the project.

Resulting documents/data		Input for
Deliverable 5.06: Identification and optimization of the most suitable in vitro methodology		Toolbox section 3.4
Factsheet 5.06		
SOP 01 LAL Assay for Nanoparticles (Annex 1 to Deliverable 5.06)		Toolbox section 3.4
SOP 02 LDH assay (Annex 2 to Deliverable 5.06)		
SOP 03 Reaction Oxygen Species Detection (Annex 3 to Deliverable 5.06)		
SOP 04 Human Lung Cell transformation assay. Long term chronic experiment (Annex 4 to Deliverable 5.06)		
SOP 05 Apoptosis/Necrosis Analysis (Annex 5 to Deliverable 5.06)		
SOP 06 Production of pro-inflammatory cytokines (Annex 6 to Deliverable 5.06)		

√ *In vivo genotoxicity of NM (D4.10)*

In the Description of Work it was foreseen to correlate the results of *in vivo* genotoxicity testing on titanium dioxide with respective in vitro experiments. Unfortunately it has not been possible to produce useable *in vivo* data.



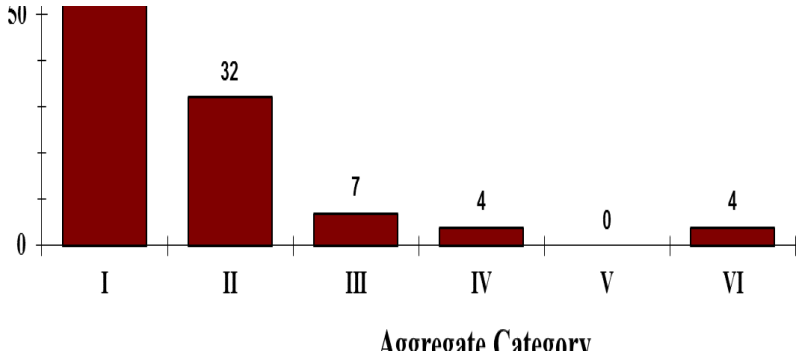
The *in vitro* experiment was performed with Caco2 intestinal cells since the intestinal epithelium represents a major barrier for TiO2 following ingestion. The internalization of TiO2 was investigated as well

as Cytotoxicity and genotoxicity. The NANoREG core material NM103 was investigated and compared to NM104, a material of similar composition and size but without a hydrophobic surface.

First the uptake and distribution inside the differentiated human intestinal Caco2 cells were analysed after an acute (24h) exposure. In a second step, the behaviour of NPs was measured after repeated exposure as well as after a period of recovery.

TEM analysis shows that, after acute and repeated exposure, NM 103 and 104 were taken up by Caco2 cells and accumulated over time inside the cells. Particles remained inside the cells even after a recovery period. ToF-SIMS data revealed that single cells had nanoparticle agglomerates incorporated.

The TiO+ agglomerates from the acquired ToF-SIMS pictures were analysed for total agglomerate counting and agglomerate size determination. All agglomerates were categorized into 7 groups: Group 1: 100 nm^2 agglomerate size, Group 2: 100–500 nm^2, Group 3: 500–1 μm^2, Group 4: 1 to 5 μm^2, Group 5: 5 to 10 μm^2, Group 6: 10 to 30 μm^2, Group 7: >30 μm^2.



Overview of the observed agglomerate numbers in NM-103 exposed Caco-2 cells

No cytotoxicity (Neutral Red Uptake) was observed with NM103 on differentiated Caco2 following an acute or a long time treatment followed or not by a recovery period (up to 1 week)

NM103 did not induce IL8 release, an indicator of inflammatory reactions, on differentiated Caco2 cells following an acute or a long time treatment followed or not by a recovery period. Even if some increase can be observed punctually, no dose-response was noticed. The absence of any change in IL8 release maybe due to the change of medium inclusive of nanoparticles every second or third day. This may have caused a reduction of inflammatory effects that would have been expected following repeated exposure.

Test	NM103	NM104
Comet assay	Negative	Negative
Comet assay+FpG	Negative	Negative
Micro nucleus assay	Negative*	Negative*

**due to the interference with the assay, it cannot be concluded that no induction of micronuclei occurred at the highest concentrations tested.*

Aside the NANoREG project, the genotoxicity of NM103 and NM104 was tested *in vitro* on Caco2 cells for concentrations up to 256 µg/ml during a bilateral ANR/DFG project between France and Germany (SolNanoTox). The results are shown in the table

No positive result was obtained in genotoxicity testing of TiO2 during the *in vitro* experiments conducted in the project.

Resulting documents/data		Input for
Deliverable 4.10: Particle distribution of NM in hepatic and liver cell models		
Factsheet 4.10		

Task 5.6: Develop a rapid High Throughput Screening methodology (D5.07)

The adoption of HTS and HCA methods for NM toxicity testing allows the testing of large numbers of different materials at different concentrations and on different types of cells. It reduces the effect of inter-experimental variation and importantly permits to analyse human cell lines and tissues instead of rodents. It also leads to substantial savings in time and cost. On the basis of a literature survey, several of these methods have been evaluated regarding the applicability for specific biological assays and endpoints. The methods that have been evaluated are: Label-free cellular screening of NM uptake, HCA, HTS flow cytometry, Impedance-based monitoring, Multiplex analy-

sis of secreted products, and genotoxicity methods – namely HTS comet assay, HTS in vitro micronucleus assay and γH2AX assay.

Based on the literature review, the deliverable gives an overview of the potential field of application of the selected methods and assays. In general, it is concluded that the HTS/HCA clearly allow a reduction of the time required for toxicity testing while increasing data outcomes. A set of methods (see below) were selected based on their suitability for NM toxicity testing. For validation, a set of common end-points, the same batch of A549, common NM concentrations (expressed as mass/volume and mass/area) and exposure time, i.e., 24h, were employed by the partners.

As a second step, several methods for HTS/HCA have been, where relevant, further developed and applied by multiple laboratories on 10 core NMs: NM100, NM101, NM110, NM212, NM200, NM401, NM300K, NM203, NM103, and NM220.

- The following HTS methods have been set up and standardized for NM testing: real-time bioimpedance, microfluidic-chip impedance-based flow cytometry, real time RT-PCR, colorimetric assays and colony forming efficiency for measurement of viability, cell proliferation, and cytotoxicity. To address genotoxicity, comet assay, yH2AX and micronucleus assay by flow cytometry were standardized and applied to test NMs. In addition, bioanalytical techniques developed as HCA, i.e., label-free confocal Raman microscopy, multiparametric automated imaging and image analysis; quantification of fluorescence in the cell and cellular compartments were employed in order to assess: 1) NM uptake after in vitro exposure, 2) morphological modifications in cells exposed to NMs, 3) viability and identification of the modes of cellular death induced by NMs, 4) production of ROS, 5) mitochondrial structure and function, 6) inflammation and 7) genotoxicity.
- The main cell model common for all endpoints for this task was cell line A549. Additionally, TK6, CAKI-1, HEP3B, CALU-3, Caco-2, V-79, SAOS2, U937, primary gingival fibroblasts and HEPA-RG were used.

Preliminary cytotoxicity ranking in A549 cells based on real-time bioimpedance xCELLIgence results is: NM-300k > NM-111 > NM-110 > NM-302 >> NM-101, NM-103 > NM-220 > NM-203, NM-212, NM-100, NM-200 > SiO_2@IIT-25nm-green(-), SiO_2@IIT-115nm-red(+) > SiO_2@IIT-50nm-red(+), SiO_2@IIT-50nm-red(-), SiO_2@IIT-50nm-green(-), SiO_2@IIT-50nm-green(+), SiO_2@IIT-115nm-red(-).

Preliminary genotoxicity ranking based the comet assay after 3h exposure of A549 is: NM200< NM101< NM212< NM104< NM103< NM220< NM100< NM203<NM300K<NM110 <NM302<NM111 and for 24h exposure: NM104< NM103< NM220< NM101< NM203< NM200< NM100< NM212< NM300K< NM111< NM302< NM110.

An overview of the experiments performed is available in D5.07.

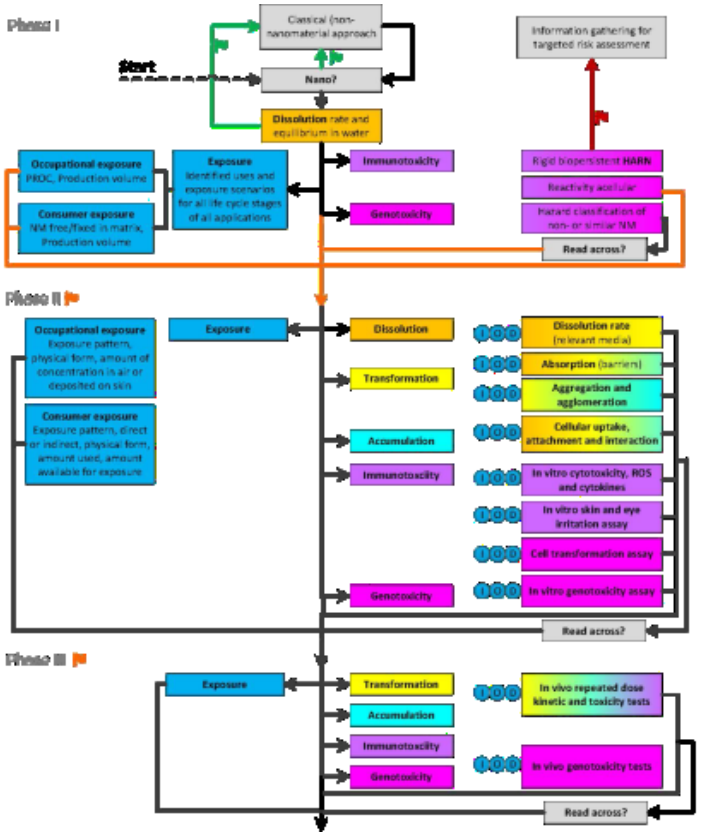
Currently data are under evaluation and several comparisons between HTS methods with the same endpoints, the same HTS methods versus standard method, comparison of several end-points with same NMs, ranking of NMs and overall evaluation, etc. are under preparation. Therefore, and to answer the main question addressed in task 5.6 “What are the most promising HTS/HCA techniques used in task 5.6”, data analysis must be finalized and methods validated to produce solid, justified recommendations.

Task 5.6 introduced the minimum characterization requirements specified in the NANoREG Guidance document; however, to answer the needs of HTS, these should be adapted and modified, so that they can be performed for a higher number of NMs in a shorter time. As they are now, they constitute a bottleneck and reduce significantly the number of tests and NMs that can be tested.

Resulting documents/data		Input for
Deliverable 5.07: Develop a rapid high throughput screening methodology to evaluate NM toxicity		.
Factsheet 5.07		
SOP 01 Label-free nanotoxicity assessment by impedance-based flow cytometry		

SOP 02 Real-time label-free impedance-based nanotoxicity assessment		
SOP 03 TaqMan real-time Reverse Transcription PCR		

Task 5.7: Develop flow chart for risk assessment (D5.08)



An efficient strategy to assess the impact of NMs on environment and human health is crucial to fully exploit the innovative and economical potential of these materials. Such a strategy should provide sufficient comfort for society regarding the safety of NMs and the products they are incorporated in. At the same time it should be cost-effective and be applicable for industry and small and medium sized enterprises involved in the production and application of NMs.

The flow chart for RA of NMs developed within the NANoREG project presents a proposal to prioritize those nanomaterial applications that may lead to high exposure or high toxic potential and ultimately high risks for human health. Six elements have been identified as most important nanospecific determinants within the RA of NMs: exposure potential, dissolution, nanomaterial transformation, accumulation, genotoxicity, and immunotoxicity. In the deliverable, the six elements are explained and an argumentation for the selection of the different elements is given.

The first objective (prioritization of applications) is addressed in the first phase of the strategy, while the second objective (identification of information) is mainly addressed in the second and further phases (see figure in the box).

The strategy gives direction to the most important information needed depending on the specific application, life cycle stage and exposure situation. Furthermore, the strategy identifies possibilities for grouping and read-across primarily based on physico-chemical properties and in vitro data.

The proposed strategy is suitable for different uses by policy makers, regulators and industry. Policy makers and regulators can predominantly benefit from using the first phase of the strategy to prioritize those applications that need to be addressed most urgently. Industry can use the first phase to get an initial impression on the suitability of the application of the nanomaterial in a specific product based on the potential of a specific nanomaterial to cause hazardous health effects during the different life stages of that product. The second and further phases can be used by regulators and industry to identify the most important information needs to address the nanospecific issues. With the current strategy it is possible to identify those situations where the use of nanospecific grouping, read across and (Q)SAR tools is likely to become feasible in the future. In addition, these phases can be used to point towards the generation data that is needed for scientific justification, which may lead to regulatory acceptance of nanospecific applications of these tools.

The flowchart is an important outlook scenario for the Framework for the safety assessment of nanomaterials (see section 3.1.1 above and NANoREG Framework or D1.11).

Resulting documents/data		Input for
Deliverable 5.08: Flow chart for risk assessment of nanomaterials (Towards a nanospecific approach for risk assessment)		Frame work part 2 Toolbox section 4
Factsheet 5.08		

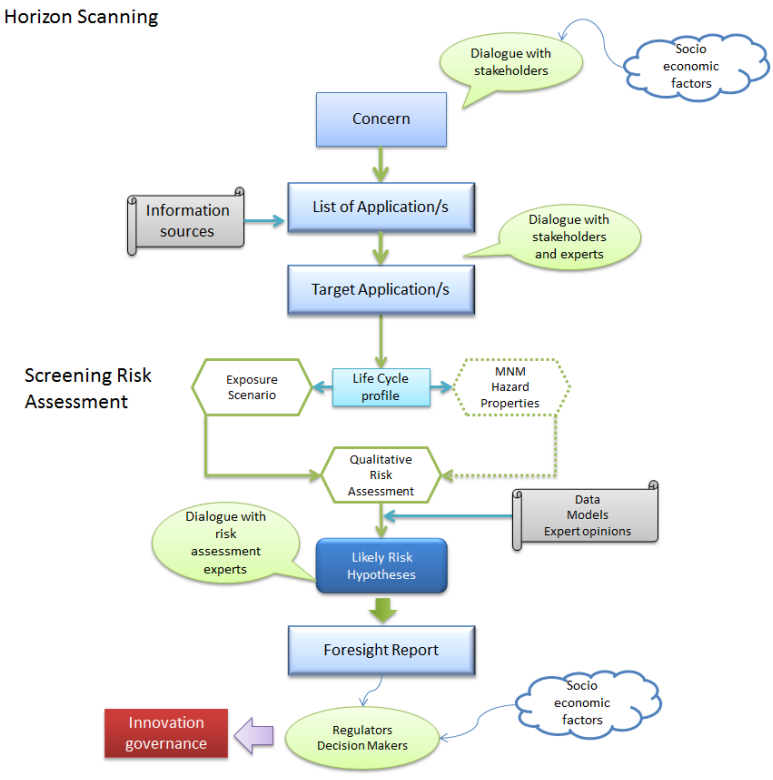
3.1.6 Work package 6

Results	*Impact*
Lessons learnt from drug design	*Awareness when and how RA for toxicity of nanomaterials can benefit from data rich environments like in drug design;*
NANoREG Safe by Design approach	*Awareness that safety research should be tuned to the innovation process;*
Concept of Risk potential	*awareness for how nano specific safety issues can be addressed from early stages of innovation onwards*
Literature database on physico-chemical toxicity data	*Systematic approach to facilitate searches for finding relationships between physico chemical characteristics and toxicity endpoints, based on qualitatively sound data*

Task 6.1: Linking risk analysis into innovation

√ *NANoREG foresight system (D6.01)*

A NANoREG Foresight system was developed to monitor innovation and evaluate the potential adverse impacts of NMs and their likely applications in a time horizon of 5 to 10 years. It is a combination of Horizon Scanning (HS) and Screening Risk Assessment (SRA).



The picture below shows the overall framework, with the different phases, input, outputs, and the role of different stakeholders and experts.

The proposal focuses on regulators. However, industry can benefit from the use of the NANoREG foresight system to assess the potential uses of the application and the related risks.

The NANoREG foresight system has been applied in a case study: a specific application of graphene to water filtration membranes. This example was selected because it is at low TRL stage with a time to market around 5 years and has a high beneficial potential for society and environment. At the same time, the water filtration membrane can cause direct and indirect exposure to graphene for workers, consumers and environment.

The assessment of the application of graphene family material for the water treatment and purification resulted in the identification of main issues to be addressed to evaluate early on its potential impact.

Resulting documents/data		Input for
Deliverable 6.01: Proposal to monitor innovations in new nanomaterials and their applications		
Factsheet		

√ *safety assessment issues and new approaches to research and governance (D6.02)*

To get a better insight in the causes and remedies for the increasing gap between innovation and risk regarding manufactured NMs, an analysis has been made on:

- Social and technical issues currently inhibiting robust safety assessment of NMs;
- Key bottlenecks inhibiting the ability of researchers to deliver answers to regulatory questions;
  It also usefully explores ways to overcome these challenges by providing a review of:

  - HTS and organ-on-a chip as new approaches to safety testing, including a discussion of their challenges and limitations;
  - Safe-by-Design (SbD) and Responsible Research and Innovation (RRI) as new approaches to the governance of NMs, including a discussion of their challenges and limitations;
  - How the identified challenges, obstacles and risks may be turned into business opportunities.

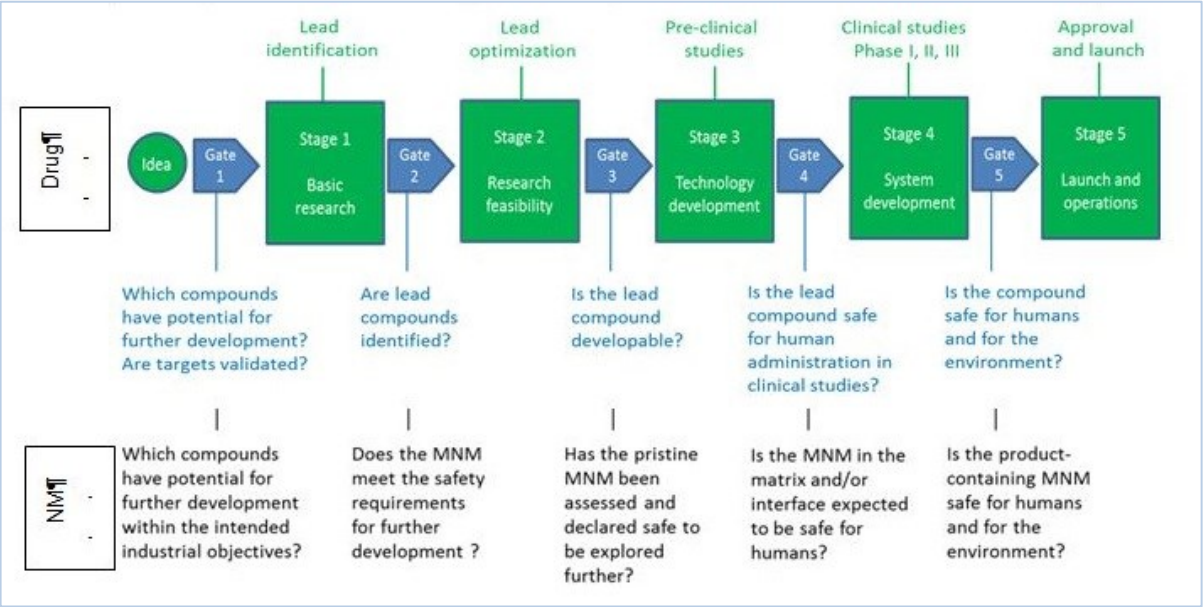
This analysis has been the basis for the NANoREG SbD approach.

Resulting documents/data		Input for
Deliverable 6.02: Inventory of safety assessment issues and new approaches to research and governance		NANoREG Safe by Design approach.
Factsheet 6.02		

Task 6.2: Safe by design: lessons learned from drug development testing.

√ *Safe by design concept (D6.03)*

The safe by design concept has gained interest over recent years as it aims to reduce potential health and environmental risks at an early phase in the innovation process. In addition to its development and use in construction and engineering sectors, this concept has also had a long history of successful deployment in the domain of drug development. In early phase drug development, new chemical entities are screened in parallel for both their efficacy and their potential for toxicity. High throughput testing in this type of research serves the question of whether benefit-risk ratios will likely to be positive. Such an approach might therefore include relevant building blocks for the



uptake and development of the safe by design concept for NMs.

In Deliverable 6.03, the safe by design concept has been described. As a basis for the description of the development of both drugs and NMs, the deliverable defines an “Innovation model” based on the widely implemented “Stage-Gate idea-to-launch” model. The key questions that were addressed are:

1. What are the similarities and differences in toxicity testing aims between drug and NM development?
2. What are the critical safety questions in the drug development process and do they apply to the NM process?
3. Could the toxicity tests for drug development be applicable for NMs also

The critical safety questions in both drug and NMs development processes were linked to the “Gates” in the Innovation model

Resulting documents/data		Input for
Deliverable 6.03: Comparison on toxicity testing in drug development and in present NMs safety testing		
Factsheet 6.03		
NANoREG Safe by design concept		

√ *Toxicity testing (D6.04)*

Regarding the applicability of the toxicity tests used in drug development for NMs development it is concluded that it is not possible to draw conclusions in general. What has been concluded is that some aspects of drug development may be further developed for a screening of risk potentials for NMs. The following risk potentials for NMs have been proposed:

Solubility/dissolution	Genotoxicity/carcinogenicity	Accumulation
Stability of coating	Immunotoxicity/Inflammation	Ecotoxicity

Following up on the results of deliverable 6.3 (Comparing the development process of drugs and NMs; see below), the concept of “risk potentials for NMs” was elaborated. The result is a screening strategy for the first stages of the innovation process based on these risk potentials. It is aimed at gaining insight into the potential for nano specific risks of a nanomaterial in an efficient way.

The risk potentials as proposed in deliverable 6.3 have been further elaborated regarding their relevance and the availability of test methods for their applicability in a regulatory context. The arguments for the suggested potentials are based on a logical reasoning thereby presuming a certain order of addressing the potentials. The figure below visualizes this order and demonstrates that it is important to first address the question that relate to whether a material is to be considered as a NM. This is the starting point of the flow chart, followed by addressing the risk potentials in case of a NM. For each of the steps in this flow chart the deliverable describes the key parameters and the methods to determine these key parameters.

The information is helpful in two ways:

- Supporting decisions by innovators on how to continue further development of the material,
- Supporting identification of already marketed materials for their potential for health risks.

The ideas on how to come to safe innovations can fuel directly into NanoReg^2 for the development of a Safe Innovations Approach, aiming at reducing the hazard of a nanomaterial as much as possible from early stages of innovation on.

Resulting documents/data		Input for
Deliverable 6.04: Inventory of existing regulatory accepted toxicity tests applicable for safety screening of NMs		
Factsheet 6.04		

Task 6.3: Safe by design: practical approach and examples.

√ *impact of phys-chem properties on (eco)toxicological endpoints (D6.05)*

The possibilities designing NMs taking into account the effects of the design on adverse effects on EHS, requires information on the relation between physico-chemical properties and toxicity.

A database has been developed and filled with information from peer reviewed articles with information on physical chemical properties and toxicity. It is designed to store information on both the properties of pristine NMs (e.g. particle's composition) as well as their interaction with biological and environmental components; this is done to keep track consistently of the particle's history and identify the influence of biological and environmental conditions on nanomaterial's toxicity (e.g. the effect of the biomolecular corona on the cellular uptake and toxicity of nanoparticles).

The database includes 3 types of data on nanomaterial characteristics and toxicity:

1. Parameters that define the intrinsic characteristics of NMs;
2. Measurement on nanomaterial properties under specific conditions
3. *In vitro* and *in vivo* (eco)toxicity endpoints.

Peer reviewed literature entries	
Studies Entered	1387
Number of nanomaterials entered	131
TiO2	55
Ag	43
SiO2	31
MWCNT	25
ZnO	23
Number of *in vitro* endpoints	1889
Number of *in vivo* endpoints	1086

The information in the relational database can/will be used to identify physico-chemical properties related to the fate and toxicity of NMs, develop structure-activity relationship (SAR) models, derive grouping principles and contribute to the development of a safe by design strategy (deliverable 6.6 of the NANoREG project) by defining a "safe window" of (eco)toxicological parameters.

This database should be seen as a first step towards identification of which and how physico-chemical properties of nanomaterials are related to fate and toxicity. The database will therefore be transferred to NanoReg^2 in order to be further built on.

Resulting documents/data		Input for
Deliverable 6.05: Database on manufactured nanomaterials physical chemical properties related to (eco) toxicological endpoints		
Database with nanoEHS data from literature.csv		
Database with nanoEHS data from literature.sql		
Instruction on how to use the database (included in Deliverable)		

√ *A first attempt to link physico-chemical properties to functionalities (D6.06)*

One of the big challenges in the field of nanosafety is to determine the relation between phys-chem characteristics of NMs, their functionality and their toxicity. Knowing this relation is pivotal for designing NMs taking into account their potential adverse effect.

One of the first attempts to identify the main key properties of the NMs that in relation to their functionalities play a dominant role on the safe by design concept. This proposed data model (also called data structure) is considered as one of the possible approaches towards a decision tree strategy for (re)designing safe NMs for humans and the environment. The optimal design of safe NMs is a challenge with multiple compromises between functionality and safety characteristics.

Resulting documents/data		Input for
Deliverable 6.06: A first attempt to link physico-chemical properties to functionalities as a contribution to a Decision tree strategy for the Safe by Design of NMs		

4 Evaluation, observations and recommendations

Over 80 collaborating partners, the need for regulatory focus in a scientific environment, top-down research instead of a "let 1000 flowers bloom" approach, an outer ring of national coordinators and advisors: all factors that made the NANoREG project a rather unique endeavour. Looking back, we can be satisfied. Thanks to the effort and commitment of all involved partners, and thanks to the work of Task Leaders and members of the Management Committee, we have been able to implement the project description of work, within the planned schedule or with relatively minor delays. We have submitted the agreed deliverables and realised a substantial scientific as well as policy-level impact (see section 2.2.5). But, at the same time, we must conclude that we could have been more efficient and effective in several fields.

This chapter elaborates these "lessons learnt" and turns them into recommendations for the EC, Member States and new nanosafety projects.

4.1 Collaboration needs facilitation

There is a growing awareness within the nanosafety community that collaboration is key to effective nanosafety research. Without an active exchange of the results obtained by individual nanosafety projects with others, including the underlying experimental data, there is a serious risk of reinventing wheels and starting from scratch every time.

In NANoREG we experienced serious formal and practical hurdles when trying to get access to the results of other projects and when opening up our own data. The nanosafety community would gain much if these hurdles could be removed and if measures would be taken to create a solid base for "*advanced information management*", comprising:

- Project results (deliverables and experimental data) accessible and available for partners outside the project.
- Assurance of basic quality and comparability of experimental data.
- Uniform language or ontology.

To create this advanced information management, the following recommendations should be properly taken on-board of any new initiative or overall programmatic approach, such as the implementation of the EU Framework Programme for Research and Innovation.

4.1.1 Legal aspects

Uncertainties regarding the legal aspects of IPR and confidentiality as laid down in the standard Grant Agreement and Consortium Agreement, make partners reluctant to share their foreground. Taking away these uncertainties by formalising the opening up of data has proven to be an arduous process, not the least because such agreements need the approval of all partners.

- *EC and Member States: eliminate legal constraints for opening up information generated by running projects and new projects by:*
  - *Opening up results of nanosafety research as condition for funding projects by Member States and EC; this includes uploading of experimental data in a standardised way.*
  - *Adjustment of the standard Grant Agreement and Consortium Agreement with respect to IPR and confidentiality;*
  - *Creativity for running projects.*

Explanation: differently from information related to innovation, nanoEHS information in general has no competitive value. Giving access to the results of a project as a condition for funding is therefore certainly justified. This condition should be mentioned in calls as evaluation criterion. Only in cases where the nanoEHS data have a commercial value, such as in characteristics of commercial products relevant for functionality, should the possibility to make a reasoned exemption be built in. The EC Model Grant Agreement and Consortium Agreement should be modified in the same way.

For running projects, partners can agree to an addendum to the Consortium Agreement stating that, notwithstanding the “confidentiality provisions” in Grant Agreement and Consortium Agreement”, they agree to open up the results. NANoREG has proven that this is possible and feasible. (NANoREG example also includes setting the copyright license conditions). The mandatory opening up of information should, as far as it concerns experimental data, include logging the data in a standardised way (ISA-TAB nano).

4.1.2 Infrastructure

At the start of the NANoREG project there was hardly any experience with the management of nanoEHS data. There was no standardised way of logging (recording) experimental data and ontology systems were generally unknown to the nanoEHS community. Facilities for data uploading, storage and curation were lacking, too.
Thanks to the FP7 eNanoMapper project, several facilities have been created. However, that project has ended, too, and the maintenance and further development of those facilities came to a halt, or became depended on other temporary projects having an interest in carrying on some elements of the system developed by eNanoMapper.

- *EC supported by MSs: take the responsibility to allocate the development and maintenance of a sustainable system for advanced nanoEHS data management, including providing or organising structural funding.*

Explanation: crucial for exploiting nanoEHS data by other parties than the generator of the data is a harmonised use of terms (ontology) and the assurance that the quality of data meets a certain minimum standard (data curation). Also, the accessibility of the data after a project has ended is crucial. This demands structural considerations and components that cannot be provided by projects with a limited duration or by partnerships on a voluntary basis, like the EU NSC. Such components can only be provided or organised when the responsibility is allocated to an organisation that has or gets structural, long-term funding for such a task.
Given its position as supra-government organisation, it is obvious that the EC is best placed in the European R&I arena to take the responsibility to organise such a system of advanced nanoEHS data management. It should comprise: facilities for data storage and data curation, the further development of the (eNanoMapper) ontology and the improvement of instruments for data recording (templates for experimental data logging and/or a data entry tool).

- *EC supported by MSs: take the responsibility to create a storage facility for (and access to) deliverables, etc.*

Explanation: to make deliverables, SOPs, guidance documents, etc. accessible for third parties outside a project, they have to be stored in a publicly accessible document repository on the internet. For reasons of efficiency, this could be combined with the advanced information management system (see 4.1 above). In the case of NANoREG, the Dutch Ministry of IenM has taken the responsibility to preserve the project legacy.

4.2 NanoEHS testing programme

Many of the NSC projects end up with the conclusion that there is a lack of reliable, comparable and exchangeable nanoEHS data. Grouping, categorisation, decision-support systems, exposure scenarios, safe(r)-by-design approaches: in all cases usable results will only be achieved when a robust set of data is available to validate the models and to translate the theoretical models into practical applications.

- *EC supported by MSs: take the responsibility to define a 'nanoEHS testing programme'*

Explanation: to be able to make the switch from ideas and theory to practical application, it is essential to create a robust set of nanoEHS data. Hence, the development and execution of a nanoEHS testing programme, aimed at filling the gaps in nanoEHS data, should be considered. Other than the research-oriented projects carried out so far, such a testing programme should have a “directive character”. Materials, parameters, testing methods, quality parameters, etc. should be prescribed upfront.

Annex I: List of partners

The table below gives an overview of partners involved in NANoREG, be it as a Consortium partner (partners 1-71) or as partner on the basis of a collaboration agreement.

Partners in greyed font terminated their participation before the end of the project. Partners in italic fonts are "third parties" linked to a NANoREG partner.

1	MINISTERIE VAN INFRASTRUCTUUR EN MILIEU	Min I&M	Netherlands
2	JRC -JOINT RESEARCH CENTRE- EUROPEAN COMMISSION	JRC	Belgium
3	BUNDESANSTALT FUER ARBEITSSCHUTZ UND ARBEITSMEDIZIN	BAuA	Germany
4	DET NATIONALE FORSKNINGSCENTER FORARBEJDSMILJO	NRCWE	Denmark
5	RIJKSINSTITUUT VOOR VOLKSGEZONDHEIDEN MILIEU	RIVM	Netherlands
6	BUNDESINSTITUT FUER RISIKOBEWERTUNG	BFR	Germany
7	CENTRE NATIONAL DE LA RECHERCHE SCIENTIFIQUE CNRS	CNRS CEREGE	France
8	AIT AUSTRIAN INSTITUTE OF TECHNOLOGY GMBH	AIT	Austria
9	INSTITUTE OF OCCUPATIONAL MEDICINE	IOM	United Kingdom
10	TEMAS AG TECHNOLOGY AND MANAGEMENT	TEMAS	Switzerland
11	FUNDACION GAIKER	GAIKER	Spain
12	NANOTECHNOLOGY INDUSTRIES ASSOCIATION	NIA	Belgium
13	BASF SE	BASF	Germany
14	THE PROVOST, FELLOWS, FOUNDATION SCHOLARS & THE OTHER MEMBERS OF BOARD OF THE COLLEGE OF THE HOLY & UNDIVIDED TRINITY OF QUEEN ELIZABETH NEAR DUBLIN	TCD	Ireland
15	KAROLINSKA INSTITUTET	KI	Sweden
16	NORSK INSTITUTT FOR LUFTFORSKNING	NILU	Norway
17	ISTITUTO SUPERIORE DI SANITA	ISS	Italy
	ISTITUTO NAZIONALE ASSICURAZIONE INFORTUNI SUL LAVORO	*INAIL*	*Italy*
18	AGENZIA NAZIONALE PER LE NUOVE TECNOLOGIE,L'ENERGIA E LO SVILUPPO ECONOMICO SOSTENIBILE	ENEA	Italy
19	STATENS ARBEIDSMILJOINSTITUTT	STAMI	Norway
20	ACONDICIONAMIENTO TARRASENSE ASSOCIACION	LEITAT	Spain
21	INSTITUT NATIONAL DE RECHERCHE ET DE SECURITE	INRS	France
22	UNIVERSITE DE NAMUR ASBL	UNamur	Belgium
23	COMMISSARIAT A L ENERGIE ATOMIQUE ET AUX ENERGIES ALTERNATIVES	CEA	France
24	*GEOCHEM RESEARCH BV*	*GeoChem*	*Netherlands*
25	DEPARTMENT OF HEALTH	DH-PHE	United Kingdom
26	CENTRUM VOOR ONDERZOEK IN DIERGENEESKUNDE EN AGROCHEMIE - CODA	CODA-CERVA	Belgium
27	UNIVERSITAT AUTONOMA DE BARCELONA	UAB	Spain
28	FONDAZIONE ISTITUTO ITALIANO DI TECNOLOGIA	IIT	Italy
	UNIVERSITA DEGLI STUDI DI ROMA TORVERGATA		Italy
29	ASOCIACION DE INVESTIGACION DE LAS INDUSTRIAS DE LA CONSTRUCCION	AIDICO	Spain
30	STICHTING WAGENINGEN RESEARCH	DLO-RIKILT	Netherlands
31	CONSIGLIO NAZIONALE DELLE RICERCHE	CNR	Italy
	UNIVERSITA DEGLI STUDI DI TORINO		*Italy*
32	ASSOCIATION SAINT YVES	UCO	France
33	NEDERLANDSE ORGANISATIE VOOR TOEGEPAST NATUURWETENSCHAPPELIJK ONDERZOEK TNO	TNO	Netherlands
34	INSTITUT NATIONAL DE L ENVIRONNEMENT ET DES RISQUES INERIS	INERIS	France
35	AGENCE NATIONALE DE LA SECURITE SANITAIRE DE L ALIMENTATION DE L ENVIRONNEMENT ET DU TRAVAIL	ANSES	France
36	BIONANONET FORSCHUNGSGESELLSCHAFT MBH	BioNanoNet	Austria
37	LUNDS UNIVERSITET	LTH	Sweden
38	GENOK - SENTER FOR BIOSIKKERHET	GenØk	Norway
39	UNIVERSITETET I BERGEN	UIB	Norway
40	EIDGENOESSISCHES DEPARTEMENT DES INNERN	FOPH	Switzerland

41	VENETO NANOTECH SOCIETA CONSORTILEPER AZIONI	VN	Italy
42	INSTITUTO TECNOLOGICO DEL EMBALAJE, TRANSPORTE Y LOGISTICA	ITENE	Spain
43	UNIVERSIDAD DE LLEIDA	UdL	Spain
44	STIFTELSEN SINTEF	SINTEF	Norway
45	UNIVERSITAET LEIPZIG	ULEI	Germany
46	NPL MANAGEMENT LIMITED	NPL	United Kingdom
47	LABORATOIRE NATIONAL DE METROLOGIE ET D'ESSAIS	LNE	France
48	LABORATORIO IBERICO INTERNACIONAL DE NANOTECNOLOGIA	INL	Portugal
49	TYOETERVEYSLAITOS	FIOH	Finland
50	NORGES MILJO-OG BIOVITENSKAPLIGE UNIVERSITET	UMB	Norway
51	FUNDACION TEKNIKER	TEKNIKER	Spain
52	CHALMERS TEKNISKA HOEGSKOLA AB	Chalmers	Sweden
53	INSTITUTO DE SOLDADURA E QUALIDADE	ISQ	Portugal
	INSTITUTO NACIONAL DE SAUDE DR. RICARDO JORGE		*Portugal*
	INSTITUTO PORTUGUES DA QUALIDADE I.P.		*Portugal*
	MINISTERIO DA SAUDE - REPUBLICA PORTUGUESA		*Portugal*
54	TURVALLISUUS JA KEMIKAALIVIRASTO	TUKES	Finland
55	BAYER MATERIALSCIENCE AG	BMS	Germany
56	ARKEMA FRANCE	ARKEMA	France
57	STORA ENSO OYJ	Stora Enso	Finland
58	UPM-KYMMENE OYJ	UPM	Finland
59	SP SVERIGES TEKNISKA FORSKNINGSINSTITUT AB	SP	Sweden
60	UNIVERSITY OF LEEDS	UnivLeeds	United Kingdom
61	ENVICAT CONSULTING SPRL	Envicat	Belgium
62	COMET BIOTECH AS	CBT	Norway
63	INSTITUT PASTEUR DE LILLE FONDATION	IPL	France
64	HERIOT-WATT UNIVERSITY	HWU	United Kingdom
65	HEALTH PROTECTION AGENCY HPA (= PARTNER 25)	HPA	United Kingdom
66	USTAV EXPERIMENTALNI MEDICINY AKADEMIE VED CESKE REPUBLIKY VEREJNA VYZKUMNA INSTITUCE	IEM	Czech Republic
67	VYSOKA SKOLA BANSKA - TECHNICKA UNIVERZITA OSTRAVA	VSB	Czech Republic
68	INSTITUTE OF PUBLIC HEALTH OSTRAVA	ZUOVA	Czech Republic
69	FOUNDATION FOR RESEARCH AND TECHNOLOGY HELLAS	FORTH	Greece
70	NATIONAL CENTER FOR SCIENTIFIC RESEARCH "DEMOKRITOS"	NCSRD	Greece
71	ECAMRICERT SRL	ER	Italy
72	CENTRO DE TECNOLOGIAS ESTRATÉGICAS DO NORDESTE	CETENE	Brazil
73	EMPRESA BRASILEIRA DE PESQUISA AGROPECUÁRIA	EMBRAPA	
74	INSTITUTO NACIONAL DE METROLOGIA, QUALIDADE E TECNOLOGIA	INMETRO	
75	UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL	UFRGS	
76	UNIVERSIDADE DE SÃO PAULO DEPARTMENT: SISNANO-USP	Gnano IFSC USP	
77	UNIVERSIDADE FEDERAL DO RIO GRANDE ; DEPARTMENT: INSTITUTO DE CIÊNCIAS BIOLÓGICAS (ICB)	FURG	
78	UNIVERSIDADE FEDERAL DE MINAS GERAIS DEPARTMENT: INSTITUTO DE CIÊNCIAS BIOLÓGICAS (ICB)	UFMG	
79	UNICAMP – UNIVERSIDADE ESTADUAL DE CAMPINAS; DEPARTMENTO DE QUÍMICA INORGÂNICA NANOBIOSS/INSTITUTO DE QUÍMICA	NanoBioss	
80	MINISTÉRIO DE CIÊNCIA, TECNOLOGIA E INOVAÇÃO	MCTI	
81	HANYANG UNIVERSITY	HYU	Republic of Korea
82	KOREA RESEARCH INSTITUTE OF STANDARDS AND SCIENCE	KRISS	
83	SUNGKYUNKWAN UNIVERSITY	SKKU	
84	HOSEO UNIVERSITY; INSTITUTE OF NANOPRODUCT SAFETY RE-SEARCH,	HSU	
85	KWANGWOON UNIVERSITY	KWU	
86	UNIVERSITY OF SEOUL	UOS	
87	DONGDUK WOMEN'S UNIVERSITY	DWU	
88	MINISTRY OF ENVIRONMENT/ NATIONAL INSTITUTE OF ENVIRON-MENTAL RESEARCH	MOE/NIER	
89	MINISTRY OF FOOD AND DRUG SAFETY	MFDS	

Evaluation and update of the IMPACT Chapter DoW

This annex evaluates the impact of the NANoREG project so far on the basis of the chapter 3 of the DoW part B that gives a comprehensive overview of the expected impact at the time of writing the DoW.

	Chapter 3 DoW Impact	Achieved impact(s)	Next steps, remarks
3.1.1	**Seamless collaboration among authorities of MS Governments**		
	Collection of their questions and needs in workshops, consolidating the data as input for WP1 which will set priorities and elaborate a solution path for each agreed study to be performed within the WP2-WP6.	• Questions and needs have been identified (Del 1.1) and linked to Tasks. • Strategy has been established to link the results of WP2-6 to the Q&Ns. • Deliverable 1.09 presents the answers to most of the questions.	
	Organising the transfer of the answers and recommendations to the national Authorities with dedicated workshops on a case-by-case basis.	• Dissemination strategy for NC established • Coordinators' workshop on Policy and regulation (October 2016) • ProSafe – OECD Scientific Conference and NANoREG final conference Nov-Dec 2016 • Large number of national events	• Continuation of dissemination activities at national level. • Integration of results in ProSafe White Paper • Continuation of dissemination activities coordinator
	Building and strengthening the relationship with the relevant industrial enterprises and their organizations. Obtaining their opinions on the feasibility and practicability of the recommendations to the regulatory and legislative authorities.	• Impact is below expectations due to the difficulties to involve industry in the project's NICC. • However during several industry oriented meetings like the NICC meeting organised by NIA and Solvay, 31 January 2017 in Brussels, the results of the project have been intensively discussed	• Activities will be continued in ProSafe context (White Paper)
3.1.1.1	**Solid mechanism for networking**		
	Memorandums of Understanding (MoU) or Letters of Intent with the national and international regulation, legislation and standardisation organisations.	See Final Report Part 2; task 7.1 • Informal agreements with OECD and ECHA. • Formal agreement with CEN and ISO.	
	Membership in EU's networks NanoSafety Cluster and NANOfutures	• Participation in meetings of the NSC. • Several formal and informal collaborations with NSC projects (NanoDefine, SUN, Cost Modena, eNanoMapper).	Formal aspects of collaboration are difficult to arrange; EC could facilitate this by establishing a standard (and mandatory) collaboration agreement between projects.
	As partner (EU authorized) of the "Research community between Europe and USA"	See Final Report Part 2; task 7.1 • Participating in SRA collaboration. • Chair regulatory WG of EU-US CoR. • Collaboration with US partners on topics like data management. High Throughput techniques under the umbrella of ProSafe.	Continuation of activities in the context of ProSafe.
	Agreements on a case-by-case basis with other interested companies supported by NANoREG's service desk as an entry point for interested new organisations and authorities.	See Final Report Part 2; (task 7.3) • Several new partners from different countries established a formal relationship with NANoREG. • Under the umbrella of ProSafe several new collaborations have been established such as with Greek partners and US Universities.	

	Chapter 3 DoW Impact	Achieved impact(s)	Next steps, remarks
3.1.2	Collaboration with Global organizations	See Final Report part 2 (task 7.1) • Active role of several MC members in OECD, CEN, ISO, etc.	Activities regarding harmonisation should be coordinated and continued under the umbrella of the NSC
	Linking projects joint EU-US nanosafety research initiative.	Under the umbrella of ProSafe several contacts have been established with US organisations resulting in • Collaboration WP3 partners with a number of US universities. • Close cooperation with DUKE University on data management.	
	NANoREG participation of countries.	See Final Report Part; task 7.1 • 25 partners from CZ, GR, ROK and BR became NANoREG partner. • Contacts with EC and non EC countries have been expanded under the umbrella of ProSafe (SPDG).	Further exploitation of these collaborations.
	Liaisons with OECD, CEN, ISO, EU dossier requirements, ICSU/CODATA	See above	
	NANoREG's impact on standardisation: a) OECD Harmonized Templates), b) Standards of CEN and ISO, c) IUCLID data structure and endpoints	The NANoREG activities on this field have been described in Deliverable 7.3.	
3.1.3	**Knowledge required for appropriate risk management**		
	Reduction of the gap of uncertainties to perform risk assessment on MNMs.	See Final Report • Great number of scientific Deliverables submitted including SOPs • Advance system for Risk Assessment	Dissemination of results partially under the umbrella of ProSafe.
	Tests and Value Chain Case Studies (WP1/Task 6) NANoREG will overcome the problems initiated by many novel methods which are breakthroughs in scientific terms, but are poorly evaluated from a regulatory perspective.	Due to the late availability of NANoREG results it has not been possible to use the Value Chain projects to evaluate the results from a regulatory perspective. As such, the projects have been fruitful to get a better insight in the potential of Value Chain Studies	
	The "NANoREG Tool Box" will provide the regulators and legislators for assessment with decision supporting instruments for the short to medium term.	Deliverable 1.10 and its annexes provide the NANoREG Toolbox. It includes SOPs, strategies etc. developed under the umbrella of the NANoREG project as well as other projects	
	a) Gathering data and pilot risk assessment, exposure monitoring and control, for a selected number of nanomaterials used in products.	• Exposure scenarios for the selected NMs have been defined and prioritised (D3.01). • Data gaps identified and filled up by, among others, field measurements and tests in mesocosms (D3.04, D3.05, D3.06, D3.07, D3.08, D3.09). • Methods, results and data will be valuable in and outside the NANoREG project.	
	b) development of new testing strategies adapted to a high number of nanomaterials where	Final report Task 5.7; Deliverable 5.08 The strategy presented in the deliverable provides a more efficient and effective approach of	Strategy will be one of the main elements of the ProSafe White Paper

	Chapter 3 DoW Impact	Achieved impact(s)	Next steps, remarks
	many factors can affect their environmental and health impact supporting the long-term behaviour NANoREG will deliver a decision tree for the risk assessment of nanomaterials using all existing information on exposure, stability and toxicity of MNMs.	risk assessment of nanomaterials.	
	The possibility of extrapolation from data of similar nanomaterials in order to avoid unnecessary dossier requirement.	Final report Task 5.1; Deliverable 5.01 • The approach with respect to grouping and categorisation described in the Deliverable has been developed in close cooperation with ECHA in order to realise a large impact.	
	c) close collaboration between authorities and industry with regard to mutually acceptable datasets and risk management practices.	Final report; several deliverables • A great number of deliverables present proposals for harmonised methods to characterise and assess NMs thus fulfilling part of the prerequisites for mutual acceptable datasets by setting basic conditions for the reliability, comparability and exchangeability of nanoEHS data. • The ProSafe Joint Document gives an overview of regulatory relevant and acceptable test methods.	Results will be included in White Paper (process)
3.1.3.1	**Toxicity testing, a substantial impact on toxicity assessment of NANoREG will be given by**		
	Prioritization criteria for physico-chemical properties and minimal set of toxicity tests, preferably in vitro, and validation of in silico methods and computational toxicology, as tools for toxicity assessment.	Final Report; Task 6.3; Deliverable 6.05 and 6.06. • Task 6.3 has delivered a database with structure-toxicity information that can be used to identify relevant relations between phys-chem properties and toxicity *Deliverable 6.05. Unfortunately it has not analysed the data in depth so far. • In Deliverable 6.06 a first attempt has been made to identify the physico-chemical parameters of NMs that may influence the functionality in terms of cell uptake, optical properties, electronic properties and catalytic activity/bio-recognition to physico-chemical parameters	The data set is available for further analyses
	Suitability of in vitro assays to connect to the in vivo experiments or specific regulatory questions. For several toxicological endpoints the most suitable assays from a regulatory point of view will be identified and optimized for their predictive value for regulatory purposes.	Final report; task 5.3, 5.4. 5.5 and 5.6; deliverables 5.03, 5.04, 5.05, 5.06, 5.07 • The deliverables mentioned provide information on the feasibility of several types of *in vivo* tests • Based on the results of the different tasks, Deliverable 5.06 comes forward with a scheme providing a first line strategy to collect hazard information at early stages of product development (see figure). Information collected following the proposed scheme may assist product developers in collecting relevant safety information on their potential products, taking into account their final use (route of exposure).	
	Providing guidelines on how to prepare and deliver the test	• A Guidance document has been developed and training on how to follow it has been pro-	• Results of all activities will be reported and

	Chapter 3 DoW Impact	Achieved impact(s)	Next steps, remarks
	substance for any given regulatory toxicity test.	vided to all users. Benchmark data have been generated and made available. • Development of (other) SOPs and guidelines has been finalised.	where appropriate be included in the evaluation by the ProSafe Task Force.
	Rapid high throughput screening methodology for MNMs toxicity in in-vitro chambers in order matching endpoints tested elsewhere.	Promising results of applying HTS and HCA techniques (see D5.07) Results are promising.	Great number of publications on the results of task 5.6 foreseen
3.1.3.2	**Exposure control**		
	Scenarios developed and tested in NANoREG include: • operational conditions, • risk management measures and • estimated exposure.	NANoREG Final report 3.2.3 all tasks • Deliverables have been submitted with information on exposure scenarios and their prioritisation and simulation approaches for aging, weathering, data on effectiveness of PPE, data of field measurements, etc.	Document integrating results WP3 in preparation.
	A map of critical exposure scenarios (in terms of economic importance and regulatory gaps) across the three domains will support the regulation authorities.	NANoREG Final Report 3.2.3 (task 3.1); • Critical exposure scenarios have been identified (Deliverable 3.1).	
	The NANoREG guidance for the measurement and characterisation of exposure of MNMs for the identification and assessment of hazard and risks is based on tested and validated reference materials and standardized methods.	• Great number of SOPs for exposure measurement have been developed and made available via the NANoREG Results Repository (e.g. Mesocosms, PPE, release from products, etc.)	
3.1.4	**Expected breakthroughs**		
	The NANoREG toolkit with a set of tools for risk assessment and decision making instruments for the regulators.	NANoREG Final Report part 2 D1.11 • Toolbox includes tools and instruments generated by NANoREG as well as other projects • Available as JRC publication and via the NANoREG Results Repository	It would be beneficial if the NSC could come to an agreement on the maintenance and expansion of the toolbox.
	NANoREG Data storage conversion supporting the regulators IUCLID data base of ECHA.	NANoREG Final Report 3.2.1 (Task 1.5) • Standardised way of data logging developed in collaboration with eNanoMapper. • Data available for other projects via the NANoREG – eNanoMapper instance.	Experimental data will feed into NanoReg2 and caLIBRAte
	Correctly place nano-testing in the context of REACH, other relevant legislation.	NANoREG Final Report part 1 several sections • Deliverable 2.12 comes forward with recommendations for adjusting several REACH guidances to the characterisation and testing of nanomaterials • Deliverable 3.02 Contributes to the evaluation and standardisation of CEN standard on dustiness • Proposals for adjusting OECD test guidelines for eco-toxicity testing (D4.12)	
	Procedure to determine the number size-distribution according to the EC definition of a MNM using the minimum di-	NANoREG Final Report 3.2.2 (Task 2.2) • The achievements in this field have been extensively described in the Final Report. They include the development of test methods	

	Chapter 3 DoW Impact	Achieved impact(s)	Next steps, remarks
	mension of a nano-object.	and the interlaboratory comparison of results. (D2.10)	
	Procedure for determination of the Volume-Specific-Surface Area as a secondary qualifier for the NM according to the EC definition of a MNM.	NANoREG Final Report 3.2.2 (Task 2.2) • Deliverable 2.11 has been submitted.	There is a marked disagreement between project partners regarding the science at the basis of the results presented.
	Correlation between in vivo and in vitro results, and extrapolation possibilities.	NANoREG Final Report 3.2.5 (Task 5.5) • Due to the late availability of results of *in vivo* experiments the correlation between *in vivo* and *in vivo* has only been evaluated to a limited extent. Deliverable 5.05	Other projects will build on the *in vivo – in vitro* data generated by the project
	Guidelines on how to prepare and deliver the test substance for any given regulatory toxicity test.	NANoREG Final Report 2.2.1 and 3.2.2 • An important step has been set by establishing the Guidance Document and associated SOPs and probe-calibration, which is key for harmonizing the dispersion energies applied.	No remarks
	Agreed test methods for well-defined hazard and exposure.	• Several tasks are developing and evaluating SOPs. One of the major achievements is the submission of the draft deliverable 2.6 (Validated protocols for test item preparation).	No remarks
	Common metrics for characterization of MNMs.	NANoREG Final Report 3.2.2 tasks 2.2, 2.3 and 2.4	No remarks
	Guidance on physical/chemical properties of MNMs that either increase or decrease the hazard of MNMs.	Data to determine the relationship between physic-chemical characteristics and hazard are now available. NanoReg2 and caLIBRAte will evaluate these data.	No remarks
	A high throughput, rapid screening methodology to study MNMs toxicity.	NANoREG Final Report • Task 5.6 on High Throughput Screening has almost been finished. • Deliverable 5.07 will be submitted at short term.	Positive forecast
3.1.5	Focus on questions and needs of regulation and legislation authorities.	All tasks of the project are linked to Q&Ns.	Working groups per category of Q&Ns will monitor, coordinate and integrate the results.
3.1.5.1	Data platform IUCLID standard	NANoREG Final Report 3.2.1 • NANoREG has chosen other way of data logging. • Standards for data logging have been established. • Partners have all uploaded their experimental data.	Implementation of NANoREG recommendations for EC and MSs for advanced data management.
3.1.6	Industry participation, the participation of industry is essential with respect to:		
	– Communicate their needs to NANoREG.	Industry has contributed to Deliverable 1.1 (Questions and Needs)	
	– Initiator of topics for Value Chain Case Studies and the implementation of such studies using real cases of their daily business.	Several Value Chain projects have been initiated in which industry participates. The scope of the projects however was rather small. Due to the late availability of NANoREG results the project could not build on these results (D1.06, D1.07	

	Chapter 3 DoW Impact	Achieved impact(s)	Next steps, remarks
		and D1.08)	
	– Evaluating NANoREG's answers and solutions for the regulation and legislation authorities with respect to the practical implementation and the effect on the day to day businesses.	Deliverable 1.09 has only been issued recently. Results of the NANoREG project has been discussed with industry during a NICC meeting initiated by NIA and Solvay in January 2017	Activities will continue under ProSafe
	– Giving advice to NANoREG as a member of the Industry Consultation Committee.	Involvement of industry with respect to shaping and execution of the NANoREG project has been limited due to disappointing participation of industry in the NICC and the late availability of results of the NANoREG project	
3.1.7	**Innovation dimension**		
3.1.7.1	Impact on the innovation process, safer design.	NANoREG Final Report 3.2.7 • NANoREG Safe-by-Design concept (SbD) as part of the Safe Innovation Approach has been elaborated by RIVM (WP6) and TEMAS (WP7).	SbD concept will be further elaborated and tested in NanoReg2 Results of NANoREG (data as well as framework and toolbox) will contribute to the implementation of SbD.
3.1.8	Economic impact – new business opportunities.	All activities of NANoREG are aimed at this goal: reducing uncertainties on nanoEHS aspect, NANoREG toolbox and Safe by design thus creating a basis for new business opportunities.	
3.1.9	European Dimension	As mentioned in the DoW the project proves to have more than a European Dimension (accession of non-EU countries; cooperation with International bodies like OECD, ECHA, CEN, Duke University)	