Development of reference methods for hazard identification, risk assessment and LCA of engineered nanomaterials

Final Report Summary - NANOVALID (Development of reference methods for hazard identification, risk assessment and LCA of engineered nanomaterials)

Executive Summary:
Nanotechnology is considered as a cutting-edge technology with the potential to revolutionize many other key technology areas, such as IT, biotechnology, medical care, food, cosmetics, or automotive industries. The groundbreaking innovation that this new high technology can bring about is based on the fact that material properties can dramatically change at nano-scales. Recent research has shown that it is this change of fundamental physicochemical (pc) properties at the 1-100 nm scale, which also may change the human health and environmental behavior of nanomaterials. Their increasing production, commercialization and use may in addition increase exposure of man and the environment to these new materials. However we still lack a scientifically sound understanding of what really happens, when
nanomaterials with these novel properties will enter man and the environment. This lack of knowledge has triggered world-wide a tremendous amount of research during the last decade, to predict the behavior, fate and possible risks associated with these unique materials. What we know today is that current measurement and testing methods often fail to reliably cover and assess the variability of the properties of engineered nanomaterials (ENM) and in particular of associated biological effects. These uncertainties have provoked the urgent need to develop reliable methods that accurately identify and measure the intrinsic properties and possible associated risks. The EU FP7 NanoValid project has addressed this need and mobilized the critical mass of expertise to find answers to this question, which is vital for the full exploitation of this new technology. Besides new measurement instruments, such as a novel hot gas nano-sampler or an on-site exposure assessment device combining physical and biological assays, a variety of measurement and testing protocols has been validated and new SOPs established during the 4 year project duration by means of a series of comprehensive inter-laboratory comparisons (“round robins”). The resulting methods show a high degree of reproducibility, accuracy and robustness for measuring size-dependent properties and effects of EN on test organisms, including man. Based on these validated methods, reference methods and certified reference materials (CRM) have been developed that allow the generation of even more accurate, comparable and traceable physicochemical (pc) and biological data, which is so urgently needed to make current risk (RA) and life cycle assessment (LCA) schemes more applicable to ENM. To even further increase method robustness and applicability, NanoValid has shared validated standard operation procedures (SOPs), cell cultures and test materials also with other relevant EU projects, such as NANO REG, MARINA or QualityNano. NanoValid has strongly contributed to relevant standardization efforts on measurement and testing of nanomaterials going on within various national and international bodies and committees, such as DIN, CEN /ISO or OECD WPMN, which resulted also in a joint workshop with CEN in November 2014 fully dedicated to the identification of SOPs and test materials generated within NanoValid that are candidates for further standardization. All tested and validated methods and materials, but in particular the developed reference methods and certified reference materials (CRM), will assist practitioners and end-users, such as material and instrument manufacturers, technology users, regulators or policy makers, to improve practices to minimize risk and maximize safety when handling nanomaterials or to upgrade existing regulatory approaches to predict possible risks that may occur along a product’s lifecycle from manufacturing to use until final disposal. Upgrading current RA and LCA tools by using methods validated within NanoValid will allow to early identify critical processes or life cycle stages, and so help to re-design and engineer materials in a way that will not cause any harm to man or nature (“safety by design”).

Project Context and Objectives:
The rapid development of novel engineered nanomaterials (ENs) is based on new ground breaking material innovations that revolutionize cutting-edge applications in key areas such as medical treatment, energy generation or food supply. This in turn is creating an enormous potential for future economic and sustainable growth. The strong innovation potential of nanomaterials is mainly based on the fact that material properties can dramatically change at nano-scales. Also the increasing production, commercialization and use of nanomaterials may increase exposure of man and the environment to these novel substances. Moreover, recent research has shown that it is just this change of fundamental material (pc) properties at the 1-100 nm scale, which is also changing their human health and environmental behavior. Despite huge efforts not only in Europe but world-wide during the last decade to assess the safety of nanomaterials, our present knowledge on key properties, mechanisms and factors that are
crucial for their behavior is still hampered, as current methods developed for conventional chemicals are often inappropriate to reliably assess possible associated hazards, exposure and risks associated with nanoparticles. Based on the lack of proper methods, our scientific understanding is still insufficient to know or predict what is really going on, when nanomaterials enter living systems. Before this background, there is an urgent need for new reliable measurement methods and instruments that can help to detect, prevent or minimize possible harmful effects along the life cycle of nanomaterials, or to assist in designing safe material properties. Protocols and tools derived from testing conventional chemicals often do not match the great size dependent variability and impact of properties of ENs, and of resulting biological effects, or to indentify the mechanisms that control their interaction with organisms. The resulting data uncertainties challenge any true risk assessment and provoked the urgent need to develop validated methods that are applicable to nanomaterials in a standardized way. It was exactly here, where NanoValid came into the game with the primary goal to meet this need and to help to overcome existing method limitations and data uncertainties, and so help to improve current risk (RA) and life cycle assessment (LCA) schemes. In 2010, a group of outstanding European and international scientists from various research fields, both from Europe and abroad, came together to mobilize the critical mass of expertise needed to tackle this question. At the end, a concept and strategy was designed and suggested to implement a 4 years research program that is able to integrate the different disciplines and tools into a master plan required to develop validated methods applicable to nanomaterials. Based on the development of validated methods, the ultimate goal of NanoValid was to contribute to the safe exploitation of the benefits of nanotechnologies by developing robust reference methods and reference materials that help to reliably assess hazard properties and exposures of man and the environment to engineered nanomaterials. To achieve this overall goal, the following specific objectives have been defined:

• to provide and distribute well-defined test materials to all project partners for method testing, development and validation
• to run comprehensive inter-laboratory comparison (ILC) studies at various levels of pc characterization, labeling, dispersion control and in vitro/in vivo testing, supported by extensive literature reviews, to update and validate our current knowledge and to integrate the validated methodology into current RA and LCA schemes
• to contribute current method and material standardization and harmonization by proposing new work items (NWI)
• to design and generate specific reference materials, in particular certified reference materials (CRMs), to improve data comparability, reproducibility and precision of toxicological testing
• to document the robustness and applicability of validated methods under real conditions.

Project Results:

NanoValid has successfully completed an ambitious and comprehensive work plan with the ultimate goal to establish validated methods for the measurement and testing of commercially and environmentally relevant engineered nanomaterials (EN). To achieve this goal, a tiered and bottom up approach was designed and used consisting of various tasks, deliverables and milestones organized around 5 vertical (scientific/technical) and 3 horizontal (managerial/dissemination) work packages (WP) such that they allowed a stepwise implementation of the planned work (see Figure 1).
established to ensure close cooperation between the various work packages and emphasizing their close interconnectedness required to implement such a complex and large-scale project. 30 different partners came together from 17 different countries (Europe, Canada, India, the US and Brazil) to mobilize the critical mass of expertise and the number of expert laboratories needed to successfully implement the comprehensive inter-laboratory method and materials testing and validation work.

Figure 2: Overview on the coherence and interactions of the different WP, tasks and subtasks

A short description of the main work performed within each technical WP and across WPs is presented below, followed by a description of main S&T results/foreground achieved within each WP during the 4-year project duration.

Main work implemented during the 4 year project duration

In WP2 a first pre-selection and pre-testing of commercially available and newly produced relevant nanomaterials and of current measurement and testing methods was performed to early identify candidates that are most promising for further upward testing and validation. A total number of 13 regulatory relevant materials has been in-house fabricated or partly procured from external sources, pre-characterized and pre-tested. 9 of these materials were identified as suitable for further method development and distributed to subsequent work packages, in particular WP3 (validation) and WP5 (reference methods and materials) (see Table 1). As part of the preliminary testing in WP2, selected materials were also used to test the performance of biological screening methods to generate a first toxicological profile of the test materials and so help to indicate their biological significance. Another main outcome in WP2 was the built up of a project-specific database to serve as a repository for all relevant analytical and biological results produced, including methods and materials that have been validated, tested and applied as reference tools in upward work packages.

Table 1: Materials selected and distributed in WP2. (PC=physicochemical characterization)

In WP3 a series of comprehensive inter-laboratory comparisons (ILC) has been conducted, to study the methods and materials selected in WP2 in more detail, and to validate their suitability, in terms of accuracy and reproducibility, to become reference methods and materials (in WP5). A variety of pc and in vitro / in vivo protocols has been tested, validated and further developed, and a great number of new standard operation procedures (SOPs) established for characterization, hazard and exposure assessment of a panel of well-defined nanomaterials. Based on the outcome of the extensive inter-calibration and inter-comparison campaigns carried out in WP3, a range of methods and materials showed a high degree of precision and accuracy in particle size, shape or surface measurement and in assessing size-dependent hazard properties and effects in organisms and human cells. These validated methods were identified and suggested as good candidates to become further developed as reference methods and reference materials in WP5.

The suitability of the developed and validated methodology to improve the applicability of current risk (RA) and life cycle assessment (LCA) schemes to nanomaterials was examined in WP4, mainly by comparing the newly produced hazard, exposure and effect data generated by the newly developed methods with data from the most recent literature, and their integration into new approaches and strategies. The potential of existing toxicity tests to define, if a nanomaterial is toxic or not, was critically assessed.
Recommendations derived from the evaluation of the performance of test methods were put into a broader risk assessment framework by proposing a new test strategy based on decision trees and flow charts guiding risk assessors through the sound pc characterization of nanomaterials (see Figure 3). A thorough characterization of the properties of nanomaterials, such as dispersion/solution or agglomeration/aggregation, prior to biological testing, proved to be a crucial step to receive comparable results and hence to improve current RA schemes. Also a life-cycle assessment of nanomaterials was performed uncovering significant data gaps (especially in manufacturing data) and data inconsistencies that are challenging the life cycle inventory and the calculation of characterization factors, which are needed to make current LCA approaches applicable to nanomaterials.

Figure 3: Decision tree on test scenarios and application to testing of CuO NP in a stabilized and a non-stabilized state (UFZ)

A more stringent method and material testing and validation approach was used in WP5 to develop reference methods and materials. These reference tools are characterized by an estimate of the statistical uncertainty of results. They can help to assess the quality of other methods, to characterize reference materials and to determine reference values. A variety of reference methods have been established for the (1) pc characterization of nanomaterials and the development of related certified reference materials (CRM), and for (2) human and environmental exposure and impact assessment. 2 types or classes of reference materials have been produced: a) calibrants for pc characterization methods, such as size measurement (Au and SiO2) and specific surface area measurement by BET (anatase TiO2), and b) well characterized reference materials (2 different SiO2, TiO2) that are suitable for big batches required for toxicological tests. SOPs for reference methods for sizing and size distribution measurement (mean equivalent spherical particle and hydrodynamic diameters as measurands), surface charge (expressed as zeta potential) and specific surface area measurement (BET method) have been established by comprehensive ILC and characterized by uncertainty considerations and traceability. Based on these results, validated SOPs have been established and agreed convention parameters or endpoints obtained. Other reference methods (SOP) developed include: (i) dispersion control in various test media, such as water, KNO3 and DMEM+FBS (has been submitted for standardization under ISO TC 201), (ii) quantification of labeled nanoparticles (lanthanide-doped Y2O3 NPs) in various matrices, (iii) measurement of uptake and distribution of nanoparticles (SPIONS, nano-Ag, nano-Au, nano-whiskers, C60, SiO2, CuO, ZnO, TiO2) in the human body, including in vitro toxicity assays such as ATP, Propidium Iodide, Mitochondrial Dehydrogenase, and the NRU OECD GD 129 assay, and (iv) eco-toxicological testing, such as microbiological tests (bacteria Sinorhizobium meliloti), terrestrial tests (plant test according to OECD 208 and the Porcellio scaber test), or aquatic tests (the Daphnia magna (freshwater) and Artemia salina (seawater) test, which all showed a high potential for standardization. Also the NanoValid “ENP Specification Form” to define properties of test materials required for toxicological testing has been created and also already integrated into a new ISO standard document.

WP6 has assessed the robustness and applicability of the developed methodology under real conditions by means of 6 case studies dealing with problems of the working environment and in natural systems, and including real-life on-site work place exposure measurements, studies in water, sediment and soils, and the modeling of a transport accident simulating an explosive release of nanoparticles. A great deal of methods developed in WP2, WP3 and WP5 were taken outside the laboratory and employed in the field, which is a challenging but necessary step. Case study 1 analyzed nanomaterials collected in an industrial
location as well as intentionally mixed with realistic contaminants. The experiments helped to assess the robustness of methods and give insight into health effects of particles under real conditions. Case study 2 developed a novel online exposure system combining physical and biological assays for airborne nanoparticles. Case study 3 tested ecotoxicity of collected nanomaterials already used in case study 1 and compared the performance of toxicity assays in actual lake water with those applied in conventional artificial lake water. Case study 4 modelled a transport accident leading to a massive release of nanoparticles. Based on field study tests at 4 different locations, and on available regulatory considerations, case study 5 developed a guideline on safe working with nanoparticles. Case study 6 analyzed nanoparticle release during a work process in the automotive industry.

Main results/foreground achieved
Selection of methods and materials (WP2)
A total of 13 prototype materials have been prioritized, selected and distributed within the consortium (see Table 1). Also existing protocols were reviewed and optimized in WP2 for “in-house” synthesis of some of these prototypes. This resulted in 6 out of 13 prototypes being internally supplied by NanoValid partners: 3 silica (SiO2) NPs (of which one dispersed in water) by NLAB, 2 titania (TiO2) NPs by CCMB, and 1 dispersion of gold NPs in water by INMETRO.

Among the seven prototypes that were procured externally, a dispersion of silver (Ag(H2O)) NPs in water and carbon nanotubes (CNTs) were supplied by MARINA partners Colorobbia and Nanocyl, while zinc oxide (ZnO) NPs was supplied by Nanogate (partner in NanoSustain). A dispersion of palladium NPs in water (Pd (H2O)) and fullerenes (C60) was procured from Plasmachem, a fourth SiO2 NP prototype was purchased from Evonik, and copper oxide (CuO) NPs were provided by Intrinsiq Materials Ltd. For various reasons, two of the SiO2 prototypes (including the SiO2 dispersed in water) as well as the Pd (H2O) and C60, were discarded from further use at an early stage. In WP2, the nine remaining NP prototypes (2 SiO2, 2 TiO2, Ag(H2O), Au(H2O), CNTs, CuO and ZnO) have been fully pre-characterized by following OECD standards including most important parameters, such as size, structure, shape and porosity and by using XRD, SEM, DLS, BET, z-potential, TEM and ICP (see Table 2).

Table 2: PC characterization techniques used in WP2

| 4 draft SOPs on characterization methods (XRD, TEM, DLS and BET) have been prepared, and 2 methods (for DLS and BET) subsequently validated by WP3 as potential candidates for reference methods. One of the two SiO2 prototypes was very promising to serve as a good reference material candidate for BET measurements (in WP5). To support the identification and validation of appropriate fabrication and characterization methods and the selection of suitable test materials, the expertise available within the NanoValid consortium on toxicity testing has been reviewed and the most promising methods tested for their potential to serve as screening methods (toxicological profiling) and to provide a preliminary hazard analysis of the selected nanomaterials (SiO2 NNV-001, SiO2 NNV-002, Ag NPs NNV-003, Au NPs NNV-004, TiO2 NNV-005, MWCNTs NNV-006, CuO NNV-011, ZnO NNV-012 and TiO2 NNV-013). In the case of soluble metallic NPs (Ag NNV-003, CuO NNV-011, ZnO NNV-012), the respective metal salts were tested in parallel. Toxicological profiling was performed by using and testing 16 different bioassays with 12 different organisms/cell types. The applied toxicity screening battery included eco-toxicological assays with species of different food-web levels, medically and hygienically important microbial strains as well as in vitro tests | 6 of 24 |
with mammalian cells. As a rule, the toxicity pattern of nanomaterials was similar, whatever the assay.

Toxicity decreasing in the order: Ag>ZnO>CuO>MWCNTs>Au=SiO2=TiO2.

Based on the obtained results a refined test battery was suggested including 3 assays for the initial toxicological screening of test nanoparticles that follows the 3R’s principle:

- bacteria Vibrio fischeri 30-minute bioluminescence inhibition assay,
- aquatic crustacean Daphnia magna 48-hour immobilization assay and
- 48-hour NRU assay on murine fibroblasts in vitro, is suggested.

This test battery involves prokaryotic and eukaryotic organisms/cells, particle-feeding organisms (Daphnia) and assumingly particle ‘proof’ organisms (bacteria). All three assays are standardized at the OECD and/or ISO level. Obtained results reflect very well the amplitude and variability of possible responses that may occur when nanomaterials are released and end up in ecosystems.

To collect, organize and make the newly generated data available for all project partners, a online available project-specific results database has been developed as a repository to collect, store and evaluate the vast amount of data and SOPs generated and make it available to other projects and stakeholders. Data generated in WP2, WP3, WP4 and WP5 both on pc characterization and toxicology have been uploaded to facilitate data sharing between partners and work packages and avoid unnecessary duplicate work. The database is available at the protected part of the NanoValid website (www.nanovalid.eu) and gives access to a total of 57 files related to methods (SOP) and materials developed for the pc characterization, exo-toxicological testing, in vitro / in vivo assays and LCA assessment tools (see Table 3). The uploaded files cover the 9 NNV test materials provided and tested within the project: NNV-001 (SiO2), NNV-002 (SiO2), NNV-003 (Ag), NNV-004 (Au), NNV-005 (TiO2), NNV-006 (CNTs), NNV-011 (CuO), NNV-012 (ZnO), NNV-013 (TiO2) (in addition to non NanoValid specific test samples).

Table 3: Structure of the NanoValid results database

The database structure allows the rapid search and access of reports according to chemical composition, NNV material code, WP or type of study, to easily find the right information (see screenshot in Table 4).

Table 4: Example illustrating the structure of the project-specific database

Method validation (WP3)

Main objective of WP3 was to generate a set of validated SOPs on measurement and testing of NP that can help to improve current RA and LCA schemes (in WP 4), develop reference methods/particles (in WP5), and to perform case studies to assess their feasibility under real conditions (in WP6). SOPs were built through a set of inter-laboratory comparison (ILC) studies aimed to (a) characterize nanoparticles and to (b) conduct cytotoxicity studies using various cell lines and end point assays. To monitor the release of NP at work places or from accidents, a specific NP aerosol particle generator and a self-cleaning dispersion room have been tested and measurements performed. Also a variety of fate and effect studies in aquatic and terrestrial systems have been undertaken to validate tests used to assess uptake, bio-persistence and bioaccumulation of EN in laboratory models.
A panel of validated SOPs has been generated in WP3 to reliably measure and assess relevant material properties (size, size distribution, shape, surface charge, surface chemistry, purity, surface area, hydrodynamic size etc.). For this, 5 types of ENM (gold, MWCNT, zinc oxide, copper oxide and titanium dioxide) selected in WP2 have been used and characterized to validate a variety of methods by several comprehensive inter-laboratory studies and by using SOPs tested in WP2 (see Figure 4 below). As a result, a set of 5 validated SOPs on pc characterization was established. Also methods for assessing cytotoxicity of Ag and Au NP were validated by ILC and by using several cell lines (A549, THP-1 and HepG2) and endpoints (cytotoxicity, apoptosis, inflammation) resulting in a set of 7 validated SOPs on toxicity testing (including MTS assay, ATP assay, caspase 3/7 assay, ELISA, A549 cell culture, THP-1 cell culture and cell growth rate and viability) (see Figure 5 below). Likewise, relevant eco-toxicological methods have been validated by ILC to measure bioaccumulation, bioavailability and biodistribution of NPs in organisms at main trophic levels (see Figure 6 below).

Figure 4: TEM images of ENMs used in WP3 for method validation: (a) Copper oxide, (b) Zinc oxide, (c) titanium dioxide

Inter-lab comparison of cytotoxicity tests have been conducted by several partners to develop validated SOPs for several cell lines, and selected assays (e.g. MTS, ATP, caspase, cytokines). Experiments conducted were using gold and silver nanoparticles (see Figure 5). In total, 11 SOPs have been created to assess the effect of cell culture conditions, the growth of A549, THP-1, and Hep-G2 cells. In addition, SOPs for different end points (cytotoxicity, inflammation, oxidative stress and hemo-compatibility) have also been developed, keeping in mind the interferences occurring in the assay from the nanoparticles themselves.

Figure 5: Variability among participating laboratories conducting round robin to validate MTS assay using silver nanoparticles on (a) A-549 cells and (b) THP-1 cells.

Eco-toxicological testing (eco-toxicity and biological fate) of ENMs has been completed by and various SOPs developed on selected model organisms to cover both, aquatic and terrestrial systems. Quantitative uptake studies using LA-ICP-MS were developed as a viable method for the visualization of nanoparticle uptake and distribution of particles within tissues (see Fig. 6).

Figure 6: Visualisation and quantification of nanoparticles in Daphnia magna using LA-ICP-MS (University of Ljubliana)

Aerosols of SiO2 nanoparticles have been obtained by using a state-of-the-art fluidized bed aerosol generator (FBAG), in which both airborne nanoparticle concentration and particle size distribution can be effectively controlled. The aerosol generation from FBAG is based on a long-lasting detachment of 100-nm nanoparticles upon fluidization at different flow rates. Also, a novel self-cleaning test chamber for the dispersion of ENMs aerosols was developed to simulate the behavior of NP aerosols in either particle-free or controlled environments, and to simulate indoor conditions at different locations. Equipment and places for the dispersion of ENP aerosols have been made available to validate the performance of aerosol characterization methods, such as CPC + SMPS, OPC and NPS500.

Applicability of validated methods to RA and LCA (WP4)

Main objective of WP4 was to test the suitability of validated methods to improve current risk (RA) and life cycle assessment (LCA) schemes. For this, results achieved from relevant work packages (WP2, WP3, WP5 and WP6) and data from the relevant literature were used, evaluated and integrated to critically estimate their potential to identify hazards posed by ENM to humans and the environment within current RA strategies, but also to support the life cycle assessment and life cycle impact assessment of ENM. Existing RA strategies specifically developed for nanomaterials were critically reviewed and hazard
assessments on specific nanomaterials (e.g. CNT, Ag, CuO) was based on data obtained from the most recent literature. Also, the suitability of several toxicological endpoints used in WP2 and WP3 was examined and evaluated, and short reviews provided for each method. Further, hazard assessment test methods developed and applied mainly within WP2 and WP3 were critically examined for their applicability to nanomaterials. In order to detect and identify nanomaterial hazards in a reliable way, most methods could be classified as applicable to nanomaterials but still require specific amendments. A review paper has been published on NM hazard identification for human health and on identification of methods most applicable for human health hazard identification.

The integration and evaluation of the vast amount of toxicological data obtained by various partners in various biological test systems has focused on results obtained for nanosilver within the project. The data were compared with regard to toxic effect levels in different organisms, as well as to their physical-chemical characteristics over time (aging).

A second main outcome achieved within WP4 involved the creation of decision trees and flow charts (see Figure 3). The decision tree approach aims at harmonizing the developed and validated test procedures for nanomaterials by guiding in the selection of a nanomaterial specific characterization and testing regime. Specifically, material identity, physical-chemical characterization, choice of test conditions and test systems, and behavior during the tests are considered. The decision trees were further refined and extended by integrating the relevant data and knowledge produced by the methods developed and validated within NanoValid. With the help of DIN, the approach has been submitted for further standardization. In line with that, a list of criteria for good quality nanotoxicity data to be used for RA and LCA was developed and continuously updated. The criteria relate to four different data categories: (1) physical-chemical characterisation, (2) sample preparation (3) toxicity testing, and (4) general aspects. These criteria were chosen in order to represent all relevant nanomaterial properties and nano-specific behaviours to be considered when subjecting them to toxicological tests, as a material may exist at the same time in various, substantially different nanoforms. The diversity of the nanoforms of a substance may hence lead to different testing requirements. Accordingly, for data evaluation and judgement of appropriate test procedures, several parameters specifically applying to nanomaterials have to be taken into account. The decision tree approach was also proposed as a new work item proposal (NWIP) for standardization and presented to the DIN committee “NA 062-08-17-03 UA Health and environmental aspects” to prepare and initiate standardization. The procedure developed was published in Science and Technology of Advanced Materials.

The performance of LCA and life cycle impact assessment required a comprehensive collection of relevant data for each selected nanomaterial and their compilation in data collection sheets. Cradle-to-gate manufacturing data have been collected for 6 nanomaterials and resulted in the life-cycle inventory datasets. This data collection was actively supported by all NanoValid partners that have produced LCA relevant data. The calculation of new characterization factors (for the fate, exposure and effect of selected nanomaterials) has been realized by using models for ENM that have been developed for conventional chemicals, but also by cooperating with the developer of the “SimpleBox4nano”, which considers nanomaterial specific processes and characteristics. Based on the received modeling data, a case study on nano-TiO2 in sunscreen has been implemented to show the applicability of LCA methods developed and to assess the impact of manufacturing and use of TiO2 on environmental and human health (see Figure 7).

Figure 7: Sun screen life cycle stages considered in the LCA case study (QUANTIS)
A paper describing the developed Life Cycle Impact Assessment method for nanomaterials, with detailed
description of the proposed fate, exposure and effect models has been prepared including the generation of characterization factors for fate and toxicity estimation required for the case study. All nanotoxicity data obtained within the project (focusing on nano-Ag), and the relevant literature data on the toxic potential of a particular NM, has been compared and a review published in Environment International. Major obstacles and bottlenecks in nanomaterial testing within a RA and LCA perspective still occur at the interface between nanomaterial characterisation and toxicity assays and were identified and elucidated. Recommendations regarding testing of nanomaterials with regard to the interference with dissolved ions were given.

Developing reference methods and materials (WP5)

Methods identified under WP2 and validated under WP3 were evaluated in WP5 for their potential to be developed as reference methods. Test materials selected and/or newly fabricated in WP2 and validated in WP3 have been used to develop appropriate RMs and CRMs. A principal milestone was the preparation and characterization of stable nanoparticle suspensions that can be directly used for in vitro testing, analysis of the particles behavior in physiological media and environmental matrices, and for verification of these approaches by various size measurement methods. Materials supplied by WP2 have been characterized in WP5 by means of T-SEM and XPS in terms of size distribution and chemistry. To develop best practice methods and validated methods for pc characterization of ENM, certified reference materials (CRMs) are needed, in particular to support toxicology studies. Specific procedures were launched to develop new CRMs and reference methods, in particular to synthesize CRMs of nano-Au, nano-SiO2 and nano-TiO2 (anatase), and to implement metrologically sound ILC for measurands, such as size distribution, zeta potential, BET specific area, dispersion in various test media and labeling of ENPs. Validated SOPs have been established with test materials from WP2 to assess the suitability (reproducibility, uncertainty budget etc.) of current human toxicology in vitro and in vivo assays and protocols to measure fate and effects in aquatic and terrestrial systems, and finally to become further developed as reference methods. New protocols for cell cultures used for eco-toxicity testing and a new soil-plant test have been also developed as well as new human toxicity models and their applicability to ENPs delivered by WP2 and validated in WP3, which include an angiogenesis model, a chronic rhinosinusitis model, a SDS-page technique to determine proteins in urea, and a nasal model. Results indicate that the developed models (tests) are applicable to a wide range of ENPs (see Figure 8).

Figure 8: Validated assays for human toxicological testing (University of Tampere)

Together with WP2 and WP3, WP5 developed at the beginning an internal set of standard criteria for the specification of test samples that can be used for toxicity testing. This resulted in the NanoValid specification form that was based on a template developed by the German NanoGem project and that has been used for all NPs tested within the project. The document was presented at a ISO/TC 201 meeting and will be considered for standard development [www.nanovalid.eu/templates].

In addition, a report on the definition of reference methods and a standardized protocol for inter-laboratory comparison of pc measurement methods was prepared. A literature study on existing methods for the dispersion of nanomaterials in water as well as in cell culture media was prepared (see WP3) and results of validation measurements that use different dispersing methods for standard nano-TiO2 and nano-SiO2 have been obtained. Based on this, a SOP on dispersion has been developed and a draft protocol and appropriate strategy for determining the uncertainty of measurement as part of an inter-laboratory comparison on dispersion was established resulting in a validated SOP for dispersion of NPs in water and other test media. This will ensure proper handling of NP
in toxicological and environmental media and particle size measurements in appropriate concentrations, e.g. by using DLS and qualified by zeta potential determination.

NanoValid developed in total 2 classes of reference nanomaterials: (1) a CRM representing a well defined, “certified” parameter with an uncertainty, and measurands such as size, BET surface etc., and (2) reference materials (RM) useful for eco-toxicity testing. These 2 types of RM are supported by a set of relevant specification data, which are valid and delivered together with the NanoValid ENP Specification Form.

The following reference materials (RM) have been produced: a BAM nano-SiO2, a nano-Au prototype prepared by INMETRO (Brazil), and titania. Also RMs available for bigger batches and useful for ecotoxicity testing were prepared. Due to the homogeneity in size and shape of the synthesized BAM nano-SiO2, this prototype was submitted to the certification procedure developed by BAM to become a CRM. For certification, traceable SAXS measurements are applied. The Au NPs synthesized by NanoValid partner INMETRO were characterized by SEM, MET, DLS, Zeta, XPS, AFM (see Figure 9). An inter-laboratory comparison on size determination was performed (in WP3). The homogeneity of the batch has been investigated and found to be sufficient. The potential CRM candidate titania has been tested and found to be sufficiently homogeneous in terms of surface area. The batch has been tested in a round robin with measurand surface area and will become certified.

Figure 9: TEM image and particle size distribution of gold particles (INMETRO).

To establish reference methods for NPs labelling, different optical labelling methods of NP have been tested. Synthesis methods were modified to reduce particles size but maintaining the capacity of labelled ENPs detection. After analysing the labelled nanomaterials developed within the project, lanthanide-containing Y2O3 nanoparticles were selected as candidate for ILC. The conditions for the preliminary tests have been defined to quantify the labelled NP and to set up threshold detection limits. A SOP for labelling has been prepared and validated by a round robin. Also hollow Au nanospheres and SPIONs have been synthesized and tested as contrast agents (imaging) to evaluate the biodistribution and effects of NPs in the human body. To assess the usefulness of these labeled NP as type 2 reference material, the batch homogeneity has been tested after several synthesis sets and found to be sufficient.

To establish reference methods for assessing uptake and distribution of NP in the human body, the nanotoxicity of the 2 SiO2 prototypes and the Ag delivered by WP2, as well as of carbon nanowhisker (from MARINA) were tested in vitro and evaluated by using WST-1 (or MTT), ATP, and nuclear membrane permeability method and propidium iodide assays. To study changes in the function of biological barriers, such as skin, mucosa and brain, nano-Ag, SPIONs, nano-Au, the MARINA nanoparticle-carbon nanowhisker, and C60 were tested in vivo by using the inner rat ear as a multifunctional model and evaluated by imaging, auditory function and histology evaluation studies. The distribution of SPIONs and nano-liposomes containing LPS+Gd-DOTA in the body could be visualized by MRI. DMSA@SPIONs enter the inner rat ear more efficiently than POA@SPIONs after intratympanic administration and stayed in the inner ear for at least 7 d. Hollow Au NPs impaired the middle ear mucosa but not the inner ear barrier, while uptake and distribution of Ag NPs in the body were detected by micro CT (see Figure 10). It was also possible to track uptake and distribution of ENMs in the body using near infrared (NIR) imaging in vivo and the concept was proved in mouse ear using NIR dyes. In addition a method was developed to evaluate possible toxicity of ENM on kidney filtration. In vitro tests proved to be more than 1000 times more sensitive than in vivo studies.

Figure 10: Distribution of Ag NPs in the ear after transtympanic injection shown by micro CT. (from: Zou et al., J Nanobiotechnology, 2015, 13 (1) 5)
Also various inter-laboratory comparisons of eco-toxicity tests have been conducted to test the suitability of aquatic (Artemia salina, Daphnia magna) and terrestrial (Porcellio scaber) organisms for nanomaterials’ ecological hazard identification. The P. scaber in vivo feeding assay was initially considered for toxicity screening of nanomaterials but withdrawn from the final test battery as it was rather time consuming and better suited for further Tier 2 toxicity evaluation. Protocol templates specific for (1) particle synthesis/production, (2) particle characterization, and (3) toxicity testing have been compiled and preliminary validated SOPs provided by adopting the template available from the EU FP7 Nanommune Handbook.

Based on these tests, the following conclusions have been drawn:
- micro-biological tests proved to have the potential to flag possible risks posed by NP to soil microflora with evidence for nano-TiO2 to induce acute cytotoxicity to the agronomically beneficial nitrogen fixing bacteria Sinorhizobium meliloti
- a review identified toxicological and ecotoxicological key organisms that are relevant for nanotoxicity testing.
- a modification of the OECD test protocol 208 (“Terrestrial Plant Test: Seedling Emergence and Seedling Growth Test”) was developed that reduces the amount of materials required by more than 60 %. Comparison with the standard protocol demonstrated that although results at toxicant concentrations causing less than 20 % reduction in growth (EC20) are more variable, there is no loss of power at EC50 concentrations. This modification is recommended for preliminary toxicity tests in situations where nanomaterials are costly or available in only limited quantities.
- a priority list of methods suitable for ENP exposure assessment (biological and non-biological) according to the environmental compartment (human exposure including occupational exposure, environmental exposure including soil and aquatic exposure) was compiled.
- A novel approach was developed to measure the size of NPs attached onto a surface by using a quartz crystal microbalance with dissipation (QCM-D) monitoring and the applicability of this method was demonstrated for studying disruption of lipid bilayers by engineered NP.

The 2 certified reference materials nano-Au and nano-TiO2 have undergone formal certification as calibrant materials at INMETRO and BAM with the aim to improve measurements for pc measurands Size and Specific Surface Area (BET), respectively, and for human and ecotox testing applications. CRMs will help to solve problems caused by insufficient metrology of the testing methods used in the characterization of ENPs and the assessment of their risks as required by European legislation and industry to increase its competitiveness through sound regulation and product safety.

The 2 reference materials nano-SiO2 (BAM) and nano-SiO2 (NLAB) have been prepared in big batches to be commercially used for future human and ecotox testing applications. New and improved measurement capabilities in the field of routine and valid human and eco-toxicological testing will be facilitated by commercially available reference materials. This will increase data reliability and comparability as required by REACH regulation and needed for method validation.

Valid SOPs for dispersion of nanoparticles have been prepared taking the specific energy input for dispersion, kind and amount of dispersant aids and particle concentration and fluid composition into account. Dispersion SOPs as part of controlled nanoparticle sample preparation for human and eco-toxicity testing will help to solve problems caused by insufficient metrology of the testing methods used in the characterization of ENPs and the assessment of their risks as required by European and national regulatory bodies and concerned industries. Less uncertainty will increase competitiveness of industries and enhance product safety as demanded by European regulation bodies.
taken up for drafting new CEN/ISO standards. Reference methods (SOPs) for pc characterization endpoints Size, Size Distribution, Surface Charge (expressed as zeta potential) and Specific Surface Area (BET method) have been developed as a result of metrologically strengthened ILC based on information on uncertainty budget. ILC can help to solve problems caused by insufficient metrology of the testing methods used in the characterization of ENPs and in the assessment of possible risks as required by European legislation and industry. This in turn will help to improve the market position and economic competitiveness of European industries, due to increased product and consumer safety, and the higher data comparability and reliability will support standardization, which has been already initiated.

Reference methods (SOPs) on cell-based, tissue-based and in vitro and in vivo assays, including new models (e.g. the rat inner ear model) and new visualization methods for the distribution of ENPs after different type of exposure to industrially relevant nanomaterials have been developed (e.g. by successful bilateral comparisons), which help to deliver more valid protocols and reliable data needed for risk assessment. SOPs developed on base of these validation activities will help to solve problems caused by data uncertainties due to insufficient metrology of the testing methods used in the assessment of risks as required by European legislation and industry, and so help to increase market competitiveness due to increased product safety and application, and data comparability due to better standardization, which has been already initiated.

Reference methods (SOPs) for assessing environmental exposure and effects of nanomaterials to freshwater and terrestrial organisms, all tested by using selected and well characterized WP2 test materials, have been established by ILC relying on metrologically underpinned protocols. These SOPs will help to minimize the observed variability of toxicity testing results. As an early impact, selected validated SOPs for eco-toxicological testing have been streamlined and submitted to international standardization (CEN/ISO) with the help of the national (Slovenian) standardization body.

Case studies (WP6)
Case studies were used to translate the knowledge gained through validated methods in the laboratory to the real world. The studies were differently structured, with a focus to enhance the practical value of NanoValid methods under realistic conditions. These practical studies focused on work place safety assessment (case study 1+2), the environment (case study 3), accidents (case study 4.), reducing risks for occupational handling (case study 5), and monitoring NPs in the automotive industry (case study 6).

One challenge was to take the real world situations with so many unknowns into account, why laboratory practice needs to be replaced by situations that correspond to actual exposures. It is widely accepted that laboratory methods using highly controlled materials are essential for unraveling molecular mechanisms, but need to be complemented with more robust assays that are able to deal with actual exposure scenarios. Main efforts were devoted to link optimized test materials and validated methods developed in WP2-WP5 with real world situations, in which particles are not clean, not always well characterized, and may behave in ways not anticipated in the laboratory. The case studies included nanomaterials in mixtures (resembling real life situations), TiO2 and SiO2 particles collected in a paint factory, and non-toxic gold particles combined with bacterial compounds and allergens.

The work started with a code of procedures (case study 5) that was resulting from an open workshop (Berlin 2012) to ensure safe and standardized handling of NPs in all partner labs. The proceedings of this workshop are available as “best practice” example at the project website (www.nanovalid.eu). A standardized procedure for field studies to assess the feasibility of the developed guidelines has been prepared and field studies have been implemented at various research and small production sites to
assess the occupational exposure to NM during manufacturing and handling (see Figure 11). The outcome was directly fed into the practical guidance document (“Nano to go”) provided to improve and verify current procedures based on practical experience (see Figure 12).

Figure 11: A setup for the production of carbon nanotubes (CNT) located at BAUA. This system is part of the practical studies in case study 5.

Figure 12: Guidance document “Nano to go!” for the safe handling of nanomaterials (BAUA 2015)

Monitoring work place environments was done by using collected materials and on-site testing. Samples were collected in a paint factory in Denmark and distributed to partners for testing. Another approach produced mixtures of clean NPs with selected compounds (the pro-inflammatory bacterial compounds LPS, MDP, ieDAP, CpG-DNA and flagellin, plus the allergenic proteins Bet v 1, Phl p 5 and Der p 1). In this case the type and extent of contamination is known and the mixture can be used to validate the robustness of biological assays (see Fig. 13).

Artificial mixtures. Bacterial compounds bound to nanogold still elicited inflammatory responses. LPS emerged as the main compound of concern, with two others having reduced effects after binding to particles. In contrast, 2 out of 3 allergens tested were able to induce higher allergic reactions, identifying safety of allergy patients as an area of concern. Specific uptake pathways into lung cells were analyzed, which improves our present understanding of molecular mechanisms. First report shows increased allergenicity of a common indoor allergen, after attachment to nanoparticles, which should prompt further investigations of allergic persons as a possible risk group. The uptake study suggests the use of positively charged gold nanoparticles for drug delivery, which will be further investigated.

Figure 13: AU NPs, from left to right 1) as synthesized, 2) coated with LPS, and 3) coated with flagellin. Average size by TEM is 64, 79 and 97 nm, respectively, suggesting homogenous coating with a single layer. This is confirmed by chemical analysis (University of Salzburg)

Factory collected material. Real life material collected in a paint factory was tested with human cells, invertebrates and plants. It proved to be fairly harmless in all these tests. Publications are in preparation on the new findings that will be relevant for work place safety assessment.

Hot gas nano sampler. A novel hot gas nano sampler was developed which has desired properties missing in existing instruments. It is also suitable for room temperature and allows the on-site measurement of nanomaterials in the gas phase (see Figure 14). The device avoids the problems associated with existing instruments (such as isokinetic sampling needed, conventional flow splitters do not work for large particles, particle losses at bendings). Marketing and commercialization is planned by the partner creating the prototype.

Figure 14: The novel hot gas sampler (Grimm Technologies)

For the purpose of direct on-site testing (in case study 1+2), a panel of reporter cell lines was developed expressing Red Fluorescent Proteins (RFP) under control of promoters indicating cell stress or inflammation, i.e. unspecific and early signals of the body indicating irritation. The reporter cells are based on the human lung epithelial cell lines A549 and developed and used in a novel on-site measurement device (“Navetta”) also produced and tested within the project (see Figure 15). A modulation to make these tests more realistic was the use of human lung fluid. Such fluid was collected during some clinical procedures (bronchoalveolar lavage fluid, BAL), and tested to show if the addition of human BAL gives different effects than the conventionally used FCS, which of course contains different proteins and other compounds compared to human lung.

Navetta. The novel device (Navetta) was developed in which human lung cells can be exposed to nanoparticles via the gas phase (see Figure 15). The prototype mimics some aspects of nanoparticle
inhalation better than all systems reported so far. A panel of novel reported cell lines specifically designed for Navetta is now available and has already been used for several in vitro studies within and outside of the project. The Navetta itself is a prototype that will allow further development, aiming specifically at making it simpler and more cost effective to analyze biological reactions to nanoparticles on site. The problem of depositing substantial amounts of nanoparticles directly from air (mimicking inhalation) was solved by using an electrical field.

Figure 15: Novel device for combining biological and physical on-site measurements (University of Salzburg)

Ultra-weak light sensor. A new sensor for ultra-weak light emissions was developed of on-line testing, but technical problems prevented so far the intended combination with the Navetta device. The sensor was successfully used to follow development of a stress response in various human cells. It is intended for combination with Navetta, where it can provide data nearly in real time (minutes after exposure). It is also attractive to use with environmental test species since the stress response is evolutionary extremely conserved.

In the “environmental” case study 3, three methods were explored: (1) a particle measuring device to characterize the properties and fate of selected ENPs in various natural and standardized test media in relation to dispersion, agglomeration, aggregation and solubility under realistic conditions; (2) a novel in vitro multi-compartment fish epithelial barrier model to determine bioavailability, persistence and toxicity of ENMs to fish; and (3) an in vivo isopod-based assay to assess the uptake of ENM via food in a representative terrestrial organism. Performance of these methods was assessed and validated by testing the behavior and impact of well characterized NP under environmentally realistic conditions. Significant differences have been observed comparing natural lake waters and standardized laboratory water. Real lake water. Possible effects of NPs in real lake water and artificial media were compared in fish and Daphnia toxicity tests. Real water affected results due to its natural organic matter (NOM) and chloride contents. This calls for analyzing environmental effects in a more realistic experimental setup. Several existing models (terrestrial crustaceans “Porcellio scaber”; aquatic crustacean “Daphnia magna”; and aquatic ciliated protozoan “Paramecium caudatum”) were used and tested for their applicability to nanomaterials. The effect of various types of nanoparticles collected in a paint factory was studied in an invertebrate model using terrestrial crustaceans, without finding significant toxicity and similar findings were made in a plant model that was tested in parallel. Possible effects of NP in real lake water and in artificial media were compared in fish and Daphnia standard toxicity tests. Real water affected results due to its contents of natural substances, such as organic matter and chloride, which calls for analyzing environmental effects in a more realistic setup, which may also involve different bodies of water, as two lake waters induced clear differences.

Trout gut model. A novel fish gut barrier model was developed by using trout cells. This allows comparing in vitro / in vivo effects in an important environmental sentinel species. Impact: The model allows testing aspects that are otherwise not well accessible, like transmigration. It also allows replacing some animal tests with in vitro tests (strengthening the 3 R’s principle).

Transport accident scenario. A transport accident scenario was elaborated based on mathematical simulation with a novel simulation program and with experiments on controlled release. The study gives important information on management of accidents where large amounts of nanomaterials are released. The novel tools – a computer model and a dedicated exposure test chamber located at the responsible partner laboratory remain available for further studies. A study was performed on the distribution and fate of nanoparticles due to a massive transport accident, like a destroyed truck by using a novel software and
by experiments using release of nanoparticles within a fully enclosed test chamber. Conclusions from these studies will contribute to the further development of European regulations on accident management.

Nano to go! A handbook on safe handling of nanoparticles was developed in the first year of the project. This handbook was tested and consequently revised in four case studies, performed at locations where nanoparticles were either produced or production was just set up (see Figure 11). The full handbook with field study results is freely available from NanoValid including the data on on-site applications of the guideline. The fact that the handbook is field-tested makes it a “best practice” document that is valuable for everybody handling nanomaterials (see Figure 12). The entire guide is freely available as brochure and has been distributed on several occasions, including the last Marina-NanoValid conference and the last NanoSafety Cluster meeting in Paris (2015), and will be further distributed through the BAUA, NSC and NanoValid website.

Automotive study. The automotive case study 6 carried out by CRF-FIAT focused on nanocomposites, where NPs are embedded within polymeric matrices in order to give them new functionalities for improving the performance of automotive applications. The case study was evaluating the environmental impact of NPs during the life cycle of a vehicle and included the following materials:

- new modified e-coating for improving corrosion resistance of metallic automotive components.
- new modified adhesives activatable by radiofrequency (RF) fields with reversibility properties.

To modify the traditional systems by NPs to supply new functionalities and improvements of performances to final applications, experimental work was performed for coating and adhesive applications. A detailed selection of potential NPs was done and further specific indications of parameters elaborated for the final choice of NPs. Also dispersion tests of NPs within the e-coating were performed to optimize the final product. For the adhesive application, different typologies of characterization were carried out on unaltered adhesive and on NPs in order to define the main process parameters for innovative adhesive manufacturing. The challenge was to integrate carbon nanotubes (CNT) into composite surfaces under conditions that still allow using coating technologies relying on an electrical field, while ensuring at the same time that CNT do not present a hazard at the work place. The results of this study have direct implications for the automotive industry using CNT.

Release of nanomaterials during steps of preparation, use and discarding of nanomaterials containing composites used in the automotive industry was followed in a time- and space-dependent mode. Release is limited to short distances with few defined release points in the production process. The results will help to implement the new technology process safely in the production line. The release of nanomaterials applied in the car industry has been studied by using methods tested and developed within the project. The study shows that particles are released, but only during one step of the process, at defined sites, and over a rather short range (25 cm). There is no long-term or long-range distribution that can be distinguished from background values, why very specific release- and dust-control measures will be effective to safely handle airborne nanomaterials released from similar automotive-specific product sites.

Potential Impact:

NanoValid was the first European “flagship” project that was entirely devoted to the development of validated SOPs for a large range of physicochemical measurement and biological test methods. The ultimate aim was to develop reference methods based on these validated methods that help to remove existing data uncertainties and hence improve our present understanding of the main factors and mechanisms that control the complex interaction between nanoparticles and the living systems. More reliable data will help to make current risk (RA) and life cycle assessment (LCA) schemes more applicable.
to nanomaterials.

Beside the development of a panel of well characterized reference tools, including certified reference materials (CRM) and validated and reference methods (SOPs) that will assist risk assessors in industry and regulatory bodies to recognize, understand and manage/reduce/prevent possible risks arising from nanomaterials, NanoValid also created an enormous wealth of new scientific knowledge on the behavior of these new substances.

In particular, novel insight was produced on critical dose-response relationships, toxic pathways and endpoints as well as on processes that govern the release, distribution, bioavailability and uptake of nanoparticles into man and the environment along various life cycle stages. NanoValid generated also a variety of practical and commercially relevant products, such as new instrument and material prototypes, new exposure models and experimental equipment specifically designed and developed for the generation and simulation of real exposure concentrations. In particular the development of a new hot gas nanosampler and of a novel online exposure device that combines physical and biological assays for airborne particles has to be emphasized. In cooperation with the European and International Standardization bodies CEN and ISO, and with the help of national bodies and metrology experts, such as DIN, BAM and INMETRO, NanoValid could provide strong input into current standardization initiatives, such as the CEN/TC 352 “Nanotechnologies”, ISO/TC 229 “Nanotechnologies” and ISO/TC 24/SC 4 “Particle characterization”, by proposing a variety of new work items for method standardization developed within the project. Another highlight and direct practical outcome of the project is the preparation of a guidance document and training manual for the safe handling of nanomaterials that can be used by SME industries, including start-up research companies, materials and instrument manufacturers or enforcement labs, to prevent, reduce and minimize any occupational risks.

The newly developed methodology, in particular the validated SOPs on particle measurement, dispersion control, labeling and in vitro / in vivo testing, will assist and strengthen future method developments and contribute to risk research efforts going on in other relevant projects, and hence contribute to the generation of a more reliable database, which is urgently needed to improve the performance of current RA and LCA tools and of current legislation, such as REACH, CLP or the Biocides directive. Upgrading current RA and LCA tools by using methods validated within NanoValid will allow early identification of critical LC stages and processes, and so help to (re-) design material properties in such a way, that they will not cause harm to man or the environment (“safety by design”).

Most of the results generated during the project have been and will be still published in peer-reviewed journals or presented at international conferences and workshops, and will in this way strongly impact our present understanding on the behavior of engineered nanomaterials.

Potential impacts from results achieved within WPs:

Besides fabrication, selection and distribution of test materials, and the design and operation of the project specific database, one practical outcome of WP2 was the development of a test battery for the preliminary screening the (eco-) toxicity of nanoparticles. This new approach includes the following OECD and ISO standardized assays:
TEST BATTERY FOR TOXICOLOGICAL PROFILING OF NANOMATERIALS
- bacteria Vibrio fischeri 30-minute bioluminescence inhibition assay
- aquatic crustacean Daphnia magna 48-hour immobilization assay, and
- 48-hour NRU assay on murine fibroblasts in vitro.

The test battery involves prokaryotic and eukaryotic organisms/cells, particle-feeding organisms and
assumingly particle ‘proof’ organisms and follows the 3R’s principle.

Another practical outcome of WP2 is the fabrication of 2 nanomaterials that have been further developed
in WP5 as reference materials and that will be commercialized by NLAB and INMETRO:

- silica (NNV-001) was developed as class II reference material to support validation and standardization
of eco-toxicological test procedures
- gold (NNV-004) is being developed as certified reference material (CRM) with particle size as
measurand to be used as calibrant for (eco-) toxicological testing.

In WP3, 11 SOPs have been generated by using and characterizing 5 nanoparticles as test materials. The
comprehensive ILC resulted in 5 validated SOPs (TEM, DLS, Zeta, BET, XRD) and 6 non-validated SOPs
(NTA/AFM, ICP-MS, XPS, Dispersion, Solubility). The validation was a requirement for the development
of reference methods in WP6. Results have been uploaded to the NanoValid database (Data and ENP
sheet). In addition, 5 SOPs on cell culture have been produced: A549 cells (respiratory tract); THP-1 cells
(immune system); HepG2 cells (digestive tract, liver); Normal Human Epidermal Keratinocytes (skin). In
addition 7 SOPs for in vitro assays have been developed and validated for different end points
(cytotoxicity, inflammation, oxidative stress, hemocompatibility, genotoxicity) have also been developed
and validated. From the ILC, both MTS and ATP assays showed good reproducible results for both
positive control STS and Ag NP. Caspase 3/7 did not provide a coherent data set. Caution should be
applied for the ATP assay as Ag NP interference was observed with the assay.

Another major outcome in WP3 was the development of ecotoxicological assays suitable to predict the
hazard (or lack of hazard) of nanoparticles (Ag, ZnO, CuO, TiO2, Au and SiO2), but not of MWCNT. A
cost efficient test battery for screening the nanotoxicity has been established that consists of three acute
assays:
- Bacteria Vibrio fischeri 30-min bioluminescence inhibition assay;
- Daphnia magna 48-h immobilization assay
- 48-h murine fibroblasts neutral red uptake test in vitro.

Most results obtained in WP4 have been continuously disseminated to the scientific community by a wide
range of peer-reviewed publications including journals, book chapters and conference presentations, and
these activities will continue after the end of the project. One major outcome was the critical review and
synthesis of existing data on risk assessment in comparison with the data produced by the methods
developed for a variety of nanomaterials within the project. Another review on nano-silver was prepared of
all nanotoxicity data produced within the project. The developed risk assessment decision tree and flow
chart approach was already published in a scientific journal and also proposed for further standardization
as new work item submitted to the DIN working group NA 062-08-17-03 UA: Health and environmental
aspects. The procedure was presented to that committee during a first meeting in Berlin on 23 June 2015. Also the newly developed LCA methodology was applied to nanomaterials and demonstrated by means of a specific case study on TiO2 in sunscreen, which was an important first step forward to further improve the existing LCA methodology and to enable its application to a wider range of nanomaterials and products, and the integration of other factors (such as use of resources, recycling) into sustainable product development. Critical literature reviews have been prepared on hazard data and the evaluation of the suitability of methods tested in WP2 and WP3 to improve human and environmental hazard assessment, also to provide again feedback to WP5 and WP6 on the practical performance of these methods.

Driven by the urgent need to address safety issues that may arise from nanotechnologies, activities in WP5 were launched to solve problems caused by existing data uncertainties caused by the testing methods used in characterizing nanomaterials and in the assessment of their risks, as required by the European Commission and concerned industries to improve existing regulation and increase competitiveness, respectively. Existing measurement capabilities have been clearly improved by the development of reference methods (SOPs) validated by a variety of inter-laboratory method comparisons. These reference methods are unique as they are supported by comprehensive statistical uncertainty and traceability measurements. A great deal of the reference methods developed within NanoValid will have a strong impact on future method standardization. For example, the SOP developed and validated within the project to control NPs dispersion in various test media already had an early impact on ISO TC 201, as the knowledge developed was used and submitted through DIN, the German standardization body, for the preparation of a new draft standard “ISO/WD 20579-4 on Surface Chemical Analysis: Sample handling, preparation and mounting, Part 4: Nanomaterial reporting requirements, analysis challenges and solution extraction methods.

In addition to reference methods, new reference materials have been produced in WP5 for both the calibration of existing characterization methods used for ENP size measurement and for human and eco-toxicological testing. The developed reference materials will be commercialized in 2016 by the project partners INMETRO, BAM and NLAB (see Figure 8).

The new improved instrument measurement capabilities established in the field of human and eco-toxicology that are based on validated reference methods (SOPs) will provide more accurate information on the comparability of the data produced and the methods used by different testing laboratories. The SOPs established will help to improve the harmonization of methods and hence the comparability of results and to minimize data variability that is still a problem due to the use of not-validated methods. Some of the validated SOPs developed for eco-toxicological testing have been already proposed for international standardization with the help of national standardization bodies. As in other WPs, most of the knowledge produced in WP5 has been already disseminated through many publications in peer reviewed journals, or as presentations at international conferences, or to national industrial stakeholder associations (including consultancies), but will serve also for training purposed through international workshops (summer schools).

In WP6, a panel of novel reporter cell lines was produced, which is suitable to quickly and cheaply test cell stress and inflammation (suitable for medium-to-high throughput). Also allergens have been identified as a problematic contaminant, as increased allergenicity was found in combination with nanoparticles.
Surprisingly, factory-collected material was found to be rather harmless, both in human and ecological toxicity testing. It also contained extremely low level of endotoxin, which suggests that pro-inflammatory properties may be less pronounced for materials handled in bulk. One practical output with a high potential for commercialization was a novel hot-gas nanosampler and a novel prototype for biological on-site measurements developed during the course of the project. Not less important for future exploitation was the development of a new cell barrier model derived from trout gut cells, which will allow in-depth tests relevant for this sentinel species without animal testing. Modeling and analysis of a transport accident scenario gave insight into proper accident simulation, prediction and management. Another practical outcome was the preparation of a new guideline on safe working with nanomaterials, which is a freely available, field-tested and up to date guidance document for many practitioners, such as research and materials and instrumental manufacturers, or enforcement and regulatory laboratories. The automotive case study makes it possible to set up safe working places at automotive sites, when introducing and using nanomaterials in the production line.

One of the strongest impact of NanoValid will be on current method and material standardization efforts. To transfer and translate achieved project results into method and materials standardization, NanoValid has signed a project liaison with CEN and was represented by BAM and DIN and other WP5 partners at various CEN and ISO/TCs meetings. A project-internal survey identified possible work items and TCs that appear most interesting, including CEN/TC 352 Nanotechnologies, CEN/TC 137 Assessment of workplace exposure to chemical and biological agents, CEN/TC 201 Surface chemical analysis, ISO/TC 202 Microbeam analysis, ISO/TC 229 Nanotechnologies and ISO/TC 24/SC 4 Particle characterization. A dedicated workshop on standardization (led by DIN) held at CEN in Brussels in November 2014 has identified the following methods with a potential for future standardization, including SOPs and results of inter-laboratory comparisons:

1. MTS-assay: FUNDP will participate in the development of ISO 19007 “Modified MTS assay for measuring the effect of nanoparticles on cell viability” which was being developed by ISO/TC 229. DIN will contact the Belgium Standardization Body NBN and announce that Jean-Pascal Piret will participate in the national mirror committee to ISO/TC 229 and contribute to the development of the standard.
2. ATP-assay: DIN together with FUNDP will prepare a NWIP. DIN will contact the Belgium Standardization Body NBN and announce that Jean-Pascal Piret will participate in the national mirror committee to ISO/TC 352 and that he intends to propose the New Work Item to CEN/TC 352.
3. ROS: PLUS will participate in the development of several standards, which were being developed by ISO/TC 229. DIN will contact the Austrian Standardization Body ASI and announce that Albert Duschl will participate in the national mirror committee to ISO/TC 229 and contribute to the development of the standards.
4. Decision trees: DIN together with UFZ will prepare a NWIP. UFZ will participate in the DIN national mirror committee to CEN/TC 352 and DIN will propose the New Work Item to CEN/TC 352 with Dana Kühnel as Project Leader.
5. Feeding of NPs to isopods: DIN together with UNILJ will prepare an NWIP. DIN will contact the Slovenian Standardization Body ESV and announce that ESV will participate in the national mirror committee to ISO/TC 147 and that intends to propose the New Work Item to ISO/TC 147.
6. Dispersion: FHG will participate in the development of a future document which is just under preparation within ISO/TC 201/SG 1 “Nano-materials characterization”. Annegret Potthoff will participate in the
German mirror committee to ISO/TC 201 at DIN and actively contribute to the development of the document and aim to include a clause about dispersion stability.

7. Dispersion: FHG will participate in the development of a future document “Colloidal Stability Measurements for the Nanoparticles in Aqueous Media” which was proposed by South Korea and is just under preparation within ISO/TC 229. Annegret Potthoff will participate in the German mirror committee to ISO/TC 229 at DIN and actively contribute to the development of the document.

8. Sizing: There are several standards on sizing available from ISO/TC 24/SC 4. BAM will participate in the DIN national mirror committee to ISO/TC 24/SC 4. During the next ISO/TC 24/SC 4 meeting on 11 June 2015 in Paris, BAM will present the new method and discuss with ISO/TC 24/SC 4 the best solution how to integrate it into the existing standards. Later on, DIN together with BAM, will prepare the relevant NWIP for the revision of the relevant standard. DIN will then propose the New Work Item to ISO/TC 24/SC 4 with Wolfgang Unger as Project Leader.

9. Zeta-Potential: BAM and UOB will participate in the development of a future document ISO/PWI 19997 which is just under preparation within ISO/TC 24/SC 4. BAM will participate in the German mirror committee to ISO/TC 24/SC 4 at DIN and actively contribute to the development of the document. DIN will contact the UK Standardization Body BSI and announce that Superb Misra will participate in the national mirror committee to ISO/TC 24/SC 4 and contribute to the development of the standards.

10. BET: BAM and UOB will participate in the possible revision of ISO/PWI 9277 within ISO/TC 24/SC 4. During the next ISO/TC 24/SC 4 meeting on 11 June 2015 in Paris, BAM will present the new method and discuss with ISO/TC 24/SC 4 about the possible revision of ISO 9277. Later on DIN together with BAM will prepare the relevant NWIP for the revision of ISO 9277. DIN will then propose the New Work Item to ISO/TC 24/SC 4 with BAM staff as Project Leader.

11. DLS: UOB will participate in the development of ISO 22412, which was being developed by ISO/TC 24/SC 4. DIN will contact the UK Standardization Body BSI and announce that Superb Misra will participate in the national mirror committee to ISO/TC 24/SC 4 and contribute to the development of the standard.

12. TEM: ISO/TC 229 is performing round robin tests to develop a proposal for a future standard. UOB will participate in the development of the proposal. DIN will contact the UK Standardization Body BSI and announce that Superb Misra will participate in the national mirror committee to ISO/TC 229 and contribute to the development of the proposal.

13. Non-isotopic labelling: nanoSTAIR with the help of UOB and BSI is preparing a proposal for a future standard. Superb Misra will contact his colleague Iseult Lynch at UOB and contribute to the development of the proposal.

Obtained project results (foreground) have been made available to relevant stakeholders (academia, industry, regulatory, EC, public) mainly by means of peer-reviewed publications, conference or workshop presentations, exhibition stands, flyers, brochures, regular project newsletters and via the project-specific website. Internally, a specific partner intranet has been installed at the website to allow the password protected uploading/downloading and exchange of all relevant documents among partners, such as deliverable or milestones reports, presentations and minutes from meetings or legal documents, such as amendments of the grant agreement etc.

All partners contributed to the dissemination of the results they produced both by preparing technical deliverable and milestone reports and by publishing in peer-reviewed scientific journals or at conferences.
or workshops. A list of main dissemination activities is presented in Template A2 and of peer-reviewed publications in Template A1 of the Final Report. Altogether, NanoValid has already produced almost 100 peer-reviewed publications and more publications are still in progress after the end of the project.

In the final project year, NanoValid has engaged the wider community through a range of different activities and events, such as those organized within relevant conferences, e.g. within the ENF2015 international conference or the Marina-NanoValid final meeting at the OECD in Paris (2015), but also by initiating and participating in minor meetings or workshops, such as the Salzburg or Ljubliana workshop in 2015. In addition, a continuously updated project specific website with an integrated database of project results and relevant news sections made the wider community aware of the resources being developed within the project. Relevant initiatives, organizations and individuals could register and receive regular output through a quarterly newsletter, regular reports (to all partners targeted to different user groups, including policy makers and industry.

The results, new knowledge, methods and materials generated during the project have been mainly used to develop and propose new standards (DIN, BAM), e.g. under CEN/TC 352 “Nanotechnologies”, ISO/TC 229 “Nanotechnologies”, or ISO/TC 24/SC 4 “Particle characterization, or for the preparation and adoption of new technical guidance of the EC’s Chemical Agents Directive (CAD) (BAUA). Also instrument prototypes, such as a hot gas nano-sampler or an online exposure measurement device (the “Navetta”) have been established for commercialization. A good practice guidance document has been prepared by BAUA and other partners based on results from field studies and on methods developed within the project. All data generated in WP6 from case studies 6 have been integrated into this good practice document, and tools and procedures evaluated for future exploitation.

A project liaison was agreed between CEN/TC 352 and NanoValid from the beginning of the project and a proper format for SOPs developed to ensure that the provided information will contain all necessary details. A standardization workshop was performed on 28 November 2014 and the participation in several ongoing standardization projects organized. SOPs on ATP assays, decision trees, feeding of nanoparticles to isopodes, and detection of engineered nanomaterials internalization in cells using flow cytometry, were transformed into the relevant new work item proposals including working drafts and sent to the respective national standardization bodies for discussion. A possible new work item proposal on BET was discussed in the DIN standardization committee. Results of FHG concerning the dispersion of nanoparticles were discussed in the DIN mirror committee and the content sent to ISO/TC 201. The content was then already included into the current version of ISO 20579-4 Detection of engineered nanomaterials internalization in cells using flow cytometry. DIN has given a final presentation about NanoValid at the CEN/TC 352 meeting on 26 November 2015 to promote the future new work item proposals origination from NanoValid.

A training manual “Nano to go!” was produced providing a guidance document on good working was prepared with a focus on research institutions and SME industries. Four decision criteria have been defined to support these end-users to find appropriate occupational safety measures in a feasibly way and tailored to the respective groups of nanomaterials following the precautionary approach. These measures comply with the priority list of the STOP principle: substitution, technical measures, organisational measures and personal protection measures.
Three approaches to support safe work with nanomaterials have been compared: the NanoValid “manual approach” Nano to go (2015), and the European Commission (EC) adopted “Guidance on the protection of the health and safety of workers from the potential risks related to nanomaterials at work” (2014) and the guidance “Working Safely with Manufactured Nanomaterials” (2013). The 3 approaches are fundamentally similar and consider the precautionary principle to ensure the safety and health in work with nanomaterials under conditions of risk uncertainty. Differences between the two EC approaches and the NanoValid approach refer to the level of practical specification.

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Related documents

- final1-nanovalid-young-researchers.pdf
- final1-figures-and-tables-of-the-final-report.pdf

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