Final Report Summary - NANOMILE (Engineered nanomaterial mechanisms of interactions with living systems and the environment: a universal framework for safe nanotechnology)

Executive Summary: The FP7 project NanoMILE (www.nanomile.eu) was a unique partnership of 28 of the highest calibre European and US institutes in nanosafety, dedicated to developing detailed mechanistic understanding of the interactions of manufactured nanomaterials (MNMs) with living systems. Test systems ranged from biofluids, simple unicellular through to multi-cellular organisms, whole animals and humans. Identification of conserved pathways across species and development of in vitro alternative test methods and high throughput approaches were among the core aims, in line with the European Commission’s drive to reduce animal testing (the 3Rs) and to ensure safe and responsible implementation of nanotechnologies. Focus on translating the mechanistic insights into rules for safe by design MNMs and development of in silico hazard assessment and prediction tools will be some of the enduring outputs from NanoMILE.

NanoMILE aimed to revolutionise nanosafety research and regulation through its robust and novel approaches to the selection and development of test MNMs, including environmentally aged variants to represent real-world materials, its technically and computationally advanced integration of systems biology with high throughput toxicity assessment and advanced imaging of MNM uptake and interactions, its balanced toxicological/ecotoxicological approaches, its extensive library of well-characterised MNMs including single property variants, its development of novel high throughput platforms for screening and its feedback loops for development of MNMs that are safer by design.

Additionally, NanoMILE had a strong focus on development of new methodologies for MNM hazard assessment, with industry partners developing and optimising approaches including an Air-Liquid Interface (ALI) platform developed by Vitrocell to mimic the lung exposure more effectively, Malvern who developed an auto-focussing approach for the NanoSight platform to allow assessment of MNM agglomeration and sedimentation, and Attana who extended the applicability of their biomolecules interaction platform for use with MNM to assess receptor interactions and MNM internalisation. New or refined assays developed included a material transfer model in fish, the isopod model organism was progressed towards standardisation, and approaches for single-cell toxicity assessment, and high throughput protein corona screening were developed. In silico predictive approaches were also developed by Novamechanics utilising the NanoMILE datasets and are publically available as tools for hazard and risk assessment. Together, these approaches resulted in a robust framework for classification of MNMs according to their biological impacts (Hazard assessment) that is currently being translated into policy briefings for regulators and industry.

The NanoMILE partnership, which consisted of 10 universities, 3 research facilities, 5 government bodies, 3 multinationals and 7 SMEs (3 technical consultants, 4 materials / instrumentation manufacturers), was fully committed to the goals of the project, and to the Open Access policies of the European Commission. NanoMILE has produced over 65 journal publications to date, and over 350 conference presentations, demonstrating the partnership’s commitment to dissemination. In addition, NanoMILE has initiated an ISO standard around toxicokinetics of MNMs and a CEN standard on isopod feeding for assessment of MNMs.
NanoMILE MNMs, data management tools and datasets are being built-upon and continue to be utilised beyond the lifetime of the project via Horizon2020 projects NanoFASE, NanoGenTools and ACEnano (coordinated by UoB) further ensuring return on investment. In February 2017, NanoMILE’s coordinator (UoB) were voted as the overall coordinator of the NSC for the next 2 years, with partner RIVM as a member of the coordination team.

Project Context and Objectives:
Project context: “Half of the newly designed advanced materials and manufacturing processes are built using control at the nanoscale. The structure and function control still may be rudimentary in 2015 as compared to the long-term potential of nanotechnology.”

Nanotechnology is a rapidly evolving enabling technology with the potential to revolutionise modern life. On the nanoscale, common materials can take on entirely new chemical, physical and biological properties. These properties open up new possibilities for exploitation and commercial enterprise, and indeed the value of nanotechnology global markets has been variously predicted as reaching trillions of euros in the next few years. 2012 estimates suggested that there were many hundreds of MNM-containing products on the market, mainly at the low technology end of the sector e.g. carbon nanotubes used as structural materials in tennis rackets and other products, C60 fullerenes, titania and zinc oxide MNMs used in cosmetics, sunscreens and paints, Ag used as a bactericide in fabrics and elsewhere etc. In a number of countries, environmental remediation using nanoscale zerovalent iron and other MNMs is being actively used, although the uncertainty of risks has led to a voluntary moratorium in the UK on the release of free MNMs into the environment. This is an example where a lack of sound knowledge of potential MNM hazards prevents the potential benefits of MNMs from being exploited, with negative impact on innovation in Europe.

An increasing body of scientific evidence would suggest that some materials in their nano-form may induce harmful biological or environmental effects through a variety of potential mechanisms, not all of which are fully understood or quantified as yet. Indeed, although significant research efforts have been made to make the risk assessment of nanotechnology possible, at the outset of NanoMILE a mechanistic and systematic understanding of which physico-chemical parameters, or combination of parameters, governing the toxicity of nano-sized objects was still missing. Thus, society remained unable to ensure the protection of health and the sustainable commercialisation of nanotechnology.

The overarching objective of NanoMILE was therefore to address the gaps identified above and to formulate an intelligent and powerful paradigm for the mode(s) of interaction between MNMs and organisms or the environment to form the basis of a classification scheme for manufactured nanomaterial (MNM) safety and to enable the creation of a universally applicable framework for nanosafety focussing on MNM hazard assessment.

NanoMILE Objectives: The specific objectives, placed here in chronological order were:

- Objective 1: To select and synthesise/procure MNM libraries suitable for hypothesis-driven development of mechanistic models of MNM interactions with organisms and the environment, in harmony with, and linking to existing EU funded platforms, such as the sponsorship programme of the OECD Working Party on Manufactured Nanomaterials (WP2).

Status before NanoMILE: A large number of MNMs exist, many already in industrial production. Often behaviour and toxicity of nominally identical MNMs vary, perhaps a result of poor characterisation or understanding of their structure and complexity or perhaps resulting from batch-to-batch differences or poor synthesis control. Studies of the effect of a systematic variation in properties of MNMs on biological reactivity including toxicity are virtually non-existent. A paradigm systematically linking MNM properties with biological effects including toxicity was urgently needed.

Progress during NanoMILE: The project strengthened the evidence for the following key mechanistic paradigms: (1) oxidative stress, using CeO2 MNMs doped with ZrO2, as a tool to control redox activity, and the more toxicologically potent pair cobalt-
doped Fe2O3 MNMs, which were designed such that they bandgap would overlap with the conductance band of cells. (2) Dissolution as a source of toxicity, utilising Ag MNMs with various coatings to mediate the dissolution rate. (3) Size of MNMs tested using a panel of MNMs with sizes < 30nm utilised for high content screening of mechanism of cell death. (4) Composition, using a library of ultra-small (5nm) and highly comparable PVP capped MNMs with different metal oxide cores (ZnO, CuO, CeO2); (5) Environmental ageing of MNMs using a range of MNMs and ageing scenarios, as a means to mitigate the surface reactivity and toxicity. Such carefully controlled and extensively tested libraries did not previously exist.

• Objective 2: To establish an understanding of changes in the nature of MNMs as they undergo transformations within products and biological or environmental compartments across their life cycle and critically to feed this information into subsequent research to ensure that these “aged” and transformed MNMs are tested for their biological/environmental role (WP3).

Status before NanoMILE: Many MNMs are likely to undergo significant transformations during their life cycle, following their release and as they move into different biological or environmental compartments. These transformations have received limited attention to date and predictions of MNM behaviour are currently unsupported by robust data. Exposure to MNMs in occupational, consumer or environmental settings may either be to the original, parent MNMs or to MNMs that have been incorporated into products and subsequently released, either in their original form or in an altered form due to industrial or natural processes. NanoMILE intended to investigate and quantify the alteration and transformation of MNMs in products and during their use and release into the environment or biota.

Progress during NanoMILE: One of the key questions NanoMILE addressed was whether ageing in products, during storage or in the environment following release alters the form and thus toxicity of the MNMs. Extensive ageing studies were undertaken utilising a subset of the NanoMILE MNM (Ag, ZnO, CeO2, TiO2 and CNTs) utilising a range of ageing approaches mimicking different environmental processes (e.g. weathering in air, interaction with media components (ions and natural organic matter), and effect of surfactants during washing) and the toxicity of the aged variants was tested using a high content screening approach for direct comparison with the pristine variants. NanoMILE work revealed great variability in aged products and responses as a function of ageing conditions.

• Objective 3: To establish a screening platform (WP4) based on high throughput techniques at two stages: a) at the start of the project, to screen for the most relevant MNMs and endpoints (using both classical and novel biomarkers) to provide a focus for subsequent WPs (5-8) and later, b) to screen the mechanistic discoveries from WP5-8 and develop the test methods of the future.

Status before NanoMILE: There are simply too many different MNMs to be tested by any one project or lab. Harmonisation of data across labs is a further challenge. A high throughput platform for hazard ranking is required. One of NanoMILE’s pioneering approaches is the practical incorporation of a high throughput platform, which will allow screening of a large numbers of MNMs/MNM variants at the start of the project, in order to identify “lead candidates” for subsequent work. High throughput and content screening (HT/CS) in vitro (cell culture) and in vivo (zebrafish) will therefore be established. The large volume of data generated by this work will be instrumental for the quantitative structure (property)-activity relationships (QS(P)ARs), to allow identification of no-observed-adverse-effect levels (NOAELs) and to predict the impacts from physico-chemical characteristics or “initial” corona characteristics.

Progress during NanoMILE: The screening platform was very effectively utilised in NanoMILE shedding light on key candidate MNMs for more detailed mechanistic studies, and by virtue of the size of the NanoMILE libraries and the number of MNMs variants (including aged MNMs) tested, the overall dataset produced is the largest to date allowing confirmation of findings from smaller patchy studies as well as development of Quantitative Nanomaterial Activity Relationships (QNARs) coupling the extensive MNMs characterisation datasets to the toxicity data allowing development of predictive models (objective 9). The MNMs were tested in different mammalian cell lines to link physical-chemical properties to multiple adverse effects. The cell
lines were derived from relevant organs such as liver, lung, colon and the immune system. Endpoints such as viable cell count, cell membrane permeability, apoptotic cell death, mitochondrial membrane potential, lysosomal acidification and steatosis were studied. Soluble MNMs, Ag and ZnO, were the most toxic in all cell types. TiO2 and SiO2 MNMs also triggered toxicity in some, but not all, cell types and the cell-type specific effects were influenced by the specific coating. CeO2 MNMs were nearly ineffective in our test systems. Differentiated liver cells appear to be most sensitive to MNMs. The bulk of tests carried out during the project (and underpinned by full characterisation and reproducible protocols) gives confidence in drawing generic conclusions enabling future development of read-across approaches in the near future based on the NanoMILE data.

• Objective 4: To qualify and quantify MNM interactions with environmental (humic acids, polysaccharides, clays) and biological molecules (proteins, lipids, sugars, nucleic acids) before and after uptake into biological systems to enable understanding of how these interactions alter MNM fate and behaviour in cells, organisms and animals. To generate a computational-based screening platform for bionano interactions to allow tests on a comprehensive dataset of MNMs (WP5).

Status before NanoMILE: MNMs transform upon contact with biological or environmental media, and it is likely that a layer of biomolecules or geomolecules (“corona”) cover their surface. The nature, properties and robustness of this layer and interactions between the core and the corona are currently poorly understood; it is also not clear how different environmental or biological compartments will impact on the formation of this corona. The protein corona formed around MNMs upon contact with biological fluids or living is now understood to govern the particles’ biological fate. However, even this long-lived “hard” corona evolves and re-equilibrates as particles pass from one biological fluid to another, providing a “fingerprint” of its history. An important hypothesis is that this evolution could be used to map the transport pathways utilized by MNMs, and eventually to predict nanoparticle fate and behaviour based on characterisation of the initial corona in a representative biofluid. A similar concept for MNMs exposed via aquatic or terrestrial media containing natural organic matter (NOM, initial corona) taken up into organisms (final corona) has also been shown to exist and needs to be further investigated.

Progress during NanoMILE: Linking the absorbed biomolecules or corona composition was a key goal of NanoMILE, both in the animal context and the environmental context. A range of experimental and modelling approaches were utilised, as well as novel imaging approaches to assess the exchange of the corona following MNM uptake into living organisms. New assays and approaches for assessment of receptor interactions of biomolecules bound to MNMs were developed, allowing new insights into MNM-protein-receptor interactions under competitive conditions. MNM-lipid coronas were also investigated, as well as methods for single-cell assessment rather than monolayer approaches, which can mask some of the subtleties of MNM interactions. Formation of complexes with macromolecules in the environment is strongly dependent on the subtle competitions between attractive and repulsive long-range (electrostatic) and short-range (hydrophobic) interactions, solution chemistry, MNM and biomacromolecule properties.

• Objective 5: To establish in-vitro and in-vivo reactions between MNMs and a carefully selected range of cell-lines/organs/organisms, representative of a wide range of species with increasing biological complexity, from algae to fish, aquatic and terrestrial species (WP6) and humans (WP7).

Status before NanoMILE: Although ecotoxicological studies exist for a number of different species, many such studies produce different results and there is no framework for comparisons across species and in different environmental compartments (terrestrial/marine/freshwater). It is becoming clear that MNMs react with a biota in a MNM specific manner where toxicity is one of the outcomes of these interactions. Others may include reduced energy reserves, reduced fitness and ultimately increased vulnerability. There is however currently no overarching framework for risk assessment.

Although a substantial volume of mammalian toxicological studies exist (in vivo and in vitro) a model for human toxicity has not yet emerged. While in vitro studies are very useful for identification of toxic potency and mechanistic studies, and can support the outcome of in vivo studies, the information does not fit well in risk assessment. In addition, the availability of in vivo repeated dose toxicity studies is limited, and such data are urgently needed, as are new paradigms for low doses and
closely linking toxicology and biokinetics.

Progress during NanoMILE: Hazard mechanisms were a key focus of activities addressing this objective, and a careful set of integrated in vitro and in vivo assays were performed to allow correlations of effects in a range of healthy and disease model systems (cardiovascular, Alzheimer’s model for ageing etc.) and in a wide range of environmentally relevant species including algae (Chlamydomonas), Daphnia magna and Danio Rerio (zebra fish) and others such as isopods and C. Elegans. Other mechanisms, assessed in more selective studies using a narrow range of MNMs, included genotoxicity, biodistribution and biokinetics in a range of organisms, developmental and organism ageing effects, and others.

A strong focus on method development was also central to the activities, with development of a model for maternal transfer in fish, and progress of the isopod model towards standardisation. An Air-Liquid interface (ALI) inhalation exposure unit was developed and built, and transferred to both KIT and RIVM for in vitro studies. In summary, NanoMILE produced a body of toxicological work that has advanced the state of the art qualitatively and quantitatively making a significant contribution to the critical mass of nanosafety data.

• Objective 6: To complement the above with a carefully selected range of systems biology based studies (WP8) to support the understanding and comparisons of mechanisms of MNMs activity across several species of increasing complexity.

Status before NanoMILE: Systems biology has in recent years emerged as a powerful tool for understanding biological mechanisms at the molecular level and using such information to generate predictive and mechanistic approaches in disease. These advances have yet to be applied in the field of nanosafety. NanoMILE will seek to discover and compare mechanisms and potencies of the potential harmful effects of different MNMs using an integrated Systems Biology approach, including transcriptomics, metabolomics, lipidomics and computational biology. The overall aim is to identify prototypic modes of action of MNMs, including both species-specific and evolutionarily conserved responses, with the latter likely to provide extremely powerful biomarkers in relation to assessing MNMs impacts on environmental and human health

Progress during NanoMILE: The omics approaches (transcriptomics and metabolomics) are traditionally considered as non-directed approaches in order to shed light on potential new mechanisms of action from MNMs, relative to ionic or larger particulate controls. These approaches have really come to fruition during NanoMILE and have been applied in both human cells (A549) and in three ecotoxicity model species Chlamydomonas, Daphnia magna and Danio Rerio, with extensive lists of up- and down-regulated genes and metabolites analysed for function and linked to known mechanisms / pathways as a first step towards development of Adverse Outcome Pathways. NanoMILE’s lasting mark here is enabling the streamlining of systems biology in nanosafety.

• Objective 7: To more intelligently design safer MNMs (WP9), using the previous WPs as a guide, and working towards designing out adverse effect causing features.

Status before NanoMILE: No platform exists for referencing and comparing the activity, in terms of toxic behaviour, of MNMs; no fundamental concept of safe MNM design has yet been developed. Following early work within NanoMILE which will discover systematically the precise mode of action of MNMs properties, key later activities will be carried out towards: a) practically test such features by designing them in or out (both at bench and pilot scale); b) develop models of quantitative structure (property) –activity relationships (QS(P)ARs) enabling predictive work to evolve and feed into risk assessment; and c) provide an integrated platform for hazard assessment. In order to design safer MNMs, the work in NanoMILE will involve a central iterative link between MNM properties and biological/environmental effects.

Progress during NanoMILE: Clear outcomes have emerged from the integration of the NanoMILE datasets, which are being developed as a set of design criteria for safe-by-design MNMs, linked to the mechanistic information for MNMs and the information on their ageing and transformations in the environment, to provide a useful framework for industry and regulators.
Key highlights include the fact that environmental ageing generally reduces toxicity, but shows greater diversity than previously thought, while understanding the interactions between the coating or stabilisers and the surrounding biomolecules can have important outcomes in terms of MNM uptake and internalisation and its rate of loss or exchange following MNM internalisation into organisms. NanoMILE’s achievement here was the testing and evaluation of the relative significance of key descriptors in nanotoxicology.

- Objective 8: To develop appropriate models linking quantitative structure(property)-activity relationships (QS(P)AR), established from the biological effects studies above, to population response models, thus enabling predictive work to evolve from molecular mechanisms (specific toxicity pathways and classification of MNMs according to their mode of action) to the scale of the ecosystem (WP9).

Status before NanoMILE: QSARs, perhaps more appropriately termed QPARs (as it is physicochemical “properties” rather than “structures” that need to be linked to a specific mode of hazardous activity) will form a fundamental component of NanoMILE. There are two main difficulties related to the development of nano-QSARs: The first is lack of sufficiently numerous and systematic experimental data and the second is the currently limited knowledge on mechanisms of toxic action. The former is being addressed in a number of major EU funded projects, data from which will feed directly into NanoMILE, via common project partners. The latter will be addressed within NanoMILE and knowledge acquired will transfer to WP9.

Progress during NanoMILE: Extensive work towards development of a universal QSAR model, and an in silico platform for nanosafety have been undertaken in NanoMILE, with the first publically available tools for prediction and risk assessment being made available. The extensive NanoMILE database is still being integrated as deliverable reports from previous objectives/WPs are finalised, so computational mining of the datasets will continue far into the future. Furthermore, work will continue beyond the project lifetime, in part funded via the H2020 RISE project NanoGenTools in which both UoB and Novamechanics are involved, and whose focus is on safety-by-design. Thus, NanoMILE’s dataset on hazard mechanism will be re-used and developed as a tool for MNMs safety-by-design predictive modelling.

- Objective 9: To interact closely with other EU and US funded projects and the NanoSafety Cluster, to ensure maximum integration of prior state of the art within the project and progression along and beyond paths and platforms thoughtfully designed by these projects (WP10).

Status before NanoMILE: A lot of projects operate in isolation both laterally by not interacting with other concurrent research on the same or similar topic and temporally by missing existing background and allowing the generated foreground to lapse after the project ends. NanoMILE will have a WP and team ensuring interactions with other major funded projects, to ensure recently acquired state of the art flows smoothly into the project, parallel developments from ongoing work are known to the research teams and future developments through NanoMILE flow into other projects and applications, ensuring the maximum possible impact by the project.

Progress during NanoMILE: NanoMILE has been very active in terms of interacting with, and transferring knowledge to, ongoing projects. The final meeting in Malaga, organised with sister projects NANOSOLUTIONS, GUIDEnano, SUN and eNanoMapper, was an extremely effective dissemination platform. The conference was recorded as one of the outputs from NanoMILE, and will be a fully searchable, permanent resource providing a snapshot of the current state of the art, and a durable output from the project. Dissemination activities continue beyond the project lifetime, including contributions to the multi-project workshop on Safety by Design MNMs in Bilbao in April 2017 and the EuroNanoForum meeting (June 2017, Malta) where NanoMILE is contributing to activities on standardisation and in the nanosafety session.

Over the course of the project, NanoMILE partners took several leadership roles in the EU Nanosafey Cluster (NSC), Chairing several working groups (WGs) including Hazard (WG2), Standardisation Sub-group (WG7), the new Safety-by-design (WG9), and representing WG7 (Dissemination) on the Steering Committee.

Project Results:
The overall structure of NanoMILE is shown below in the pert diagram of Figure 2. WP1 was the project management tool. Subsequent WPs (2-9) were technical in nature undertaking the core research of the project, whereas the final WP10 focused on interactions with other research activities and dissemination and exploitation of the NanoMILE outputs and research results.

There were two major foci in the structure: WP2, which was the centralised MNM source and WP4, which was the high-throughput hub that will enable a first pass testing (screening) of all MNMs available to the project, thus guiding the selection for more narrow testing to be performed in the other WPs.

The 10 industry partners have been fully integrated into the NanoMILE research activities, contributing actively to WP2 (PROM, N4I; particle synthesis / manufacturing), WP5 (Malvern, Attana; method development for characterization of particle-biomolecule and particle-cell interactions), WP6 (Eurofins, ecotoxicity of pristine and aged NMs), WP7 (BASF, Vitrocell; inhalation exposure, including a new Air-liquid Exposure device); WP8/WP9 (Biomax, Novamechanics; data management and QSAR development) and WP10 (EU-vRI; dissemination and exploitation). A report on the specific benefits of participation in NanoMILE for the industry partners in included in the impact section of this report, and the specific exploitation plans for the various project outputs are described in detail. All industry partners have gained commercial advantage and/or opportunities to influence policy via participation in NanoMILE.

Summary of outcomes from WP2: MNM selection, acquisition, engineering, characterisation

WP Leader: Douglas Gilliland, JRC
WP Partners: UoB, PROM, CEA, N4I
The objectives of this WP were to:
- underpin the work with an overall classification scheme
- procure or synthesis the engineered nanomaterials for use in WP3-8
- controllably modify engineered nanomaterials for use in WP3-8
- provide physico-chemical characterisation of NanoMILE MNMs for use in classification.

Within the NanoMILE project, WP2 was tasked with selecting, procuring/synthesising and characterising the Manufactured Nano Material (MNM) libraries necessary to develop and test hypothesis-driven, mechanistic models of the interactions between nanomaterials, organisms and the environment. To facilitate the development of these models the approach adopted for the selection of the materials was derived from a consideration of physico-chemical properties rather than the more commonly adopted approach of simple chemical compositions, industrial scale or perceived commercial relevance. The initial choice of descriptors was based on a priori knowledge of those factors considered most likely to influence the interactions of the materials with biological systems as follows: Size, Dissolution potential, Surface charge, Hydrophobicity, Redox potential, Shape and Crystal structure. To provide a comprehensive coverage of existing MNMs, a wide range of MNMs were selected from all the major classes of materials - metals, metal oxides, carbon based structures, functionalised structures and core-shell structures. The detailed selection of which materials to include from these major classes of MNMs was made so as to produce a library of materials based not only on their relevance to the mechanistic studies but also the feasibility of sourcing/synthesising them with controllable variations of the previously listed key physico-chemical descriptors.

An important novelty of the NanoMILE project which had a great influence on the execution of WP2 was the introduction of high through-put robotic systems for cell toxicity testing (WP4). These systems, having a large capacity for parallel testing of materials, placed great demands on the development strategy for the library by requiring access to an elevated number of different test materials in a very limited time scale. To meet this challenge a 3 tiered approach was adopted in which a preliminary series of materials were rapidly sourced from commercial suppliers and existing libraries such as the JRC nanomaterials library and OECD nanomaterials testing program. In parallel with this a second tier of in-house synthesised
materials were prepared by the WP2 partners using methods which were already consolidated and which could, with moderate adaptation, produce families of materials with known or predictable properties. Finally, as the project developed and new needs were identified a third tier of novel or highly tailored materials were developed and supplied on request to tackle specific problems or to test hypothesis identified during the testing of the first two series of materials. By adopting this time efficient strategy WP2 was able to rapidly populate a unique materials library with more than 200 distinct examples of nanomaterials including at least 150 were made available to the consortium by the 5th quarter of the project.

Achieving this large variety of distinct materials, particularly with regard to producing materials with systematic variations of key properties, could not be achieved by the development of individual methods but rather by identifying more generic basic synthesis methods which could produce families of materials in a way which was both robust and flexible.

Examples of such processes were the versatile and reproducible protocols for the wet-chemical synthesis of a broad range of PVP capped metal oxide NMs (UoB) which allowed variations in the size and core composition whilst keeping the capping agent constant and/or varying the molecular weight of the capping agent. Similarly, the use of size controlled silica nanoparticles activated with epoxy silane (JRC) provided a highly flexible platform for systematically varying surface chemistry properties such as charge and hydrophobicity without modifying size and solubility. In the case of carbon nanotubes (CNTs) which require specialised high temperature growth methods the basic synthesis process was used to produce batches of controlled raw materials which could be further processed using selective chemical and physical treatments to controllably vary both size and surface chemistry (CEA). In a further, very specialised, adaptation of this methodology specialists in WP2 were able to use a chemical precursor enriched in the radioactive 14C isotope permitting the synthesis of carbon nanotubes marked with an intrinsic radiotracer. In this way it was possible to make CNTs which were uniquely suited to overcoming the difficulties of detecting and quantifying carbon based particulates in carbon rich biological media- a task routinely possible using mass spectrometry for non-carbonaceous inorganic nanoparticles but impossible for purely carbon based MNMs.

While these previous examples were developed at laboratory scale and thus generally limited to gram scale quantities, WP2 has also involved commercial partners experienced in intermediate production levels such as those used for synthesis of dextran coated Ultrasmall Superparamagnetic Iron Oxide nanoparticles (USPIO) based MRI contrast agents (N4I): These materials represent not only practical examples of nanomaterials being used in critical health technology applications (biomedical imaging) but are prime examples where an understanding of the mechanisms behind any toxicological behaviour is particularly important. Finally, where maximum flexibility and potential for scale-up were needed WP2 was, through another commercial partner (PROM), able access the know-how and pilot plant scale production facilities necessary to exploit methods based on supercritical water hydrothermal synthesis (scWHS). This powerful technology exploits a relatively simple and environmentally friendly process for the production of larger quantities of individual materials while retaining great flexibility to systematically vary key properties such as size, composition, shape, capping agent and crystal phase. Important examples where this technology was invaluable, was the production of capped mixed metal oxides whose composition was systematically varied to influence critical properties such as solubility (ZnO2- Fe doped) or redox potential (CexZr1-xO2) MNMs.

To further valorise the major achievement of producing this large and varied MNM library WP2 partners ensured that all the materials underwent a minimum basic physico-chemical characterisation (e.g. size, charge, shape, concentration and composition) with the resulting data being collated and made accessible to all partners. Additionally, where relevant, the analysis of other specific properties (e.g. redox potential, solubility, colloidal stability in selected test media etc.) were specifically undertaken to provide the materials users with the data necessary to better understand the interactions with the varied biological systems studied in the other work packages. In the final stage of the project, as part of the WP9 activities in finalising the hazard assessment framework, the fully range of NanoMILE MNMs (See Table 1) were assessed for their dissolution behaviour after 2 hours at pH7 and pH4 on those of Avramescu et al.

Key publications from WP2:
Lynch I., Weiss C. Valsami-Jones E. A strategy for grouping of nanomaterials based on key physico-chemical descriptors as a
Summary of outcomes from WP3: Life cycle evolution of MNMs

WP Leader: Bernd Nowack, EMPA
WP Partners: UoB, CEA, UoGen

The objectives of this WP were to:
- identify for different MNM the main aging processes
- expose selected MNM from WP2 to different aging processes
- provide aged MNM to WP4-8.

Exposure to MNMs in occupational, consumer or environmental settings may either be to the original, parent MNMs or to MNMs that have been incorporated into products and subsequently released, either in their original form or in an altered form due to industrial or natural processes. To date, few studies have tried to establish the changes that MNMs undergo when incorporated into, and released from, products. As a result, there is major uncertainty as to the state of many MNMs following their release. The use of pristine, as-manufactured MNMs to examine environmental behavior or toxicity is convenient, but it is the aged MNMs that are much more likely to be released into the environment and it is these materials which organisms will ultimately be exposed to.

The goal of WP3 was to establish an understanding of changes in the nature of MNMs as they undergo transformations within products and biological or environmental compartments across their life cycle and to critically feed this information into subsequent research to ensure that these “aged” and transformed MNMs are tested for their biological/environmental role. WP3 has investigated and quantified the alteration and transformation of MNMs in products and during their use and release into the environment, as indicated schematically in Figure 3.

WP3 has exposed relevant MNMs selected from the NanoMILE particle library (CeO2, TiO2, ZnO and Ag) to different processes and different physico-chemical conditions in order to characterize the changes in the MNMs and delivered altered MNMs to NanoMILE partners. The wide variety of MNMs evolutions studied confirms the notion that each group of a MNM with similar properties (coating, chemical identity, mineralogical form, synthesis method, etc.) in a specific environment (medium, physical and chemical conditions) needs to be evaluated separately for its potential transformations. The different fates of MNMs during relevant processes over their life-cycle are evidence for a necessary grouping strategy in risk assessment that goes beyond the material identity and includes life-cycle aspects.

Selected MNMs were exposed to different aging processes and were characterized using various complementary techniques including microscopy techniques (TEM / SEM), XRD, dynamic light scattering, zeta potential, BET, and FFF-ICPMS / SP-ICPMS. When exposed to sunlight/temperature in the powder form, TiO2 and ZnO MNMs showed a likely alteration of their polymeric coating but no significant consequence on their morphology. Conversely, significant chemical and physical transformations were observed for MNMs aged as suspensions, ranging from changes in speciation, to surface coatings, to agglomeration and changes in surface charge.

CeO2 MNMs showed severe alteration of morphology when in contact with phosphate solution: structures resembling sea urchins or needles were observed. Zr substitution in ceria NPs decreased the extent of alteration of the particles as a result of ZrO2 resistance to transformation. Increased temperature led to partial dissolution of CeO2 NPs but the PVP type of capping
(chain length) plays a role in the changes in size at different temperatures.

Highlights of the work about transformation of MNMs with natural organic matter are related to the much better understanding of the interactions of CeO2 particles with fulvic acids (FAs). Our results confirm that FAs coating around CeO2 MNMs is stable and irreversible, and is one of the key factors that will control, even in changing pH or diluting conditions, transformation (agglomeration) and toxicity of MNMs in aquatic systems. Moreover, our results indicate that when FA-coated CeO2 MNMs are present in a system in which ionic strength is increasing (passing for example from fresh to coastal or marine waters) they will better resist to aggregation when monovalent salt is considered.

Ag MNMs were aged with washing liquids. The detergent chemistry, dominated by oxidizing agents and secondarily by the physical presence/absence of non-dissolvable solids, was a major factor in particle dissolution, surface chemistry change(s) and new particle precipitation/formation. However, in the case of nano-enhanced fabrics, degradation of Ag NPs is negligible compared to those suspended in these same detergents, which is most likely due to the protection offered by the incorporation into the fabrics themselves. Besides, based on leaching tests, the release of NPs from landfills into the environment due to disposal of nano-enabled textiles is likely small. Subsequently, when exposed to sunlight as a suspension, the size and morphology of Ag NPs are altered, with transitory-formed prisms and competition between dissolution and aggregation phenomena.

In conclusion, WP3 has significantly advanced the investigation of transformation reactions of MNMs by building its works on a life-cycle perspective of the use of the MNM in products and by investigating in detail processes that were identified as having a high relevance for selected MNMs. The provision of aged MNMs to project partners for inclusion in (eco)toxicological experiments was a major achievement of NanoMILE.

Key publications from WP3:

Summary of outcomes from WP4: Development of a screening platform for MNMs

WP Leader: Silvia Diabate and Carsten Weiss, KIT
WP Partners: UoB, UCD, JRC, LMU, UNEXE, UCLA

The objectives of this WP were to:
- carry out systematic toxicity screening of up to 100 of nanoparticles in a range of cell lines and zebrafish embryos against a range of endpoints to identify lead candidates for WP 6-8
- further develop novel, robust toxicity assays for high-throughput/-content screening (HT/CS) of MNM
- validate toxicity pathways of selected MNMs (from WP 6-8) for other NM
- identify common biomarker profiles (from WP 6-8) across multiple species
- provide reporter gene cell and fish lines for distinct toxicity pathways from objectives 3 and 4.

A range of MNMs with different chemistry and surface modifications was investigated in order to link their physical-chemical properties to multiple adverse effects in various cell lines from different organs. Microscopy-based high-throughput screening
assays were applied to enable multi-parametric analysis of adverse effects from exposure to the MNMs. While no major surprises emerged in terms of the acute toxicity of the MNMs dependent on their chemistry, the studies confirm a lot of previous data from smaller, fragmented datasets thereby increasing the confidence into the use of various HT/C assays to assess acute toxicity of MNMs. Whereas most of the investigated MNMs showed no acute toxicity, it became clear that some show adverse effects dependent on the assay and cell line. Hence, it is advised for future studies to rely on a multi-parametric approach such as HT/C screening to avoid missing signs of toxicity. Furthermore, some of the cell type specific effects should be followed up in more detail and might also provide an incentive to address potential adverse effects in vivo in the relevant organ.

Future studies on mechanisms of MNM toxicity should focus on the identification of the molecular initiating events (MIEs) such as the disturbance of lysosomal integrity, which might not only be essential for downstream acute cytotoxicity but presumably also drive other adverse outcomes in different settings. As more and more adverse outcome pathways are described, HT/C screening needs to capitalize on molecular markers monitoring these events to not only assess acute cytotoxicity but to address, as comprehensively as possible, deregulations of physiological signalling processes. In order to identify MIEs, analysis at the single cell level in real time might be superior to bulk analysis and needs to be established and assessed in the coming years.

Employment of in vitro studies including HT/C assays has been validated to predict acute toxicity of chemicals in humans (Schoonen et al., 2013). Genotoxicity of chemicals can also be reliably assessed by HT/C methods by the use of reporter genes under the control of p53 or gadd45a response elements with similarly accuracy as approved regulatory tests such as the micronucleus assay (Westerink et al., 2013). Building on this experience, more refined assays focusing on increasingly sophisticated toxicity read-outs, such as inflammation, genotoxicity or disturbance of differentiation and development, should be included in a HT/C screening format to broaden the applicability and relevance of current in vitro screening approaches for testing MNMs.

In Objective 4.1 all partners (JRC, UCD, LMU, UoB, KIT) have contributed to the toxicity screening of nanomaterials from the phase I, phase II and phase III lists of the NanoMILE particle library by high-throughput techniques (Hansjosten et al., in preparation). The MNMs in the first list were tested in different mammalian cell lines and zebrafish embryos to link physico-chemical properties to multiple adverse effects in different biological systems. The cell lines represent different organs (liver, lung, colon, immune system). Dispersion and dilution of the particles was done according to an agreed standard operation procedure (SOP, see deliverable report D4.1).

The assays based on HT/C techniques detected endpoints such as cell count (Figure 5), cell membrane permeability, apoptotic cell death, mitochondrial membrane potential, lysosomal acidification and steatosis in cells. The zebrafish embryos were tested for hatching rate, malformations and mortality (Figure 6).

Key results are that:
- Soluble metal MNMs (Ag, ZnO) were the most toxic in mammalian cells and zebrafish. Hydrophilic and hydrophobic ZnO MNMs induced similar effects.
- SiO2 and TiO2 MNMs induced cell-type specific effects which were also influenced by the specific coating.
- CeO2 MNMs were nearly ineffective in our test systems.
- The differentiated liver cell line HepaRG was most sensitive to MNMs, in particular to TiO2 MNMs
- HepaRG differentiated liver cells were more sensitive than undifferentiated HepG2 liver cells.

A reflectance imaging assay and screen of phase I NanoMILE particles was developed and performed, respectively. Reflectance imaging allows for the assessment of nanoparticle internalisation and trafficking without the need for difficult and disruptive surface modification with fluorescent probes.
With respect to Objective 4.2 4.3 and 4.4 the toxicity screening approach has been expanded and adapted to assess impacts of MNMs from a mechanistic standpoint, including a time-resolved assessment of MNM uptake, localization and degradation of the biomolecule corona. The data generated will be instrumental to establish quantitative structure (property)-activity relationships (QS(P)ARs) within WP9. The dose-response curves together with the protocols have been sent to Biomax for inclusion into the NanoMILE database. The dose-response curves have also been used to identify the NOAELs (no observed adverse effect levels) of the MNMs in the different cell lines and zebrafish embryos for different endpoints reported in deliverable report D4.3. These data will allow observed impacts to connect with the fate of the MNMs in vitro / in vivo.

Concerning the influence of the biomolecular corona various novel techniques have been developed in order to allow a detailed time-resolved analysis of the intracellular evolution and final fate of the protein corona associated with different nanoparticles and to determine if the uptake of this layer alongside the NPs has any impact on their possible toxicity (Deliverable report 4.2).

Addressing mechanisms of toxicity, four main pathways for MNM toxicity are currently recognized:

1. The release of toxic chemical constituents from MNMs (e.g. Cd from quantum dots or ionic silver from Ag MNMs) – i.e. MNM dissolution,
2. The direct effects from physical contact with MNMs, influenced by their size and shape, and surface properties and which produce interferences with important biological functions for example by altering conformation of biomolecules – i.e. MNM surface effects,
3. The inherent properties of the material, such as photochemical and redox properties resulting from bandgap or crystalline form – i.e. structure effects, and
4. The capacity of MNMs to act as vectors for the transport of other toxic chemicals to sensitive tissues – i.e. MNM Trojan horse effects.

Once a MNM encounters a cell or an organism, toxicity could occur through one or a combination of these mechanisms.

Assigning the toxic MNMs of our library to the various categories, metal-based soluble Ag and ZnO MNMs would fit into the first group and were the most toxic in all cell lines, which is in accordance with published data. Lysosomal swelling in HepG2 and RAW264.7 cells could be identified as an early marker after exposure to Ag but not ZnO MNMs in our studies. Also after incubation of cells with PS-NH2 MNMs lysosomal swelling and rupture occurs demonstrating a key role of this organelle for MNM injury pathways. SiO2 and PS-NH2 MNMs belong to the second group of surface-active MNMs. Adverse effects have also been reported for insoluble TiO2 MNMs mainly in the form of anatase in accordance with our results. These MNMs would belong to the third category i.e. structure effects dominating the cellular response.

Some TiO2 surface-modified MNMs at high particle concentrations induced cytotoxicity in several cell lines, which was studied in more detail in HCT116 colon cancer cells. TiO2-MNMs reduced cell counts and increased cell death independent of the key regulator p53. Inhibition of caspases and autophagy demonstrated involvement of these enzymes / processes in the induction of cell death due to TiO2-MNMs.

In the context of Objective 4.5 stable cell lines (transgenic A549 cells) that incorporate a fluorescent protein probe (m-cherry or GFP) linked to the nuclear Histone 2B protein (H2B) have been generated. This approach reduces potential phototoxicity induced by the excitation light over a long period of time e.g. during time-lapse studies, which has been observed when using other fluorescent nuclear dyes such as Hoechst 33342. Using this cell line it was possible to perform microscopy-based time-lapse experiments for studying MNM-induced cell death.

Key publications from WP4:
Summary of outcomes from WP5: MNM interactions with biomolecules & environmental factors

WP Leader: Marco Monopoli and Daithi Garry (UCD)
WP Partners: UoB, UoGEN, LMU, NRCWE, UNI-LJ, ATTANA, Malvern

The objectives of this WP were to:
- establish MNM dose under exposure conditions in time resolved manner, in order to correlate dose with response (in situ characterisation);
- identify the biological identity of MNMs (at contact with living systems => identify likely receptor interactions & potential uptake pathways; dependence on uptake route and consequences for final localization and impact; following uptake => final corona);
- confirm receptor interactions under in situ conditions in a high-throughput manner and develop QCM-array approach to demonstration phase;
- establish NP interactomes (signalling pathways potentially perturbed by NP-coronas – proteins and lipids) to correlate protein coronas with biological impacts observed (with WP4)
- demonstrate similar approaches relevant for eco studies, where interactions with NOM play similar roles as biomolecules do for toxicity studies.

It is widely acknowledged that the biomolecules formed on the MNM surfaces determine the biological interactions of MNM. Such biomolecular coronas are also the underlying reasons that many nanoparticle targeting strategies seem so promising when tested on cells under non-physiological conditions, but fail when tested in vivo, as multiple competitive interactions occur in vivo, and non-specific binding in many cases blocks the targeting functionality of the nanoparticles, rendering them inactive.

Despite the rapid increase in our understanding of the issues, methods by which to characterize the functioning bio-nanointerface in biologically relevant environments remains a challenge, and can be time consuming and expensive. This WP was designed to develop higher throughput approaches for identification of MNM-corona interactions / impacts, and demonstration of the general applicability of MNM-corona interactions (including for ecotoxicity assessment) as a predictor tool for classification of MNM based on their bionanointerface. A significant challenge will be to move beyond identification of the proteins and biomolecules in the corona to connecting these to receptor interactions (uptake, localisation) and signalling pathways perturbed / activated by the MNM-corona complexes.
A range of techniques were performed in order to discern a deeper understanding of how the NanoMILE MNM dispersions will appear when in contact with biological fluids and how this will impact when presented to cells. To this end, the first step in developing an understanding of how MNM present to cells assessed the impact on the size and, equally importantly, the dispersion complexity when protein adsorption occurs in biologically relevant conditions. After identifying the most abundant proteins on the surface we were able to use the newly developed high throughput fluorescence correlated spectroscopy technique to understand the dynamic relationship between how highly abundant proteins interact with bare particle surfaces. We found that highly abundant proteins such as albumin tend to cycle rapidly from the surface of particles, confirming the previous suggestion that its presence in the corona is related to its high abundance in the biological media and therefore not related to high affinity for the particle surface. Analysis of detailed molecular interactions present the advances beyond the current state of the art undertaken within WP5 in the context of mapping the thickness, stability and composition of particle coronas. Specifically, the work focused on understanding the impact of MNM polydispersity on corona determination, since previous studies had primarily focused on well-dispersed model particles rather than the broader polydispersed samples typical of industrially produced MNMs where the focus is on functionality in the specific application rather than on monodispersity. Among the interesting findings were that coating can matter dramatically in terms of the composition of the protein corona, highlighting the need to study individual surfaces.

A QCM based methodology has been developed for the screening of MNMs including in the presence of complex biological fluids for assessment of MNM and MNM-corona interactions with cell surfaces. The technique can be used to analyse cellular interactions of label free nanoparticles, which overcomes many limitations of other techniques (such as fluorescence-based detection). The hardware features of the Attana system allow performance of experiments using biological fluids such as serum. Furthermore, interactions can be monitored under continuous flow, which mimics the circulation of MNMs in the body. The real time, label free approach presented here may facilitate the understanding of mechanisms involved in MNM binding to cell surfaces, in particular identifying the binding partners at the bio-nano interface. The approach used in the final section, namely investigation of individual serum components, has been focusing on identifying selective binding of proteins to MNMs and biological receptors at cellular surfaces.

Moving forward from identifying corona composition to characterizing surface binding motifs, we have engineered a cell library in which receptor of choice is individually expressed. So far, our library hosts more than 20 receptors, including scavenger receptor family, lipoprotein receptor family, and immunoglobulin receptor family. These receptors have been speculated to be the major receptors for nanoparticle clearance in vivo. With this development, now we are in the position to individually assess the role of the receptors in cell-nanoparticle recognition, which provides the key step to close the loop. As an example, we have demonstrated that ApoB-100 is presented on protein corona derived from human serum on silica nanoparticles. The presentation of the ApoB-100 indeed leads to direct recognition by LDL receptor expressed in HEK293T cells. This cell library can be used with the Attana system with cells immobilized on chip surface, and the particle-cell interactions can be directly quantified in complex biological media.

While the biomolecular corona layer interacts with the cell surface, our studies have also shown that this interface also moves to the intracellular environment, engaging various endocytic machinery. By fluorescently labelling the corona, we have been able to follow up the corona’s endocytic trajectories and their degradation kinetics. It has been shown that in the matter of a few hours, the corona has reached lysosomes and degraded almost completely over a period of 16 hours. This highlights that we will need to further understand and evaluate the intracellular evolution of the corona to fully appreciate the signals arising from such a dynamic interface.

WP5 further explored the possibility to model the complex interactions in Task 5.5. It has been shown that we can successfully use the Metropolis Monte Carlo method and coarse-grained models to reveal the role of key physico-chemical parameters, such as solution pH, MNM surface charge density or presence of salt, driving the electrostatic complex formation between MNMs and biomacromolecules. This formation of complexes was found to be dependent on biomacromolecules conformational...
and MNM surface properties. The corona formation was of main interest in this report since the interactions (and thus the reactivity) of MNMs are widely modified when coated. With the two case studies presented in this deliverable, we have highlighted the complexity of such systems and that the presence of MNMs can significantly modify the acid/base properties of biomacromolecules, and induce important conformational changes as well as corona structure (biomacromolecule denaturation process).

Key publications from WP5:

Summary of outcomes from WP6: MNM bioavailability & biological effects in vitro/in vivo (ecotoxicology)

WP Leader: Charles Tyler (UnEXE)
WP Partners: KIT, EAWAG, IUF, UNI-LJ, EF
The objectives of this WP were to:
- identify common effects of selected MNMs across a wide range of organisms, with a focus on freshwater aquatic species (algae, daphnia, and fish (zebrafish, both embryos and adults) and a range of terrestrial invertebrates representing different ecological niches and identify the most vulnerable organisms for potential harm.
- establish which specific features of MNMs confer significant alteration in biological functioning in a biological system through the use and application of modified particles, including ‘aged’ and industrially relevant materials.
- For selected MNMs shown to be biologically active and resistant to degradation establish evidence for bioaccumulation and chronic and transgenerational health effects (focused on reproduction) for exposures under environmentally realistic conditions.
- develop a new fish model for investigating maternal transfer of MNMs.

Work package 6 sought to advance understanding on the biological effects and potencies of prioritized MNMs to a wide range of organisms covering test and sentinel species and to establish common responses and thresholds of effects. Surface modifications and ageing of selected MNPs were also assessed on bioavailability and biological effects. For materials showing toxicity or biological reactivity, we also identified body tissues accumulating MNPs. A major aim of the work in this work package was also to establish impacts for chronic exposure to MNPs and link biomarkers of response in acute exposures to these chronic health outcomes. We further set out to establish new models for studies into bioaccumulation of MNPs, adopting a terrestrial isopod, and for assess the potential for maternal transfer of MNPs, using a live bearing fish (Xenotoca eiseni). The
suitability of OECD test guidelines with terrestrial organisms for assessing the toxicity of silver NPs was also investigated.

In our work to identify common effects of selected MNPs across a wide range of organisms we established NOECs, LOECs and EC50s for a selected series of MNPs in soil invertebrate species (earthworms, Eisenia fetida, collembolans, Folsomia candida, predatory mite, Hypoaspis aculeifer, isopods, Porcellio scaber, and C. elegans) and in the aquatic species, algae (Chlamydomonas reinhardtii) and zebrafish (Danio rerio). This work included adopting OECD test guidelines, and undertaking chronic exposures. We showed that AgNP and ZnNP were the only nanomaterials of those tested with adverse effects. AgNP induced a concentration-dependent inhibitory effect on life span, reproduction, photosynthesis, hatching and neural phenotypes across the different test organisms. There was a sensitivity profile for soil dwelling organisms tested for AgNP. E. fetida was found to be the most sensitive suggesting this may be the most relevant of the soil dwelling organisms studied for application in standardised tests for MNPs. Adverse effects of AgNP and ZnNPs for chronic exposures were seen, but only at concentrations exceeding those in most natural environments.

Considering the features of MNPs that affect biological functioning, for all organisms tested soluble ions were found to comprise the toxic component for AgNP and ZnNP. We were not able to identify any adverse effects that we could ascribe specifically to the particles themselves. In studies on algae, time dependent toxicity was shown for uncoated and coated ZnO MNPs at high concentrations that could be related directly to dissolution of the Zn metal ions and its bioavailability in the exposure medium. The greater toxicity to AgNPs (NM300) in algae was also correlated with dissolution of silver ions. The rapid bioaccumulation of silver ions explained the short term toxicity of AgNPs in algae. Ageing of AgNP reduced toxicity for organisms in most culture systems, but not all. Toxicity was reduced in the freshwater algae, Chlamydomonas reinhardtii, exposed to sulfidised AgNP and there was no neurotoxicity in C. elegans at exposure concentrations less than 1.2mM for aged (sulfidised) AgNP which was not the case for non-aged AgNP, that was toxic. In zebrafish embryo toxicity assays and in zebrafish embryo-larval behaviour assays there was no effect of either aged NP or non-Aged NP for anything with environmental relevance. Interestingly, the toxicity of aged AgNP was not reduced in studies on earthworms. There was insufficient physiochemical characterisation of the silver materials, however, for the soils exposures to establish why aged AgNP retained its toxicity in this matrix.

Chronic exposures were conducted for all test organisms and for selected MNPs we established evidence for bioaccumulation and chronic health effects for exposures under environmentally realistic conditions. In C. elegans chronic exposure to AgNPs ≥ negatively impacted on the longevity and neuromuscular fitness, e.g. locomotion behavior. The behavioural defects occurred in 8 day-old adult worms (corresponding to middle age) and correlated with degeneration of axons of single serotonergic neurons. In contrast, neither ZnO nor CeO2 NPs reduced life span or neuromuscular fitness for chronic exposures, even for exposure to high concentrations (160 μg/mL). In Chlamydomonas reinhardtii chronic exposures to ZnO and Ag caused inhibition of photosynthesis and growth of the algae. Despite this, no long term adverse effects were seen indicating that C. reinhardtii is able to adapt to exposure to silver nanomaterials, at least for low exposure concentrations. For chronic exposures to metal based NPs in terrestrial isopods there were no effects on feeding behaviour or any obvious signs of adverse effects at the whole organism level, including on reproduction. In zebrafish, a multigenerational exposure to AgNPs via the diet at levels exceeding those for a possible worst case scenario for environmental exposures, no adverse effects were seen on reproduction of offspring survivor ship.

Some of the MNMs tested were shown to bioaccumulate in the different test organisms and for different exposure routes. Ag (AgNP), for example, was shown to accumulate in isopods for exposure via the diet and to accumulate also both in algae and zebrafish exposed via the water. The greater toxicity of AgNPs (NM300) in algae compared with ZnO NPs was correlated with the rapid bioaccumulation of silver ions. In zebrafish, adopting the use of isotopically labelled AgNP, we were able to detect uptake into body tissues for environmentally relevant exposures. We also identified maternal transfer for exposures to AgNP in both isopods and in fish was greater than that for in these animals when dosed with large size silver material (non nano). This was also the case for exposure of the live bearing fish Xenotoca eseni, to AgNP where there as an enhanced accumulation
of silver in the developing young compared with exposures to larger sized silver particles. These findings are important as they illustrate the greater potential vulnerability of the developing young for maternal exposure to some metal nanomaterials.

Work package 6 made good progress in the development of biomarkers for exposures to MNPs. This included the application of whole mount in situ hybridisation in early life stage fish to identify responses of some of these biomarker genes across the whole body. In isopods, activation of glutathione s-transferase was particularly marked, as a response biomarker for exposure to some of the metal based NPs. Significant progress was made also in identifying some of the mechanisms of effects for exposures to AgNP. For example, in the freshwater algae exposure to AgNP caused disturbances to the homeostasis of copper – a vital metal for normal biological function, and in C. elegans, AgNP caused neurodegeneration through degenerative effect on the axons of serotonergic neurons. Accordingly, knowledge on these biomarkers and effects can be usefully applied to future studies in assessments on the biological effects of these selected MNMs.

A major delivery from WP6 in NanoMILE has been the development (and adaptation) of test systems and development of new models for studying the bioavailability and biological effects of MNPs. This includes the adaption and validation of a standardized toxicity testing protocol for studying exposure to MNPs via feeding in the terrestrial isopod Porcellio scaber, (working with the NanoValid project) and the development of a new liquid culture testing systems for C. elegans, with direct utility for studying MNPs. In the NanoMILE project this new culture system mimics better the natural environment for this test species and extends the live span of C. elegans. The system removes cultivation stress for the animals, allows for studies into the MNP-bio-interactions and is adapted to a 96-well plate system to facilitate screening and high throughput analyses. Work package 6 has also delivered a completely new model for studying maternal transfer of MNMs in a live bearing fish, X. eiseni. We have shown this to be a robust and tractable system for investigating features of particles (including size, coating etc) that may affect maternal transport into developing offspring. This offers new potential as a vertebrate model for studies of this nature.

As a final consideration in the work undertaken in WP6 in NanoMILE for the range of ecotoxicology studies and diverse range of organisms, materials, and exposure environments, we strongly recommend that in the future the environmental matrix in which ecotoxicology studies are undertaken is given greater due consideration. This is because the immediate exposure environment has a fundamental effect on the fate and behaviour of the MNPs tested, in turn affecting their bioavailability, and thus biological effects (potencies) in the organisms exposed. This emphasises further the need also to establish greater standardisation in the test on MNPs and considering environments that are most relevant to that organism.

Key publications from WP6:

Summary of outcomes from WP7: MNM biokinetics and toxicity testing in vitro/in vivo (toxicology)

WP Leader: Wim de Jong (RIVM)
WP Partners: KIT, CEA, UU, NRCWE, UEDIN, IUF, VC, N4I, BASF
The objectives of this WP were to:
- determine the fate of MNMs after exposure via inhalation, orally or intravenous administration
- evaluate whether short term testing in combination with toxicokinetics can predict long term effects
- evaluate whether a general predictive testing strategy is possible based on common molecular mechanisms of toxicity in MNMs interaction with mammalian cells in vitro and in vivo
- evaluate, define and categorise, which physico-chemical and biological properties of NM are required for safety evaluation.

WP7 has addressed these objectives by performing both in vitro and in vivo studies in which the toxicity of model MNMs were evaluated. Supportive to the studies on respiratory toxicity an advanced air-liquid-interface (ALI) automated exposure station was developed. In addition, studies were performed to MNM uptake via the inhalation/respiratory and oral route of exposure. Various tools and methods were shown to be applicable for mechanistic research that helps understanding the relationship between exposure to MNMs and toxicity. The outcomes of the various studies can be used for safe(r) by design approaches.

A significant volume of data has been produced in WP7, both by testing the MNMs in vitro and in vivo. However, in light of the use for risk assessment, very few data appeared to be adequate for benchmark dose modelling. The studies performed within WP7, were very much focussed on the understanding of biological mechanism of action, as one of the main objectives of NanoMILE, in order to demonstrate how mechanistic understanding of MNMs-induced toxicity data can provide a basis for predictive modelling of MNMs toxicity and risk, and thus reduce reliance on animal testing in accordance with the 3R principles (reduce, reuse and refine). One of the central approaches used in risk assessment is the establishment of dose-response curves followed by the application of benchmark dose modelling in order to determine safe exposure limits. However, this requires that there is an identifiable toxicity in vivo, which was not observed for most of the MNMs tested within NanoMILE WP7. Toxicity was, at least, not observed at realistic exposure doses investigated within the ethically approved concentration range: note that in WP8 subtle effects such as up- or down-regulation of genes were observed in vitro and in aquatic organisms (algae, Daphnia and Danio Rerio). The lack of dose-response data and thus the inability to perform benchmark dose modelling is an important finding in itself though, and will be fed forward to ECHA and others involved in the risk assessment of MNMs. Thus, the identification of common molecular mechanisms of toxicity in MNM interaction with mammalian cells in vitro and in vivo could not be achieved.

The following major results were achieved in WP7 of NanoMILE
• Modified SPIONs used as medical guidewires were shown to be biocompatible, which resulted in a Technology Readiness Level (TRL) 7 of the devices (from a pre-NanoMILE TRL of 3).
• An advanced air-liquid-interface (ALI) automated exposure station for use as a more realistic in vitro lung exposure model was upgraded to Technology Readiness Level 7.
• Changing the redox potential of poorly soluble low toxicity manufactured nanomaterials (MNMs) was found to have limited effect on biological responses in in vitro and in vivo assays. The studies on generation of reactive oxygen species (ROS) demonstrated that several different forms of MNM could generate free radicals in suspension in the absence of cells/biological material. The extent of free radicals generated depended on the type of free radical under investigation and the buffer used to suspend the MNM. CeO2 and Zr-doped CeO2 MNMs were chosen to study the influence of the redox potential on mechanisms of toxic activity. For example, depending on MNM material dose-related and redox-related dendritic cell maturation and inflammasome activation could be observed.
• CeO2 MNMs did not change the atherosclerotic burden in mice, but did increase the macrophage content of atherosclerotic plaques by a redox-dependent mechanism. Increased inflammatory cell content of plaques is associated with plaque instability in man.
• Establishing the relationship between redox modification of MNMs and allergic responses in the lung, associated with asthma, was a key objective of the NanoMILE project. We have, for the first time, established the role of redox modification of MNMs on their adjuvant activity, i.e. on the degree of allergic response induced by the MNMs.
• Based on a panel of MNMs, genotoxicity was only observed for silver MNMs, most likely related to the solubility and resulting ions, and for TiO2 MNMs and Multi-walled carbon nanotubes (MWCNT) to a lesser extent and at high doses only. For TiO2,
oxidation is the main driver of genotoxicity. For MWCNT, the surface area is the main driver of inflammation, whereas diameter drives genotoxicity. In other words: thin MWCNTs promote pulmonary inflammation that is linked to risk of cardiovascular disease, whereas thick MWCNTs promote genotoxicity, a risk factor for cancer.

• Establishing the relationship between redox modification of MNMs and inflammatory processes in the lung, associated with fibrosis, was a key objective of the NanoMILE project. The size of CeO2 MNMs plays a dominant role in NLRP3 inflammasome activation. Moreover, the effects of length and surface modification (MWCNT) established above, were confirmed. Most importantly, we have, for the first time, shown the effect of redox modification on NLRP3 inflammasome activation.

• Length, more than diameter, is a key parameter in determining the extent of MWCNTs translocation from lung to spleen. In addition, modification of MWCNTs with carboxylic acids did not change the fate of translocation process. Shape dependent tissue distribution of MWCNT in lung (clearance) and in spleen (accumulation) is observed.

• Rats are more sensitive than mice for lung inflammation after inhalation of poorly soluble low toxicity MNMs (e.g. CeO2 MNMs). However, effects in rats are rather persistent after termination of the inhalation exposure.

Physicochemical properties that affect toxicity

While there are clear associations between ROS generation and the toxicity of MNMs, comparisons between MNMs are complicated by the use of different core chemistries, as well as different crystal structures or redox state between MNMs of the same chemistry, that will exhibit differences in many physicochemical properties apart from redox activity. In the current study, we applied chemical doping (intentional substitution of one element by another while maintaining the lattice crystal structure and arrangement) of a single type of MNM in order to specifically investigate the influence of redox activity on ROS formation, and the associated induction of oxidative stress responses in mice in vivo after inhalation exposure. CeO2 MNM was selected as a MNM which acute and subacute toxicity data from inhalation exposure was available,7 providing a solid scientific rationale for its selection, as well as ensuring baseline in vivo date for comparison and ground-truthing of the experimental data generated within NanoMILE.

To modify the redox activity, CeO2 MNMs were doped with different amounts of Zr4+ (Zr contents in the doped MNMs were 27% and 78%). The CeO2 MNMs crystal lattice consists of a cerium core enveloped by an oxygen lattice. These MNMs have an interesting feature, namely coexistence of Ce3+ and Ce 4+ ions and the ability of oxygen vacancies formation on their surface, which enables them to interact with and modulate free radicals. Addition of Zr4+ during the synthesis of the CeO2 MNMs would result in a lower Ce3+/Ce4+ ratio and therefore less redox activity and a lower oxidative capacity of the doped MNMs compared to the undoped CeO2 MNMs, which according to our hypothesis, would reduce the observed pulmonary and cardiovascular effects resulting from exposure to identical doses of the doped and undoped MNMs. Thus a series of CeO2 MNMs doped with increasing amounts of Zr4+ into the lattice were prepared by PROM utilising their supercritical fluid approach to produce 3-5 weight % particle solutions of high purity and good size distribution. The doping ranged from ~2.5% through to 100% and indeed the physical chemical properties of the particles remained constant (size, shape etc.) with the exception of the ability to produce ROS, as demonstrated using a H2O2 assay spectroscopically.

However, what was not foreseen was that the base CeO2 MNMs would be essentially non-toxic in the animal studies and thus the reduction of redox capability of the MNMs was not observed to reduce the toxicity as the CeO2 MNMs showed a rather low toxicity and didn’t generate ROS in vivo. Also in the in vitro studies limited cytotoxicity was observed only at the higher exposure concentrations above 80 µg/mL.

To address the lack of inherent toxicity via oxidative stress in the CeO2 doped MNM, a second series of doped MNMs was prepared while ensuring that at least some members of the MNM family would have a band-gap overlapping that of cells, which has been shown to result in MNM toxicity via oxidative stress (Zhang et al, 2012). To this end, Fe2O3 and Fe3O4 were selected as dopant, and given their close relation to Co in terms of atomic mass and size it was doped into Co3O4 MNMs. The doping was performed by PROM utilising their supercritical fluidics synthesis approach and the resulting particles were characterised by UoB, and assessed for their toxicity by RIVM. The prepared Co3O4 MNM doped with 25% Fe3O4 resulted in a
cytotoxic effect starting at the 20 µg/mL concentration.

Risk assessment

The short-term inhalation studies (STIS) have been shown to be a valuable tool for comparing MNM toxicity, and follow a standardized procedure, having a 5 day 6 hours/day exposure period followed by a 3-4 week recovery period. Within the NanoMILE project, two different CeO2 MNMs were tested, NM-212 received from the OECD sponsorship programme for safety testing of manufactured MNMs and PROM Cerium(IV)oxide MNM. The in vivo studies are extensively described in Deliverable 7.2 of WP7. Table 1 provides a summary of the regulatory-relevant outcomes from the STIS, such as the no observed adverse effect concentration (NOAEC).

The inhalation exposure to PROM Cerium(IV) oxide MNMs led to changes of several lavage parameters in rats of test groups 2 and 3 respectively exposed to 2 mg/m³ and 5 mg/m³. The effects were still significantly increased after the recovery period. At these concentrations, absolute and relative neutrophils were increased in blood, as well as monocyte counts. This finding indicates that local inflammation in the lungs was ongoing during the recovery period and a systemic inflammation started at that time. Adverse histological findings were only observed at 5 mg/m³ after the recovery period. The findings were consistent, because retarded lung clearance was observed at 2 and 5 mg/m³ (see Deliverable Report D7.5 on MNMs biodistribution). Thus, the no observed adverse effect concentration (NOAEC) was 0.5 mg/m³ for systemic and local effects under the current study conditions in rats exposed to PROM Cerium(IV) oxide MNM. Mice turned out to be less susceptible to these MNMs. The exposure to PROM Cerium(IV) oxide MNMs did not lead to any changes of clinical pathology parameters in blood or in bronchoalveolar lavage up to 5 mg/m³. Nor were there any treatment-related adverse findings in the histopathology analysis of the exposed mice immediately after exposure or after the 4 weeks of post-exposure monitoring.

Key publications from WP7:

WP8 - Systems biology approaches to reveal mechanisms of MNM activity

WP Leader: Mark Viant (UoB)
WP Partners: EAWAG, RIVM, UNEXE, BIOMAX
The objectives of this WP were to:
- measure the potentially harmful effects of multiple MNMs of differing physico-chemical properties using ‘omics approaches (transcriptomics, metabolomics and lipidomics);
- characterise classic toxicological (or phenotypic) outcomes to MNM exposure alongside molecular measurements, including
growth, reproductive output, morphological defects and mortality rates.
- employ computational modelling to identify signatures within the ‘omics datasets that represent adverse outcome pathways (AOPs), i.e. mechanistically based molecular biomarker signatures, which will be implemented into diagnostic screening assays to identify and characterise the impacts of MNMs on environmental and human health (in WP4).
- identify both species-specific and evolutionarily conserved molecular responses within the 4 species and cell line investigated.

To more deeply understand the damage, at a molecular level, that MNMs can cause, powerful new computational methods have been developed to analyse the toxicity data. These methods are called graph model algorithms and they have been used to analyse very large “omics” datasets. Omics datasets comprise of thousands and thousands of measurements of the biological molecules in a cell, including the expression of genes and the levels of metabolites, and they describe how cells respond to a stress such as can be caused by exposure to a nanomaterial. When used to analyse the omics data, our algorithms first built a graph model by linking the pairs of genes that shown a similar expression pattern across samples, i.e. which genes responded in a similar manner to a particular nanomaterial, which resulted in a so-called gene co-expression network. Figure 8 shows the co-expression network that was obtained after a small freshwater animal called the waterflea (Latin name Daphnia magna) was exposed to zinc oxide nanomaterial (NM111). Our algorithm then automatically identified co-expression modules, i.e. highly connected parts in the gene co-expression network, which consists of a set of genes that have a similar function or are involved in a common biological process. Figure 9 shows one of the co-expression modules identified from the co-expression network of Daphnia after exposure to the same zinc oxide nanomaterial. We found that this module consists of genes that are involved in oxidation-reduction processes, which tells us that the animal is responding to the MNM in the same way that it responds to metals. In turn this helped us to conclude that the ZnO MNM causes similar toxicity as zinc metal.

Key publications from WP8:

WP9 - Data integration/QPARs, risk assessment, safe MNM designs

WP Leader: Antreas Afantatis (Novamechanics)
WP Partners: UoB, KIT, CEA, DU, N4I, PROM, BASF
The objectives of this WP were to:
- coordinate with earlier WPs and identify data gaps; harmonise and combine data
- develop a robust, validated predictive nano-QS(P)AR model.
- computationally test the QS(P)AR model on new MNMs outside the project library.
- propose, design and test novel MNM structures and functionalities displaying reduced toxicity.
- develop a computational risk assessment platform, based on project results, which can act as a tool for virtual risk assessment, with the ultimate aim to be used by risk assessors/regulators but also by industry for self-regulation.

NanoMILE partner Novamechanics has succeeded in integrating two open science platforms: KNIME,9 that combines a rich graphical workflow environment for integration of diverse analytics and Enalos Cloud Platform for hosting and publishing models directly on the web, thereby allowing researchers to virtual screen and/or design novel MNMs. The development of the web service ensures immediate access to the tools and allows maximal opportunity for exploitation of the model’s results. To demonstrate the usefulness of the models, NanoMILE has also proposed a virtual screening framework that could be used to
identify novel potent structures. To further enhance accessibility to the NanoMILE QSARs and hazard assessment tools, tutorials and YouTube videos have been prepared. Two examples are presented below.

**Enalos QNAR Iron Oxide Toxicity Platform**

Enalos QNAR Iron Oxide Toxicity Platform is a web tool developed by NovaMechanics Ltd., which enables online toxicity predictions for iron oxide nanoparticles (NPs). The platform is freely available by Enalos Cloud Platform and is ready-to-use by all interested parties without any need of prior programming skills. The web service provides the functionality to virtually screen a set of NPs of interest based on the validated model, and thus yielding a preliminary in silico testing. The user must provide only the values of four physicochemical parameters for a set of MNMs and must select the coating of each MNM from among three alternatives. The platform provides two alternative ways for the input of the data; either an online form or a file submission. After entering the data by clicking on the appropriate button predictions for each NP are generated, as well as an indication on the reliability of predictions, based on the model’s domain of applicability limits.

The above functionalities are explained in a brief tutorial where screenshots and instructions give a clear overview of the web service’s required input as well as the output format and results.


Moreover, we have prepared and made available in YouTube a video demonstrating step by step an example on the use of this web service, available at: [https://www.youtube.com/watch?v=8Rxo_dhmD34](https://www.youtube.com/watch?v=8Rxo_dhmD34).

**Enalos Cloud Platform: Prediction of MNPs Uptake in PaCa2 Cancer Cells**

Online toxicity predictions for coated iron oxide MNMs are made available through Prediction of MNPs Uptake in PaCa2 Cancer Cells web service of Enalos Cloud Platform, developed by NovaMechanics Ltd. Via a user-friendly environment, researchers without any need of programming or computational skills are enabled to provide one or several structures of compounds (small molecules that modified NP coating) and get a predicted cellular uptake value for PaCa2 cancer cells along with an indication of its reliability based on the model’s domain of applicability limits. The user must insert a set of structures by drawing the chemical structure of interest, by entering the SMILES notation of the compounds in the appropriate field or by uploading a file with the SMILES notation of the compounds. The results include the predicted cellular uptake value for each structure entered, and an indication of their reliability.

The above functionalities are explained in a brief tutorial where screenshots and instructions give a clear overview of the web service’s required input as well as the output format and results.

This tutorial is available at: [http://www.novamechanics.com/Prediction_of_MNPs_Uptake_in_PaCa2_Cancer_Cells_Tutorial.pdf](http://www.novamechanics.com/Prediction_of_MNPs_Uptake_in_PaCa2_Cancer_Cells_Tutorial.pdf)

Moreover, we have prepared and made available in YouTube a video demonstrating step by step an example on the use of this web service, available at: [https://www.youtube.com/watch?v=SKenRzr1Fbl](https://www.youtube.com/watch?v=SKenRzr1Fbl)

Key publications from WP9:

*Melagraki, G., Afantitis, A., Enalos InSilicoNano Platform: An online decision support tool for the design and virtual screening of nanoparticles. RSC Advances 2014, 4, 50713-50725.*


WP10 - Dissemination activities

WP Leader: Benoit Hazebrouck (Eu-VRI)
WP Partners: UoB, RIVM, N4I, UNI-LJ, EUVRI, BIOMAX, Malvern

The objectives of this WP were to:
- operate dedicated communication channels with other projects and the main stakeholders to make sure that the results of NanoMILE will be used and implemented as soon as they are available.

The NanoMILE results to be disseminated to stakeholders have been summarized as follows:
- Mechanisms
- Assays / methods refined or developed for MNMs
- Datasets
- Quantitative models and hazard assessment tools
- Equipment: ALI, NTA, QCM
- Design rules for safer nanomaterials
- Hazard Assessment framework

The main stakeholders interested in the NanoMILE outputs and the main channels to reach them have been identified as indicated in Table 3.

NanoMILE partners have been extremely active in terms of disseminating their scientific achievements throughout the project. Table 4 shows the cumulative dissemination activities, which include over 55 papers (with as many again submitted or in advanced stages of drafting) and >350 conference presentations. NanoMILE partners chaired several NanoSafety cluster (NSC) working groups (WGs) including Hazard (WG2), Standardisation Sub-group (WG7) and the Safety-by-design (WG9), as well as representing WG7 Dissemination on the NSC Steering Committee. In February 2017, UoB were appointed as overall coordinator for the NSC, and will deliver several new initiatives including collaboration with the EU NanoObservatory.

This intense dissemination activity is further made available to all audiences on the informative NanoMILE website. At the end of the period (28.02.2017) the website included:
- 13 NanoMILE reports (PU deliverables), more to be shared as publications are released
- 51 (links to) scientific publications
- 30 presentations and posters
- 30 news items

By mid-February, the NanoMILE website had received 177,000 external clicks, i.e. clicks from non-logged visitors (i.e. external visitors).

For up-to-date progress, please go to the “outputs” page on NanoMILE’s website [http://nanomile.eu-vri.eu/].

Potential Impact:
In accordance with the dissemination plan prepared at the outset of NanoMILE, the project has successfully delivered the impacts specified in the call as follows:

(i) Increased understanding of the role of nanoparticle-biomolecule interactions in nanoparticle-induced impacts in living systems;

The main conclusion derived from activities related to investigation of biomolecule interactions with MNMs is that, as has been
increasingly recognized, the biological identity of MNMs appears to be conferred by the strongly adsorbed biomolecules. In principle then, early interactions between different organs and nanoparticles are expected to be driven by the corona interacting with receptors located on the cells of those organs.

Each organ can then be analysed to identify the relevant receptors, and this made into a library. In particular, it is now possible to measure the interactions between cellular receptors and any MNMs, and suggest how those particles could interact with organs that have those receptors. This could then be validated in vivo. Subsequently the original corona is degraded off and this early identity is lost.

The potential to directly measure interactions between particles in media and receptors seems a very promising approach but the report also highlights certain deficits for which there is currently no obvious solution. Firstly, the production of a library of receptors, organ by organ, represents a considerable investment and enormous effort, and in this work (representative examples are described here) only several organs (here for example, liver, brain barrier) can be addressed, for each species. Going beyond this will be a laborious and time-consuming process. The second challenge involves the use of poorly dispersed materials where the interactions of a very wide range of particles and particle aggregates is difficult to treat with receptor interactions, because of non-specific interactions and other artefacts. Balanced against these deficits, the approach developed within NanoMILE is one of the few that can relate the coating and exposure conditions of MNMs to organ (receptor driven interactions), without recourse to animals, and consequently is likely to be widely used in future.

NanoMILE has also made enormous advances in terms of development of models and tools for prediction of environmental coronas, such as those formed with humic or fulvic acids. The ability of coarse-grained models, using the Metropolis Monte Carlo method, to reveal the role of key physicochemical parameters, such as solution pH, MNM surface charge density or the presence of salts of different valencies, driving the electrostatic complex formation between MNMs and biomolecules has been demonstrated. This formation of complexes was found to be dependent on both biomolecules conformational and MNM surface properties. The model developed within NanoMILE is evolutionary, and can be adapted for specific situations or research questions, ensuring lasting impact.

(ii) Gene/protein fingerprints or biomarkers with potential for determination of specific pathogenic mechanisms;
The ‘omics approaches that lie at the heart of Systems Biology are non-targeted and hypothesis-generating. While these traits define the central strengths of ‘omics, it must also be realised that ‘omics analyses do not address specific hypothesis and do not provide (nor are intended to provide) robust conclusions. Consequently, the maximum value of ‘omics approaches in terms of the generation of robust new knowledge can only be achieved when these ‘omics methods are incorporated into a discovery -> validation pipeline. Unfortunately, even in 2017 (and certainly for the last decade), this is very rarely achieved in research studies and publications. The norm is for the application of one or more ‘omics technologies to study a particular phenomenon, for example the stress response of an organism to a MNM, for extensive data to be collected and mined, and then a series of putative biomarkers reported. While there is great value to such an approach, the data and putative knowledge generated falls short of what can be achieved. Specifically, such an experimental design will not have validated the putative biomarkers in an independent MNM exposure study. Furthermore, while such an experiment will provide putative biomarkers to the risk assessor, the study has not provided convenient tools for the risk assessor to implement into their own work. Currently, ‘omics experiments are not being used in risk assessment, in part due to their complexity of undertaking, and also due to the challenges of the data analyses and interpretation.

The application of an omics method coupled with advanced data mining to discover putative biomarkers, then followed by the development and application of a targeted analytical method is still rare. NanoMILE developed a targeted liquid chromatography mass spectrometry / mass spectrometry (LC-MS/MS) method to measure the metabolites in the 1-carbon pathway. Should the biomarkers being measured be of regulatory interest, then the LC-MS/MS can be used as a relatively high throughput, non-omics assay for measuring the markers of interest. Within NanoMILE, the putative biomarkers discovered by the extensive statistical analyses conducted on the NanoMILE omics datasets, following exposure of cells (A549) and three
aquatic organisms (algae, water flea, and zebrafish) to selected MNMs (pristine and aged) were identified. Additionally, the ability to translate one set of biomarkers from the complex ‘omics study into a deployable assay that could be used in risk assessment has been demonstrated. This represents a significant advance beyond the current state of the art, and a key step towards bridging the gap from ‘omics to regulatory relevant assays.

A maternal transfer fish model was also developed (shown schematically in Figure 10), and utilised in gene expression studies involving silver MNMs with different capping agents. While gene expression changes were indicated in these experiments it should be noted that it is only a single measurement of a single effect. WISH (Whole mount in-situ hybridisation) and qPCR (quantitative Polymerase chain reaction) measurements target single genes in isolation, whereas fertilisation success and developmental assays are encompassing a vast array of biological process to be measured. Additionally, a change in gene expression does not automatically link to a toxicological impact; it is rather a measurement of response. At the more realistic environmental levels at which NanoMILE’s ecotoxicity experiments focused on, it is perhaps more obvious that the long-term chronic assessment will be better positioned to shed light on any underlying toxicological effects than the short term acute assessment. The developmental endpoints, which comprise a host of physiological endpoints, are buffered by natural control mechanisms which could include the increase in a certain genes regulation. The acute exposure biomarkers measured in WISH provided evidence for changes in gene expression but it is important to ensure that these sensitive endpoints are only an indicator of the molecular responses we have chosen to measure. There may be many more which are unidentified. This is particularly true for nanotoxicology, where there is no suite of ‘classical genes’ to test, no single response pathway to investigate. The complexity of the exposure; from metal type, size, coating, dissolution rate provides a swathe of variability in response mechanisms from those associated with detoxification to one of the many oxidative stress pathways. This is also species specific.

In summary, whilst some acute biomarkers can give indications of chronic health effects, these are only at the molecular level, with more physiological/observable measurement insensitive to low level exposure. Due to the fact that there are no standard molecular approaches to zebrafish nanotoxicology at ecologically relevant exposure levels, these molecular markers are still a best guess scenario of gene choice based on the current literature and knowledge state. As responses can vary in the same species with the same Ag MNMs but at different life stages, as in the case here with mt2 upregulation in WISH embryo studies, but measured expression lower than unexposed controls in chronically exposure adult fish, there is further complexity to individual gene expression analysis. As omics platforms become more and more viable options in terms of speed and cost, these techniques will shed light onto any specific pathways to perform targeted measurement upon.

(iii) New methods for systems toxicology leading to safer final nanoproducts through providing targets for engineering refinements of products;
Numerous new methods and approaches supporting more realistic exposure and hazard assessment, and increasing the throughput and harmonising workflows for omics approaches. A brief snapshot of some of these advances is presented here.

The advanced Air-liquid interface (ALI) Automated Exposure Station (Figure 11) has been developed by KIT and VITROCELL . This system can now be used to aerosolized MNM exposure under well-controlled conditions. The system authentically simulates the conditions of human physiological exposure. It offers a capacity of up to 21-cell culture compartments for exposure and 3 compartments for clean air control. All key functions for successful exposure, such as aerosol flow rates, humidity, temperature and leak test, are edited by touch-screen prior to the experiment. The respective data is shown on live graphs and stored for further analysis. The cells are exposed at the air/liquid interface on 6/12/24-well sized cell culture inserts, thus the system has a reasonable throughput. The isokinetic sampling system enables uniform delivery of the test substance (including MNMs) to the cells. High voltage charging increases deposition efficiency on the cells for optimal contact with the exposed MNMs. The main advantage of the Vitrocell ALI approach is that the cells are exposed under air flow conditions that mimic in vivo inhalation exposure. Cells are continuously exposed to MNMs in air in contrast to the bolus exposure that occurs when MNMs are added to a submerged cell culture. In summary, Vitrocell participation in NanoMILE resulted in the design and production of a novel automated exposure station for in vitro inhalation studies. Various design
improvements could be implemented during the NanoMILE project based upon NanoMILE user requirements.

As an example of approaches developed to investigate the molecular mechanisms of MNM genotoxicity, and particularly for longer term / chronic impact assessment, novel genotoxicity approaches were developed and demonstrated. For example, anatase/rutile TiO2-MNMs (NM105 from the MNM library at the JRC) were found to cause increased oxidative damage to DNA in A549 cells, as probed in the comet assay, in both its alkaline and FPG-modified versions, as well as quantification of 8-oxo-dG. Prolonged exposure to this MNM caused the number of these lesions to increase, with a maximum number of lesions at 1 month of subculture of the cells in TiO2-MNM-containing medium (see Figure 12). At 2 months of exposure, the genotoxic damage was less intense but the cells appeared to have adapted to this prolonged exposure to TiO2-MNMs, by means of reduced cell proliferation, and had been sensitized towards subsequent genotoxicity stresses.

A range of approaches have been developed in order to increase the range of end-points that can be assessed in high throughput / high content screening formats, as part of the ongoing efforts to develop a screening platform for MNMs. For example, different strategies were employed to generate transgenic A549 lung cancer cell lines. Fluorescently labelled H2B fusion proteins are suitable for imaging cell nuclei and changes in nuclear morphology in real-time. Classical dye based detection of other markers such as lysosomal and membrane integrity can be combined with the detection of the transgenic markers enabling multi-parametric read-outs. Future studies could build on our experience and extend the repertoire of additional transgenic markers to monitor also cytoplasmic events or transcriptional and signalling responses either individually or combined by the insertion of multiple transgenes. Moreover, several different cell types should serve as hosts as the response to MNMs is, as another outcome from NanoMILE’s high throughput screening platform is the finding that MNMs effects are cell type specific, with, for example, differentiated liver cells being more sensitive than liver cell lines.

The use of high content analyses of cell toxicity endpoints involving dyes, and some specific (suitable) engineered cells is most straightforward, and readily accomplished. However, the development of specific cells lines with markers requires effort on a case by case basis, and the use of more commonly available marker systems is to be preferred. On the other hand, for specific end points, the effort may still be worthwhile. In such cases NanoMILE partners have recognized that one of the main limitations is that the cell populations, as engineered, may not yield a sufficiently refined read-out for detection of the elements of a pathway. This question was explored within NanoMILE and it was determined that specific sub-populations can be exploited usefully, suggesting a general way forward in engineering relevant cells.

An important outcome from the screening studies has been that the in vitro results with HCT116 cells showed a good correlation with the in vivo results obtained from the zebrafish embryos for toxic as well as for non-toxic MNMs (Figure 13). The applied concentration for all tested TiO2, CeO2, SiO2, ZnO, Ag and PS-NH2 MNMs was 125 µg/mL in both systems. The percentage of viable cells after 24 h exposure to Ag, ZnO and PS-NH2 MNMs was 10 % or less while no zebrafish embryos survived this treatment after 5 days. In contrast, the insoluble metal oxides CeO2, SiO2, and some of the TiO2 MNMs were non-toxic in HCT116 cells as well as in zebrafish embryos. Only few TiO2 MNMs (Figure 13) induced moderate toxicity in HCT116 colon cells. Taken together therefore, toxicity to zebrafish embryos induced by silver-, zinc oxide and polystyrene MNMs are most likely explained by crude cellular toxicity.

(iv) A framework for categorisation of nanomaterials on the basis of their bioaccumulation / biopersistence and a set of risk-factors for specific end-points, pathogenesis mechanisms;

NanoMILE’s aim was to investigate the biological mechanism(s) of action underlying the toxicity of a wide range of MNMs. The main hypothesis tested was that the redox states of poorly soluble MNMs affect their toxicity. The focus was on free radical mediated oxidative stress, genotoxicity, dendritic cells maturation, inflammasome activation, cytotoxicity and release of inflammatory mediators as underlying mechanisms of lung toxicity by MNMs.

Lung inhalation was demonstrated to result in the deposition of MNMs into the deep lung. The lung burden of deposited MNMs increases with increasing applied exposure dose. Although the lung burden decreases in time over 3 weeks of recovery post
exposure, MNM (CeO2-MNM) was still present in the lung. Rats and mice show a difference in lung burden clearance for CeO2 MNMs. Rats show a limited decrease of approximately 20% at 3 weeks after exposure when compared to the lung burden directly after the 5 days repeated inhalation exposure. For mice, after a 3 week recovery period the decrease in lung burden was more substantial with a decrease of approximately 90% of the initial lung burden. The lung burden itself is dependent on the dose and the dosing regimen. A low dose with repeated prolonged inhalation exposure results after 4 weeks in the same lung burden as a higher dose with a short exposure period over 5 days. The doping of CeO2 MNM with Zr has no effect on the deposited or retained dose in the lung.

Translocation of the CeO2 MNMs to the draining (mediastinal and tracheobronchial) lymph nodes does occur and was confirmed by histopathology, but is limited. Further translocation to other organs (e.g. heart, kidney, spleen, liver) was not observed. However, some indication was present that the Zr-doped CeO2 MNMs showed migration to the kidney. Thus, the inhalation exposure to CeO2 MNM results in an organ burden that is mainly limited to the local exposed organ (i.e. lung) and its draining lymph nodes. After oral exposure of mice a dose response in CeO2 MNM burden occurs in the intestines and liver. For other organs the Ce levels are in the same order as the non-treated controls.

For MWCNT lung clearance is a gradual process that may last for more than 1 year. The length of the MWCNT is of greater importance than the width in determining clearance and translocation. Longer (4 µm) MWCNT have a slower clearance than short (2 µm) MWCNT. The main target organs in the redistribution of MWCNT after clearance from the lung are the spleen and liver. At 360 days after administration for both organs a similar MWCNT level was present in terms of MWCNT µg/g tissue. The longer MWCNT show a higher redistribution into the spleen which was still increasing at 1 year after a single administration into the respiratory tract. There was no effect of the surface modification of the MWCNT on tissue (re)distribution. In conclusion, MWCNT show a long period of lung clearance which may last for at least 360 days after a single exposure. Spleen and liver are the main target organs after clearance from the lung and (possible) systemic availability. In terms of size the length of the MWCNT is more important than the width for lung clearance and subsequent spleen accumulation.

The nematode C. elegans has a short life span of several weeks that enables the analysis of MNM exposures concerning chronic effects during the entire adult life of the test organism. The life span of C. elegans fed on OP 50 E. coli (control) or OP 50 supplemented with different MNMs was investigated. To this end MNM-suspensions of indicated particle types and concentrations were co-incubated with bacteria and worms in liquid medium in 96-well microtiter plates. Control worms were fed on OP50 supplemented with water. Adult C. elegans were cultivated at 20°C, checked every day and scored for dead animals by absence of reaction upon gentle pokes. Each experiment was performed in triplicate and included data of >400 worms/concentration (n). The results are shown in Figure 14.

All untreated controls and worms that were exposed to CeO2 MNMs (pristine or aged) in concentrations between 20 and 160 µg mL-1 lived up to 34 - 36 days and showed nearly identical survival curves (Figure 14, A-C). However, when aged CeO2 MNMs were exposed to concentrations of 300 µg mL-1 the survival curve dropped and runs similar to the survival curve of short-lived daf-16 mutants (Figure 14, C, red curve). This indicates that neither pristine nor aged CeO2 MNMs induce alterations of C. elegans life span at low concentrations. The LOAEL (lowest observed effect level) concerning a reduction of C. elegans longevity for aged CeO2 MNMs is at 300 µg mL-1 representing a 30 fold higher concentration than what is currently modelled for MNM-concentrations in European sediments or sludge-treated soils. However, as current modelling also predicts further accumulation of engineered MNMs in environmental sinks such as sediments and sewage sludge-treated soils test organisms such as soil nematodes represent relevant species for nanoeccotoxicity surveillance. The sensitivity of the life span assays in C. elegans is well-suited to detect future changes of soil quality and potential effects of MNMs on longevity of soil nematodes.

(v) A screening platform for nanomaterials as part of a ‘safe nanomaterials by design’ strategy.
A survey of the data collected by NanoMILE has shown that four MNM libraries were developed with the aim to test the hypothesis that once a mechanistic understanding of nanotoxicity begins to emerge, designing toxicity “in” or “out” of MNMs
should be feasible.

- Library 1: Zr doped ceria (redox model)
- Library 2: Fe doped cobalt oxide (redox model)
- Library 3: metal oxide core/PVP capped NMs (composition model)
- Library 4: ceria, silver & CNT ageing (ageing model)

Each library demonstrated the principle that careful control and experimentation of the synthesis conditions allows NM variants to be created with only one property changing systematically; if this property can be linked to toxicity it is then possible, in principle, to select a “safer” variant to be used in applications. Modification of the redox activity of the MNM in the project has increased the understanding of the role of redox activity in various toxicological responses including effects on the immune system.

Overall, most of the NanoMILE materials displayed modest indications of toxicity and even though the library developed in the project is one of the largest single library to contain systematically controlled variants, with a full set of characterisation data and toxicological assessment (multiple deliverables) it still represents a very small fraction of the full breadth of MNM classes available commercially. As would be anticipated from the scale of diversity of the models and techniques used here, the results obtained were complex, and highly dependent on the type of MNM, the biological system under investigation and the endpoint employed. Of the three libraries considered above: Library 1 showed that the redox paradigm, referring to NMs containing redox sensitive elements being linked to the production of reactive oxygen species (ROS) linked to toxicity is not always valid; in the case of Library 1 toxicity remained low for the entire library. Library 2 showed a case where the redox paradigm was upheld, and indeed toxicity could be reduced by doping with a less redox-active component. Library 3 showed that for a panel of identical particles, in terms of surface and size characteristics, the core metal composition is a key influence on toxicity. Library 4 tested the ageing paradigm and enabled the discovery that, unexpectedly, ageing does not always reduce toxicity; although the ageing results from NanoMILE only are not sufficient to develop a full ageing model to support safer MNM designs, it is anticipated that with further work that could ultimately be the case.

The systematic approach of the NanoMILE programme as a whole (from synthesis, characterisation, in vitro and in vivo testing) is a vital process to derive a comprehensive knowledge required for the categorisation and comparisons of MNMs, that will ultimately allow a safe-by-design approach to MNM use.

Utilising the NanoMILE datasets, a number of in silico workflows were built including all steps required for the development of robust and predictive models including descriptors calculation, variable selection, model development and validation. Data produced within NanoMILE as well as data taken from the literature were considered and a wide range of modelling techniques were performed to give insights into the critical parameters, both experimental and computationally derived, that effect MNMs behavior and correlate with their adverse effects. A number of Quantitative Nanomaterial Activity Relationship (QNAR) models have been developed and made publically available, for hazard assessment of MNMs.

For example, a QNAR model that quantitatively describes the effect of MNMs’ surface modifiers on the cell uptake of MNMs in Pancreatic Cancer cells was developed utilising a literature dataset on the cellular uptake of 109 MNMs in pancreatic cancer cells (PaCa2). Each MNM within this dataset includes the same metal core (iron oxide/NH2 cores) but different surface modifiers which are organic small molecules conjugated to the MNM surface. In an effort to computationally explore this dataset and develop ready-to-use applications, we have developed and validated a QNAR model based on this dataset for the prediction of the cellular uptake of MNMs by pancreatic cancer cells. For that, an in silico workflow has been developed that incorporates and combines the calculation of structural descriptors together with variable selection and model development and validation techniques.

Moreover the curated NanoMILE dataset has been used to derive meaningful QNAR models based on experimental data produced within the project. We have first worked towards the generation of novel nano-descriptors for MNMs included in the
NanoMILE Knowledge Base. For that we have generated descriptors based on image analysis techniques based on SEM/TEM images and Quantum Mechanics calculation to derive theoretical structure – dependent descriptors. For the QNAR model development we have used the experimental data on the full characterization of the MNMs together with the biological assessment including different endpoints in differentiated HepRG cells. This has concluded in robust and validated models that quantitatively describe the effect of the critical variables on the biological effects of the MNMs.

All QNAR models developed within NanoMILE were made publicly available as web services via the Enalos Cloud Platform4. In this way our developed models could be of help to the wider community of end users interested in MNM’s design as the interested user can easily perform online High Throughput Virtual Screening for a wide range of new MNMs. The web services needs no special computational skills and can be easily used by different groups of scientists like chemists, biologists etc or even non experts involved or interested in the biological evaluation of MNMs.

Summary of exploitation of results by industry partners

Each of the 10 industry partners in NanoMILE have benefited from, and supported the exploitation and dissemination of the outcomes from NanoMILE. A very brief summary of their exploitation activities is provided here. Based on the research performed within NanoMILE, two products were able to enter the market at TRL7, as indicted below.

Vitrocell: As described above, the VITROCELL air liquid interface exposure system was brought to market at TRL7 opening up new markets and ensuring confidence in the comparability of the data produced.

Nano4Imaging: N4I has developed a guidewire including SPION MNM that has reached the market at TRL7 based on research performed within NanoMILE. WP7 carried out extensive investigations using a panel of commercially available and custom-synthesised SPIONs in a series of in vitro and in vivo models. These studies showed that some carbohydrate-coated SPIONS had the potential to activate the blood complement system, whereas modification of the surface coating (i.e. cross-linking of dextran molecules) prevented these effects. Furthermore, the same SPIONS have been tested for thrombogenic responses in an ex vivo Badimon chamber in which blood from human volunteers is perfused over aorta strips under representative physiological flow conditions. The collective data show that cross-linked carbohydrate-coated SPIONs did not induce either complement activation or cause a thrombogenic response. Together these findings demonstrate that, for MNMs designed for intravenous administration, careful design of the surface coatings can be used to minimise undesirable interactions with the blood, and enabled N4I to obtain CE certification as well as FDA 510K approval.

Malvern: The application of Nanoparticle Tracking Analysis for time-resolved description of MNMs dose under relevant biological and environmental conditions has been developed within NanoMILE, including the development of an automated focus for the sample stage, as well as the computer code routines for time-resolved analysis of nanoparticle agglomeration were developed, and are integrated into the software. A Nanomaterials characterisation Technical note on “Time-resolved description of nanomaterial dose under relevant biological and environmental conditions” is available via the Malvern website: https://www.malvern.com/en/support/resource-center/.

BASF: BASF have demonstrated that their short 5-day inhalation exposure model for nanomaterials provides equivalent data for No Observed Adverse outcome effect levels and qualitative effects as 90-day inhalation studies with comparable MNMs. BASF are working to have the assay standardised and accepted for regulatory testing in line with efforts to reduce animal testing.

Novamechanics: The Quantitative Nanomaterial-Activity relationships (QNARs) for nanosafety assessment developed by Novamechanics have been arranged as a series of webtools available online for in silico hazard assessment of MNMs. Models are currently open access - once in widespread use / recommended by ECHA could be a commercial service providing bespoke models and data interpretation.
Promethean particles: The main outcome for PROM is the nanosafety datasets obtained for their MNMs, supporting the company in their business to business sales and supporting commercialisation of their materials. Having robust hazard data for their MNMs, particularly those being produced at >10 tonne scale is vital for ensuring commercial partnerships, so the NanoMILE datasets are hugely useful.

Biomax: Biomax developed the NanoMILE KnowledgeBase for nanosafety assessment, which consists of the characterisation information for the NanoMILE library of MNMs, as well as all the data regarding the biological end-points assessed across the difference cell lines and species, the calculated descriptors, and the underpinning protocols and overarching metadata. The Knowledgebase is currently restricted to NanoMILE partners, but exploitation plan is in place, with onward development and access provision planned.

Attana: Attana have developed new assays for MNMs interactions with cellular receptors in the absence and presence of a protein / biomolecule corona. These new assays have extended the applicability of the instrument and developed new assays for assessment of MNMs protein corona and interactions with cellular receptors and opened up new application areas. Technical notes on the applications for nanomaterials / nanosafety assessment are in development.

Eu-VRI: A guide for nanosafety researchers “How to bring your nanosafety research to standards?” has been developed which is expected to enhance the amount of research that leads directly to standardisation. The guide and associated materials is available via the Nanosafety cluster website at: http://www.nanosafetycluster.eu/working-groups/7-dissemination-wg/standardization/how-to-bring-your-nanosafety-research-to-standards-a-comprehensive-set-of-guidance.html

Eurofins: Via the application of OECD approaches for testing of MNMs in soil organisms, Eurofins have gained vital understanding of the applicability of standard OECD protocols for MNMs. Eurofins exposed earthworms, predatory mites, springtails, honey bees and bumble bees to NanoMILE materials generating vital datasets to support their customer base.

NanoMILE Cooperation with other projects/programmes

NANOSOLUTIONS (FP7) - a close collaboration between the two projects in their latter half enabled sharing of approaches and an overall strong interaction. Activities included a joint meeting at the UoB office in Brussels in July 2015, which enabled the work towards joint papers to start. Manuscripts in preparation include: 1) selection processes that the projects underwent to determine which MNMs, which model systems and which methods/assays to utilise, which will form an important part of the scientific record at the present time in nanosafety; 2) Omics approaches; 3) outcomes from the final joint meeting (see next).

The collaboration between NANOSOLUTIONS and NanoMILE culminated into a final project meeting, which was subsequently joined by eNanoMapper, SUN and GuideNano, and took place in February 2017 in Malaga. This was an important opportunity for data and knowledge sharing and a consensus paper will be published shortly very likely in a high impact journal. A lasting output of this activity are the video recordings of the entire event organised by NanoMILE (see: http://nmsaconferencetalks.eu/)

NanoFASE (Horizon 2020) - Given NanoMILE’s focus on hazard and NanoFASE’s focus on exposure and fate, there was a natural complementarity between the projects, which was harnessed through sharing of MNMs libraries (e.g. from Promethean particles and UoB, both partner in both projects), and through the extension of the NanoMILE KnowledgeBase to cover NanoFASE particles and datasets in addition to NanoMILE’s datasets. This will support integration across data for complete risk assessment in due course. NanoMILE and NanoFASE also organised a joint workshop / Training School in Autumn 2016.

SHYMAN (FP7) - There have been crossovers between NanoMILE and another FP7 project which PROM is involved in - SHYMAN (Sustainable Hydrothermal Manufacture of Nanomaterials, Grant Agreement Number 280983). As part of this project, selected materials which PROM manufactures were scale up to be produced at industrial quantities – up to 1000 tonnes per annum. It
was in the interest of the NanoMILE project to study nanomaterials which are industrially relevant, while it was in the interest of the SHYMAN project to consider the toxicological impact of these engineered MNMs on living systems and the environment (especially when considering accidental release or disposal during the production phase). Therefore, PROM acted to feed information and samples between the two projects which has resulted in toxicology data, obtained by the JRC within NanoMILE, being used in a Deliverable Report within SHYMAN (Deliverable 6.4: Identify potentially high environmental and economic impacts of the hydrothermal synthesis process to be submitted by Czech Technical University). The JRC and the NanoMILE project have been fully credited for their work.

eNanoMapper (FP7) - NanoMILE has worked with eNanoMapper in terms of developing the ontology for MNMs ageing, MNMs coatings etc., as well as supporting the development of the database and its sustainability. NanoMILE gave a stimulus presentation at the eNanoMapper databases workshop in Brussels in January 2016, and hosted a joint meeting with eNanoMapper team members as part of the M36 meeting in Edinburgh. UoB and eNanoMapper partner have jointly developed a starting communities bid for a research infrastructure (NanoCommons, currently under stage 2 evaluation) as well as a successful bid for an e-infrastructure (OpenRiskNet).

NanoDefine (FP7) - NanoMILE is actively contributing to NanoDefine’s project networking activities, including presenting the project’s activities and potential contributions to community activities at the NanoDefine meeting in February 2016. NanoMILE completed the templates and other reporting requested, and will continue to engage as new initiatives emerge.

Nanosafety Cluster: NanoMILE are playing a leadership role in the NanoSafety Cluster (NSC), as one of the larger projects running at present. To this end, we continue to contribute to NSC activities, such as editing the NSC compendium for 2014 (Iseult Lynch), the contributing to the organisation of the 2nd NSC young-researchers meeting in Visby (Profs. Valsami-Jones and Lynch are on the organising committee), as well as providing leadership of specific WGs: Flemming Cassee leading WP2, Benoit Hazebruck leading WG7 sub-group on Standardisation, Eva Valsami-Jones leading the new Safety-by-Design WG, and Iseult Lynch representing Dissemination on the NSC Steering Committee. As of February 2017, Valsami-Jones, Lynch and Cassee (all NanoMILE participants) with Andreas Falk were voted as the new leaders of the NSC.

EU-US Communities of Research - NanoMILE have also been active in the EU-US CORs, with two NanoMILE partners presenting at the 2013 CORs workshop in the US (Denise Mitrano (EMPA) and Francesco Falciani (University of Birmingham / University of Liverpool). NanoMILE partners are actively involved in the regulatory, databases & curation and exposure CORs, and will be involved in the methods and characterisation one in due course. NanoMILE has contributed to preparations of the forthcoming CoRs meeting in Birmingham in September 2017.

Nanotechnology Data Curation Initiative - NanoMILE signed-up to act as a Stakeholder Liaison for the EU-US CoR Database & Curation / NCIP Nanotechnology Working Group “Nanotechnology Data Curation Initiative”. As part of this, NanoMILE provided input and responses to six themed questionnaires which (along with the inputs from the other liaisons) collectively formed the “landscape” of nanomaterial data curation. This also helped to ensure that NanoMILE has international visibility and that the approaches pioneered within the NanoMILE knowledge base were linked to international efforts in this arena. The first co-authored paper has been published in NanoScale.

List of Websites:
www.nanomile.eu-vri.eu

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