Executive summary:

NANORETOX was an international, interdisciplinary and strategic research program, funded under the European Commission's NMP programme and addressing the environmental and human implications of exposure to engineered nanoparticles. The project started in December 2008, and lasted 4 years. Central to the NANORETOX research approach was placing equal weight on nanoparticle synthesis and characterisation as on toxicological and ecotoxicological studies, which was quite a novel concept at the outset of the project, and indeed is still a topic of considerable debate in 2013. These three research strands were constantly harmonising activities as the project progressed.

The project's overall strategy was to apply a highly multi-disciplinary approach to a focused set of scientific questions. NANORETOX aimed to investigate the potential risks of engineered nanomaterials (ENMs) to the environment and human health by comprehensively addressing five key questions:

-How does the environment affect the physicochemical properties and the bio-reactivity of ENMs?

-How does this impact on their ability to interact with and/or penetrate organisms and cells and will bioavailability result in toxicity?

-Is there a pattern of cellular reactivity and/or toxicity related to physicochemical properties?

-What combination of conditions are most likely to pose a risk to human health and the environment?

-How can this information be incorporated in a risk assessment model?

NANORETOX addressed these questions using a panel of 8 inorganic ENM classes (TiO_2, CuO, Ag, Au, ZnO, SiO_2, CdS, CdSe NPs along with aqueous and bulk counterparts) that differed in their reactivity, solubility and core composition. For each ENM, a range of sizes, shapes, coatings and structures was studied, resulting in 55 distinct ENMs in total. Each ENM was assessed for toxicity using a range of toxicity/ecotoxicity tests: 33 types of dose response in vitro tests with 6 different cell types, 23 types of in vivo dose response tests and 11 single dose responses tested under 5 types of exposure; these tests involved a total of 8 species and a range of mammalian primary cells and cell lines.

Among the most significant conclusions from NANORETOX are that:

Conclusion 1: The choice of test/assay is critical to evaluating hazard and especially risks.

-Cell lines show different sensitivities

-Species show different sensitivities

-Exposure routes and fate have major influence on the observed effects (e.g. diet versus water)

-sublethal measures (as compared to acute toxicity) are very sensitive.

Conclusion 2: Nanoparticle reactivity is not always a measure of toxicity. The order of ENM aggregation/solubility does not follow order of toxicity. ENM chemical composition is the primary variant responsible for toxicity.

Conclusion 3: Nano-effect is real, but not extreme.

Conclusion 4: In vivo, adult, acute toxicity tests, with a few exceptions, will not detect nanoparticle risks indicated by other tests. Thus, OECD protocols must adopt more complex measures than acute toxicity to properly evaluate risks.

Project Context and Objectives:

Background and overall objectives

Engineered nanomaterials (ENMs) have unique properties with an exceptional potential for commercial exploitation. However, the commercial development of ENMs is far out in front of our understanding of environmental and health implications when those materials are inadvertently released to the environment.

Inorganic metal-based ENMs (Me-ENMs) are of particular interest both commercially and in terms of environmental and health risks, both because of their rapidly growing uses in a range of commercial products, but also because the metal of which Me-ENMs are composed can have an inherent toxicity.

Project NANORETOX was an early attempt to address some of the most significant data gaps in nanosafety, notably in terms of the combined toxicity/ecotoxicity potential of Me-ENMs. The overarching goal of NANORETOX was to generate systematic empirical datasets to facilitate a re-assessment of some of the basic questions raised by the earlier reviews, and indeed by the consortium's own analysis of the knowledge-gaps at the time of writing the NANORETOX proposal in 2008.

This was to be achieved by addressing the following specific scientific and technical objectives, which mapped onto the 8 research workpackages of the NANORETOX project, as follows:

[1] To synthesise and fully characterise a library of ENMs with a range of physicochemical properties using industrial and laboratory methods (WP1).

[2] To study the abiotic reactivity (transformations) of the synthesised ENMs in simulated environmental and biological media (WP2).

[3] To investigate in vivo uptake of the ENMs by aquatic species and study mechanisms and paths of internalization (WP3).

[4] To investigate in vitro uptake and reactivity of nanoparticles and to discover putative mechanisms of toxicity by aquatic species primary cells (WP4) and by primary mammalian and human cells and existing human cell lines (WP5).

[5] To consider the genotoxicity and carcinogenicity of metal nanoparticles (WP6).

[6] To determine whether cellular responses between human cells, mammalian cells, cell lines and invertebrate cells or whole organisms are comparable or different with relevance to screening models (WP7).

[7] To establish universal approaches to risk assessment model and risk communication (WP8).

The overriding objective of NANORETOX was to contribute new knowledge to the global endeavour of addressing the scientific uncertainties related to the health and environmental effects of ENMs and to provide a body of new information and a new tool that industry and governments can use to begin to assess the risks of these ENMs.

The NANORETOX approach and specific objectives

The three principles of NANORETOX

1) Focus on engineered Me-ENMs, synthesised and fully characterised by project partners (both research and industry), to provide a coherent well defined study material with controllable properties. The project did not consider natural or other non-engineered (e.g. combustion derived) nanoparticles.

2) Use of organisms that are not currently included in standard toxicity tests, but which have (a) greater potential to be affected by ENM toxicity due to the environment where they live or their biology and (b) are potentially valuable indicators.

3) Minimal use of animal models in keeping with the 7th Amendment to the EU Cosmetics Directive (European Commission, 2003; Council Directive 76/768/EEC) which aims to reduce the use of experimental animal research.

NANORETOX examined the molecular and cellular reactivity of well characterised nanoparticles on a panel of primary human/mammalian cells and human cell lines originating from different target organs and exposure routes as an indicator of in vivo toxicity. The aim was to discover which features of nanoparticles confer reactivity with which cell types/target organs.

This objective addressed concerns resulting from the rapid expansion of nanotechnology, which has resulted in a vast array of novel ENMs, many of which are already in industrial production without any prior safety testing. Because of the wide variety in physicochemical properties amongst different ENMs it is not possible at present to predict which elicit environmental harm. However, until the mechanistic associations between ENM

characteristics and putative toxicity are understood, determination of nanorisks will not move forward. Many toxicological studies have fallen short of this; furthermore many studies have led to contrasting results and interpretations about risks, possibly reflecting the diverse sources and nature of the test materials. This illustrates the importance of studying both commercial and purpose-made ENMs that have been fully characterised before, during and after toxicity studies. Of the many different types of ENMs currently produced, industrially or in the laboratory, environmental risks from non-carbon-based nanoparticles are the least studied. This is despite the rapidly growing use of particles such as TiO2, ZnO, SiO2, Ag, CuO, and CdS. The chemical composition of metal nanoparticles may contribute to their having significant additional toxicity, but few studies address this.

NANORETOX comprehensively addressed many physicochemical properties of industrially important metal based ENMs with a potential to induce toxicity (particle size, shape, agglomeration, crystal phase, chemical composition, surface area, surface chemistry and surface charge).

Ecotoxicological studies with ENMs were almost non-existent at the time NANORETOX was written , . Most of the existing studies at the time were simple “proof of principle” tests evaluating the possibility of either toxicity, under high concentration exposures, and/or the visual penetration of cells. There was therefore a clear need for a more systematic approach to evaluating the processes that determine hazard, exposure and risk and for validated models predicting the release, transport, transformation, accumulation and uptake of nanoparticles in the environment and the human body .

Systematic generation of new knowledge in nanotoxicology was lacking, and as a result, a key aim of NANORETOX, was to develop reliable standardised tests using new simpler more relevant screening techniques.

Project NANORETOX recognised the importance of whole aquatic organism studies of metal nanoparticle toxicity and the development of a significant body of internally consistent data on a variety of organisms and ENMs. The project was conceived at a time when the literature was dominated by inconsistent results. For example, zinc from zinc oxides was bioavailable to earthworms and induced a cellular response, but quantum dots of cadmium sulfide or cadmium selenide with a zinc sulfide shell appeared to be absorbed by a daphnid with no apparent ill effects. Indications of toxicity (lysosomal destabilization) were observed in oyster cells due to CdSe quantum dots, but rapid breakdown of particles was also documented. Metal toxicity and effects were found to differ widely among biological species, but ecological risks depend greatly upon the traits that determine the exposure and response of sensitive species, and that principle was anticipated to apply also to ENMs. At

the start of NANORETOX, studies of nanorisks had not considered comparative physiology, biology and ecology.

NANORETOX aimed to compare animal models that were likely to be sensitive to ENMs: animals that pump vast quantities of water across their gills (bivalves) or ingest plants or sediments where ENMs would be likely to accumulate.

The governing idea here for NANORETOX was that a first step in understanding risks is bioavailability: can ENMs be taken into the tissues of the whole organism from either suspension/solution in the water animals pass over their gills, or when eaten in the food of the organisms? Results were ambiguous and only a few species had been studied at the start of the project. For example, no studies existed on animals that intentionally filter large quantities of water, although these would be expected to be the most vulnerable to low environmental concentrations of ENMs. Nor had bioavailability of ENMs from food been studied. These were particularly difficult questions to address at the time of project inception because there were no methods at the time to evaluate bioavailability quantitatively, and detection of uptake was technically problematic.

NANORETOX used labelled ENMs (with either stable isotopes or fluorescent dyes) to allow quantitative determination of the accumulated material. These techniques aimed at resolving the problem of determining ENM bioavailability.

At the start of the project, there were no previous studies of stable isotope labelled ENMs in ecotoxicology and labelled materials had only just become available. The project used a number of novel experimental designs to trace ENMs, including the newly available stable isotope labelled ENMs, thus generating a significant body of environmentally relevant data. By quantifying ENM uptake using labelling, verifying the presence of particles in cells with visual or light scattering approaches, and observing responses of the organism at the cellular and whole organism level, NANORETOX assembled the lines of evidence that were necessary to determine if bioavailability and toxicity are feasible expectations for metal ENMs. By systematically making such determinations using a number of different types of particles of well-defined character nanoparticle properties were tied together with their bioavailability and toxicity in unique ways.

NANORETOX used determinations of biodynamic characteristics, including rate of uptake from water, assimilation efficiency and rate of uptake from food, as well as retention (rate constant of loss), across several species and a range of ENMs. The objective of this screening approach (equivalent to a biological bioavailability probe) was to increase the number of

particle formulations and characteristics that could be compared, and indeed 55 nanomaterial variants were tested within NANORETOX. The biodynamic studies were complemented by longer term experiments with fewer particle types to verify probe results.

One of the challenges of traditional approaches to studying ecological risk was that short-term toxicity tests have severe tradeoffs that limit their applicability to natural conditions. The most important of these has been the necessity to use unrealistically high dose concentrations to elicit a response in abbreviated tests. The alternative has been to use longer term tests (≥28 days), but this approach greatly limited the number of tests that are feasible. NANORETOX applied the novel short-term biodynamic experiments to quantify the physiological characteristics that influence bioaccumulation by a species; thereby quantifying bioavailability in a manner that was directly comparable among species and among types of particles. In addition, the biodynamic parameters could be used to model the concentration of particle that would be bioaccumulated by each organism (and for each ENM) .

NANORETOX also considered the hypothesis that if the ENMs are bioavailable and reach potentially disruptive sites in cells then some cellular stress would be expected. Stress responses of aquatic organisms to ENMs were not well known, although oxidative stress was understood to be an important response of mammalian cells. For metal ENMs, oxidative stress, metallothionein synthesis, lysosomal destabilization5 and histopathology were expected to be important indicators that accumulated ENMs are inducing responses within the cells. But carcinogenicity, DNA damage and endocrine disruption could not be eliminated as possible contributory or alternative responses.

Project Results:

The NANORETOX consortium consisted of 11 partners from the EU (including 2 industry partners) and 1 US partner. The work was organised around three main research themes:

(1) synthesis, characterisation and reactivity,

(2) environmental toxicity and

(3) human/mammalian toxicity.

The main aim for work under theme 1 was to produce sets of well-characterised nanoparticles with properties that vary systematically, so that robust links to toxicity can be made. The main aim of theme 2 was to determine if ENMs are available for uptake by whole organisms and if the nature of the particle and/or the biological trait of the organism affect that uptake. The important challenge for theme 3 was to generate consistent and comparable data on human toxicity using a variety of models. A description of each partner involved in NANORETOX and their role in the project can be found at http://www.nanoretox.eu.

The NANORETOX project was divided into 8 technical workpackages and 1 project management workpackage. Each of workpackages 1-6 focussed on a specific aspect of the question of how ENMs interact with living systems in order to generate a robust set of nanoparticle characterisation and impact data, while workpackage 7 focussed on integrating all of the data generated into an cohesive whole to allow the NANORETOX team to answer the key questions of interest to the project, and to find commonalities among the different studies so as to maximize generalizations to other nanomaterials types and applications to risk assessment. The final technical workpackage (WP8) was dedicated to performing a formal assessment of nanoparticle risks based on the data generated, via the development of a conceptual model to guide evaluation of hazards and risks from nanoparticles.

NANORETOX was an extremely ambitious project that set out to identify the potential risks to the environment and human health posed by ENMs addressing a wide range of questions (presented below by WP), using a number of models and a variety of well constrained and relevant ENMs.

Results from WP1

The objective for WP1 was to synthesise and fully characterise a set of engineered nanoparticles with a range of physicochemical properties using industrial and laboratory methods

Why? To study how carefully controlled differences in physicochemical properties influence particle reactivity, bioavailability and toxicity and to see whether physical (e.g. size) characteristics override chemistry or vice versa. To generate engineered metal nanoparticles with properties that vary systematically, so that robust links to toxicity can be made.

WP1 produced a range of well-characterised sets of ZnO, CuO, TiO2, SiO2, Ag, Au and CdS/CdSe nanoparticles. Some of the particles were produced by the industry partners or purchased and some were tailor made in partners' labs to show properties that vary systematically, so that robust links to toxicity can be made. Reproducible protocols for all the syntheses have been developed and the produced sets of nanoparticles were thoroughly characterised and tested in a variety of (eco)toxicity experiments in subsequent WPs.

The NANORETOX ENMs were produced using a range of methods of synthesis (this being also a variable under consideration as a toxicity-influencing parameter) and were well characterised. The ENMs sets were tailored to display a range of physicochemical properties of interest. In order to establish that the nanoparticles tested by NANORETOX were representative of what is currently and in the future released in the environment, both top down (i.e. nanoparticles produced from bulk materials by milling) as well as bottom up (wet chemical, plasma and microfluidic synthesis) methods were used, so that many important routes of synthesis were represented. This 'in-house' 'tailored' synthesis was essential for materials of this nature, because unlike conventional chemical toxins (where a solution of a particular substance has the same properties regardless of the way it was produced or its source) nanoparticle properties can vary substantially depending on the method of synthesis and subsequent functionalisation. This approach was complementary to that of the OECD Working Party on Manufactured Nanomaterials, by placing emphasis on the controlled variation of properties. The particles tested were: TiO2, SiO2, ZnO, CuO, CdS/CdSe, Ag and Au.

The ENMs studied in NANORETOX were extensively characterised using analytical and biochemical techniques such as Inductively Coupled Plasma Mass Spectrometry (ICP-MS), Dynamic Light Scattering (DLS) and Zeta Potential Analysis (ZPA), Single Particle Tracing (SPT), Gel Filtration (GF), Fast Protein Liquid Chromatography (FPLC), Scanning Electron Microscopy (SEM), Transmitted Electron Microscopy (TEM), Atomic Force Microscopy (AFM) in both wet and dry mode, X-ray Diffraction (XRD) and surface area analysis (BET). Multicollector ICP-MS was used for analyses of labelled nanoparticle isotopic composition. Focused Ion Beam Scanning Electron Microscopy for visualising the nanomaterials produced

and their inner structure. X-ray Photoelectron Spectroscopy (XPS) and Time-of-Flight Secondary Ion Mass Spectrometry (ToF SIMS) for nanoparticle surface composition.

Highlights from WP1: Examples of the significance of characterization

Amino functionalized Au: In order to assess the effect of surface charge on the responses of organisms to nanoparticles, amine-terminated gold nanoparticles were synthesized using an existing methodology . The correct modification of the particle surface charge was verified by measuring the zeta-potential of the treated particles at pH values across the range from 2.7-8.5.

Amino functionalized SiO2: Since the amine-modified Au particles were made by a slightly different route than the negatively charged ones, there was room for concern that the different synthesis route may also factor into the different toxicities observed. Additionally, we were interested to see if any non-toxic nanomaterial could be made more toxic simply by adding a positive charge to the surface. Thus, we also made amine modified silica nanoparticles to help address some of these questions. In the case of the silica based materials functionalization of 50nm SiO2 and 50nm SiO2-Ru(bypy)3 by the use of 3-Aminopropyltriethoxysilane (APTS) was explored and optimized to produce materials with an amine functionalized surface. In this case the starting 50nm diameter particles were synthesized as previously described and then surface modified, allowing a direct comparison between the unmodified and modified variants. The effective change in surface composition resulting from treatment with APTS was confirmed by zeta-potential measurements, which show the clear shift of the isoelectric point of the particles surface from pH 4.5 to pH 7.5. The presence of amine groups was further verified by conducting X-ray Photoelectron Spectroscopy on layers of particles deposited onto a gold-coated silicon wafer. The chemical analysis of the particles show the large increase in the carbon and nitrogen content of the APTS treated materials compared to the unmodified starting material.

Outputs from WP1

Detailed outputs from WP1 are included in three deliverable reports (D1.1- Protocols for nanoparticle synthesis; D1.2 - Sets of well characterised nanoparticles for testing in WPs 2-6; and D1.3 - Guidance document to OECD Working Party on Manufactured Nanomaterials) as well as numerous publications. Protocols for nanoparticle synthesis and characterization developed within NANORETOX are available upon request, and have been already fed into a variety of ongoing EU FP7 funded projects, notably QualityNano, NanoValid and NanoMILE.

Results from WP2

Objective 2: To study the abiotic reactivity (transformations) of the synthesised ENMs in simulated environmental and biological media

Why? To monitor the behaviour of Me-ENMs in a range of simulated environmental (hard/soft freshwater, seawater) and biological (simulated body fluid, lung fluid, gastric fluid) media. To investigate if increased reactivity is an indication of potential for toxicity and whether this can then be used as a proxy for rapid hazard assessments. To couple this work with monitoring ENMs in the biological models below.

WP2 tested the stability of the particles produced in WP1 in a variety of conditions, with the aim to understand their behaviour in biological/environmental media as well as their overall physicochemical response to changes of conditions such as temperature, pH and ionic strength.

This was specifically in order to:

(1) select the optimum form and dose for in vivo and in vitro experiments;

(2) prioritise which sets of the synthesised nanoparticles to study; and

(3) elucidate nanoparticle behaviour in biological and environmental matrices.

Physicochemical properties that were specifically monitored included: solubility, surface charge, particle size and size distribution, agglomeration/dispersion, surface area, crystallinity and crystal structure.

Behaviour of nanoparticles in biological or environmental media was also monitored. It was anticipated that nanoparticles in some situations (particularly when present in concentrated suspensions) will tend to aggregate; however it is not clear whether aggregates, even when formed, behave like larger particles. Another important parameter under investigation was the stability (in terms of solubility and physical/chemical degradation) of the nanoparticles, to establish how their properties evolve in different media with time. Most physicochemical properties of the nanoparticles, notably size, composition, surface modification and even, in some cases, structure, may evolve with time. Abiotic reactivity studies of the nanoparticles were therefore carried out in media simulating environmental (hard/soft freshwater, seawater) and biological (simulated body fluid, lung fluid, gastric fluid) matrices. In these

series of experiments factors such as pH, temperature, ionic strength and the presence of organic ligands (of biological, e.g. proteins, or chemical, e.g. humic acids relevance) of the model media were investigated.

Major findings from this work include: a) dissolution results, which indicated increased ion release of metal ions from CuO and ZnO nanoparticles compared to their bulk counterparts, while TiO2 remains relatively insoluble; b) the effect of temperature which appears to be reversible, i.e. an increase in size is observed while the samples are heated, suggesting aggregation, but temperature decrease is sufficient to re-disperse the particles; c) the behaviour of nanoparticles in different media shows different patterns depending on the composition of the nanoparticles and the type of stabilisation; however a common feature is aggregation even at modest increases in ionic strength; the presence of organics, humic acid or albumin, also induces moderate aggregation.

Highlights from WP2: Protocols for storage and handling of ENMs

This document provides environmental, health and safety (H&S) information to researchers working with engineered nanoparticles, and should be incorporated as a standard operating procedure for this project, combined with H&S rules and regulations of each institution. Given the evolving knowledge base regarding health effects of nanoparticles, this document will be reviewed and if necessary updated throughout the project's duration.

In summary, a major exposure route for nanotechnology workers is through inhalation, resulting in respiratory absorption of airborne nanoparticles. Thus, whenever possible, nanoparticles are to be handled in a form that is not easily made airborne, such as in solution or on a substrate. Dermal exposure should be avoided by using gloves, especially on wounded skin. As with any material, ingestion can occur if good hygiene practices are not followed. Once ingested, some types of nanoparticles might be absorbed and transported within the body by the circulatory system. To prevent ingestion, eating and drinking and chewing gum are not allowed in laboratories and other designated areas.

Storage guidelines differ for each material and are provided individually with each delivery. Examples are shown in the relevant deliverable.

Outputs from WP2

Outputs from WP2 are included in three deliverable reports (D2.1 - Protocols for storage and handling of nanoparticles; D2.2 -Report on the abiotic reactivity characterization of CuO, TiO2, SiO2 and ZnO; and D2.3 -Report on the abiotic reactivity characterization of Ag, Au and CdS) as well as numerous publications. Protocols for nanoparticle storage and handling have been shared with NanoImpactNet (relevant deliverables developed in consultation between the two projects); ENM stability assessment developed within NANORETOX has also been fed into a variety of ongoing EU FP7 projects, notably QNano and NanoValid.

Results from WP3

Objective 3: To investigate in vivo uptake of ENMs by aquatic species and study mechanisms and paths of internalization in vivo.

Why? To determine if engineered metal ENMs are available for uptake by whole organisms and if the nature of the particle and/or the biological trait of the organism affect that uptake. To assess if selected aquatic species show stress responses when ENMs are accumulated, and to characterize the nature of those responses.

WP3 tested ENM bioavailability and toxicity on a selection of organisms: bivalve molluscs (Scrobicularia plana, Mytilus galloprovincialis, Macoma balthica), a gastropod (Peringia ulvae), two species of polychaetes (Nereis diversicolor, Capitella teleta) as representatives of the estuarine and marine environment and two freshwater species, the gastropod (Potamopyrgus antipodarum) and the zebrafish (Danio rerio, embryos), under in vivo conditions. All ENMs from WP1 were tested, and efforts were made to produce as representative results as possible from the entire ENM library. The work focused on assessing to what extent nanoparticulate metals are accessible for uptake into the tissues of organisms, quantifying the bioavailability of different types of ENMs and determining if bioavailable ENMs exert an adverse response within organisms. Tested ENMs were occasionally labelled with artificially enriched stable isotopes to quantify biodynamic uptake and loss characteristics. Bioaccumulation was modelled from biodynamics for a variety of particle formulations, characteristics and compositions. The biodynamic predictions were verified by longer-term experiments on fewer particle types. The distribution pattern of metal nanoparticles were compared with that of metals themselves, identifying target tissues for the toxic action of Me-ENMs.

Partners experimented with different organisms in order to compare implications of different biological traits. Microscopy techniques and subcellular fractionation of metals within organisms were used to assess the internal uptake and distribution of ENMs.

Oxidative stress, genotoxicity, metallothionein induction, DNA damage, lysosomal membrane destabilization histopathology and behaviour (burrowing, feeding rate) were used to represent important indicators of stress from metals. Nanomaterials themselves produce similar type responses, in vitro. If organisms show such responses to bioavailable nanomaterials, in vivo, it is unequivocal evidence that nanomaterial uptake causes the organisms to respond. Visual evidence of nanomaterials present internally, evaluation of internal dissolution and rigorous experimental design was used to determine if responses are due to internal dissolution of the metal oxide particle or due to disruption by the nanoparticle itself.

NANORETOX compared animal models that are likely to be sensitive to nanomaterials: animals that pump vast quantities of water across their gills (bivalves) or ingest plants or sediments where nanomaterials are likely to accumulate. NANORETOX used determinations of biodynamic characteristics, including rate of uptake from water, assimilation efficiency and rate of uptake from food, as well as retention (rate constant of loss), across several species and a range of particles. This screening approach (equivalent to a biological bioavailability probe) allowed a substantial number of particle formulations and characteristics to be compared. The biodynamic studies were complemented by longer term experiments with fewer particle types to verify probe results.

By quantifying ENM uptake using labelling, verifying the presence of ENMs in whole organisms, and observing responses at the whole organism level, the project assembled the lines of evidence necessary to determine if bioavailability and toxicity are feasible expectations for the metal nanoparticles.

Highlights from WP3: Example of the effect of CuO ENMs on the behaviour of H. diversicolor

For H. diversicolor, two different movements can be characterized:

(i) undulation associated with burrowing, occurring at 0.5 Hz frequency, and

(ii) head movement associated with feeding, occurring at 1 to 2 Hz.

No significant difference was observed after 7 days of exposure to both forms of Cu, whereas the two activities had significantly decreased for worms exposed during 14 days to soluble Cu and CuO NPs.

Results of feeding rates determined at day 14 showed a significant reduction in clams exposed to CuO NPs. In ragworms, no effect of Cu contamination was shown, indicating significant species-specific impacts of CuO ENMs.

Outputs from WP3

Outputs from WP3 are included in six deliverable reports (D3.1 - Protocols for in vivo ecotoxicity experiments; D3.2 - Results of sediment exposures with CuO for Potamopyrgus,with Au for Potamopyrgus and Capitella and preliminary results with Ag for Nereis; results of water exposure (medium-term experiments) with CuO and Au for Scrobicularia plana and Nereis diversicolor; preliminary results of water exposure (short-term experiments) with CuO for Mytilus and Danio; -preliminary results of biodynamic rates with Ag for estuarine snail Peringia ulvae and freshwater Lymnaea stagnalis; test use of particle labelled with an enriched stable isotope to trace bioavailability from diet in snail, L. stagnalis; D3.3 - Results of sediment exposures with CuO for Capitella, Nereis and Macoma; Results of water exposure (medium-term experiments) and food exposure with Ag for Scrobicularia and Nereis ; results of short-term experiments with TiO2 and Au and long-term experiments with CuO in Mytilus and Danio; results of longer water and food exposure with Ag for Peringia, results with Ag for Lymnaea; D3.4 - Results of sediment exposures with Au for Nereis and Macoma; with Ag for Potamopyrgus, Nereis, Capitella and Macoma'; results of sediment exposures with isotopically modified ZnO for Nereis diversicolor and Scrobicularia plana; results of short-term experiments with SiO2 and both short- and long-term experiments for Ag in Mytilus and Danio; results with Ag for Peringia; D3.5 - Results of sediment and food exposures with CdS, measured by ICP-MS for two species (to be selected based on results of 3.1-3.4). Results of medium-term exposures with CdS for Nereis and Scrobicularia; results of complementary experiments with NP selected following results of 3.1, 3.2, 3.3, 3.4 for Nereis; results of short-term experiments with ZnO and both short- and long-term experiments for CdS in Mytilus and Danio; results with ZnO and CdS for Peringia; D3.6 - Report of in vivo ecotoxicity results for all tested organisms/particles) as well a number of publications and MSc. and PhD theses. Protocols for in vivo ecotoxicity experiments developed within NANORETOX are available upon request.

Results from WP4

Objective 4: To investigate in vitro uptake and reactivity of ENMs and to discover putative mechanisms of toxicity by aquatic species primary cells.

Why? To determine cellular reactivity to, and possible uptake of engineered metal ENMs by aquatic species primary cell cultures. To assess if cell traits influence reactivity and bioavailability in vitro in aquatic invertebrate cells.

WP4 addressed the question whether ENMs induce responses that are indicative of a bioactive or potentially toxic material after the particle is taken into the cell of an organism. For example, ENMs could possibly be inert within cells, or detoxified by mechanisms in place to fend off foreign particles. In such a case no response would be expected by mechanisms that defend the cell against toxins. However, when a response was observed this was taken as evidence the nanoparticle represented a potential threat. Furthermore, responses known to be associated with metals or ENMs were considered. Understanding whether cells recognized and responded to ENMs, and how (mechanisms of response) could be efficiently and effectively addressed with in vitro cell cultures. Thus in vitro studies in this WP were complementary of the in vivo approaches in WP3 and their combination aimed to avoid false conclusions about risks from ENMs. Most importantly, in vivo (WP3) and in vitro (WP4) approaches were co-ordinated using the same aquatic organisms (mussels) and similar endpoints, thus linking interpretation of in vitro and in vivo responses.

In close connection to WP3, WP4 determined the in vitro effects of nanoparticles in primary cell cultures of mussel haemocytes and gill cells. Haemocytes or immunocytes comprise the main internal defence system in mussels. Effects on this cell type could reflect damage on the immune system, which could have consequences at higher levels of biological organisation, ie, individuals and communities. The in vitro experiments with mussel haemocytes and gill cells used the same selected set of particles as in in vivo bio-response studies (WP3). In addition to general toxicity tests (cell viability), the emphasis was on surveying a broad range of biological targets that could be damaged by nanoparticle exposure. The goal was to cover as many potential effects as possible in order to identify the most relevant biological targets. These included oxidative stress (superoxide dismutase SOD, catalase CAT, superoxide anion and hydrogen peroxide), apoptosis (tunel assay) and genotoxicity (Comet assay, micronucleus test, oxidative DNA damage). Further, specific tests for haemocytes (endocytosis, phagocytosis, damage to the actin cytoskeleton) and for gill cells (lysosomal enzyme activity, Na,K-ATPase, multixenobiotic resistance MXR transport activity) were also carried out. These studies, performed in parallel to those in WP3, allowed comparisons between in vitro and in vivo responses to ENMs in mussels. Further, as some of the tests used were identical to those used in human cells (WP5), this WP allowed some comparison of mechanisms between human cells and mussel cells.

Highlights from WP4: Cell viability (MTT assay) of mussel hemocytes exposed to Au NPs

The objective of this study was to assess cytotoxicity effects of Na-citrate stabilised Au NPs (4.7, 13.5 and 40.4 nm in size) suspensions on mussel hemocytes viability (MTT assay) and to compare cytotoxicity effects of NPs with ionic Au and bulk Au forms. Cytotoxicity of Na-citrate only was also assessed.

Isolated hemocytes were treated with 0, 1, 1, 10, 25, 50, 100 mg Au/L of Na-citrate stabilised Au NPs (4.7, 13.5 and 40.4 nm) provided as suspensions. Exposures were performed in parallel with their respective ionic Au ($HAuCl_4$, Sigma 254169) and bulk Au (Au <45 µm, Sigma 265772) forms and the cytotoxicity of the sodium citrate (Sigma 71406) was also tested. Cell viability was evaluated using MTT tests. Cytotoxicity of Na-citrate only was also assessed at the same concentrations present in the NPs suspensions (0.001 - 100 mgAu/L).

Au NPs showed relatively low toxicity to mussel hemocytes at tested concentrations. Na-citrate influenced the toxicity of Au NPs and may be the responsible for cytotoxic effects of Au NPs suspensions. Ionic Au was the most toxic Au form and bulk Au was not toxic for mussel hemocytes. No further details are presented here as the study is as yet unpublished.

Outputs from WP4

Outputs from WP4 are included in five deliverable reports (D4.1 - Protocols for in vitro ecotoxicity experiments; D4.2 - Preliminary results of bivalve cell viability with CuO and ZnO; D4.3 - Cell viability results with Ag, SiO_2, TiO_2 and CdS; D4.4 - Specific toxicity results for CuO, ZnO, Ag, SiO_2, TiO_2 and CdS; D4.5 Report on bivalve in vitro models to evaluate dose response and comparative toxicity between cells/organs relative to the physicochemical properties of nanoparticles) and are currently being prepared for publication; they have also formed part of PhD theses. Protocols for in vitro ecotoxicity experiments developed within NANORETOX are available upon request.

Results from WP5

Objective 5: To investigate in vitro uptake and reactivity of ENMs and to discover putative mechanisms of toxicity by primary mammalian and human cells and existing human cell lines.

Why? To determine cellular reactivity to, and possible uptake of engineered metal ENMs by mammalian/human cells and investigate if toxic responses occur. To assess if cell traits influence reactivity in vitro.

WP5 used in vitro models to examine cellular responses to ENMs. The work was based on the hypothesis that the cellular reactivity of the particles will critically depend both on the target tissue and the function of the cell type within that tissue. Cellular and molecular reactivity of selected metal ENMs were investigated in a) primary mammalian and human cells and b) in a panel of established human cell lines. A relatively wide range of tissue sources were covered, to include the lung, skin, immune blood cells, gut, kidney and liver.

Using in vivo models, it is becoming apparent that particles delivered via one system (e.g. lung) can reach, and have detrimental effects on, other body systems/compartments (e.g. vasculature). However, these studies utilise significant numbers of animals, are labour-intensive and are impractical for examining the comparative effects and mechanism of action of a panel of compounds. In NANORETOX we used in vitro models to examine cellular responses to nanoparticles; this approach was also in line with the 7th amendment to the EU Cosmetics Directive (European Commission, 2003; Council Directive 76/768/EEC) to avoid excessive animal testing.

We hypothesised that the cellular reactivity of the particles will critically depend both on the target tissue and the function of the cell type within that tissue. Thus, whilst some nanoparticles may be overtly cytotoxic, even at low levels, others may not, but they may adversely affect cell function, for example stimulating inflammatory mediator release or compromising epithelial barrier integrity. Conversely, the magnitude and profile of the cellular response will depend on the physicochemical properties of each type and format of particle and its exposure dose. This WP concentrated on Ag, TiO2, SiO2, ZnO, CdS (and test all different sets of nanoparticles synthesised), which were expected to have a broad range of activity for comparative purposes.

The work addressed the following questions:

1) Which cell types are most vulnerable to nanoparticle exposure?

2) Which cellular functions are affected?

3) Which mechanisms and cellular pathways are involved?

4) What is the cellular fate of nanoparticles?

5) Which physicochemical properties of nanoparticles render them more/less bio-reactive?

Highlights from WP5: Cytotoxicity of topically applied CuO NPs on human skin cell culture

This was a comparative study of micro-sized or nano-sized CuO powders on healthy and damaged skin. The particles were dispersed in double-distilled water and adjusted to 70 mg/ml CuO (7%). Suspensions were sonicated for 5 min before use. Topical treatment was performed by spreading 3µl aliquots at the centre of emerging epidermis, ensuring no leakage of applied materials into the culture medium. For 'systemic' treatment, 3µl of CuO nano- or microparticle suspension were mixed with 1 ml of DMEM and transferred to a culture well, to get a final concentration of 170 ppm (0.021%). Alternatively, a CuCl2 solution was added to the culture medium up to 2.7 mM final, in order to test the toxicity of dissolved Cu2+ ions at the same copper content (170 ppm) as CuO suspensions.

Tape stripping was performed on human skin explants using Scotch 3 M Magic tape (Scotch, Minnesota, USA), 19 mm wide and 30 cm long. The tape was applied to the skin, rubbed lightly to assure adhesion, and then pulled off with one fluent and decisive movement. This operation was repeated 30 times. This procedure compromises the skin barrier functions and allows penetration of substances through the upper layers of the skin.

The application of CuO ENMs had major effects on compromised skin: chromatin texture disappeared, presumably through nucleolytic digestion, and the nuclear membrane was disrupted or absent. Atypical folds and void areas appeared in place of intercellular contacts. The basal layer of epidermis split from the dermis, forming large blisters. Atypical organelles were seen in the cytoplasm of keratinocytes, sometimes resembling degenerated mitochondria, and sometimes lysosomal vacuoles. All these features indicate the occurrence of necrosis.

It seems reasonable to propose that topically applied NPs may be captured by the stratum corneum (SC) at accessible sites, as seen in some histological slides. Immobilization can take place at deeper levels when the SC has been superficially removed by tape stripping. There, entrapped particles experience a more acidic environment suitable to copper dissolution. They can generate soluble Cu2+ ions which may penetrated cultured cells and were subsequently included in lysosomes. In skin, CuO NPs may constitute immobilized sources of toxic soluble compounds that can diffuse toward inner tissues. Their exposition at the skin surface, as well as their partial dissolution, would make them easy to remove during sample processing. This could explain why they most often escape microscopic observation. Although this model accounts for all experimental data, more experiments are obviously needed to demonstrate its mechanism.

Outputs from WP5

Outputs from WP5 are included in five deliverable reports (D5.1 - Report on in vitro models of the dose response and comparative cellular reactivity and toxicity between cells/organs relative to the physicochemical properties of nanoparticles; D5.2 - Preliminary results of in vitro tests with CuO; D5.3 - Results of in vitro tests with TiO2 and Au; D5.4 - Results of in vitro tests with remaining priority particles; D5.5 - Report on specific cellular mechanisms and functional processes involved in particle reactivity); some results have been published, whereas further publications are currently prepared; they have also formed part of PhD theses. Protocols for in vitro ecotoxicity experiments developed within NANORETOX are available upon request.

Results from WP6

Objective 6: To consider the genotoxicity and carcinogenicity of metal ENMs

Why? To use emerging molecular tools to determine whether engineered metal nanoparticles can induce changes in DNA that might impact on health.

WP6 investigated whether metal nanoparticles possess genotoxic and carcinogenic potential; specifically:

1) Do nanoparticles induce cytogenetic changes and formation of micronuclei?

2) Do nanoparticles cause damage at the DNA level?

3) Do nanoparticles interfere with cell proliferation?

4) Do nanoparticles induce cell transformation?

5) Do the genotoxic effects of nanoparticles vary between individuals and between species?

6) Which physicochemical properties of nanoparticles render them more/less genotoxic?

Occupational and environmental exposures to metals are associated with the development of various pathologies, including cancer; however, the mechanisms of action, especially at the molecular level, are still unclear. It has been shown that exposure to toxic metals may be induced not only by absorption in micro-molecular form but also as NPs. Although metal ENMs have been demonstrated to cause pathological responses, the mechanisms of toxicity

remain explained. Metal-mediated formation of free radicals, reactive oxygen species (ROS) and reactive nitrogen species (RNS) can cause various modifications to DNA bases, enhanced lipid peroxidation, and changes in calcium and sulphydryl homeostasis and evidence indicates that such ROS and RNS play an important role in the aetiology of a number of diseases, in particular neurodegenerative pathologies and cancer. Previous studies on human peripheral lymphocytes, show DNA damage and suggest that some metal nanoparticles might be genotoxic and therefore have carcinogenic potential; one important mechanism involves increased oxidative stress.

The chosen cell models in this WP were fully characterised and were based on human leukocyte cultures (obtained from healthy volunteers), and on cell lines relevant to occupational and environmental exposure. The A549 (human lung epithelial) cells modelled the inhalation processes and the RAW264.7 murine macrophage cell line modelled the inflammatory process. In vivo studies concentrated in zebrafish liver as this small tropical fish species is a well-known model for hepatocarcinogenesis. In addition, possible carcinogenic effects were also studied in mussels, where haemic or haemocytic neoplasia and gonadal neoplasia have been reported.

Highlights from WP6: Long-term experiments with CuO in mussels (Mytilus galloprovincialis)

This study was shared between WP3 and WP6 (deliverable D6.2), since animals exposed for 3 weeks were afterwards maintained in clean water up to 5 months in order to investigate the potential long-term genotoxic and carcinogenic effects.

Regarding genotoxic effects, micronuclei (MN) frequency in mussels exposed to CuO NPs for 21 days was significantly higher than in control mussels. Temporal differences were only observed in this treatment, since mussels exposed to CuO NPs for 21 days presented significantly higher MN frequency than mussels maintained in clean water for 122 days. At 63 days of recovery after exposure, MN frequency in mussels previously exposed to bulk CuO was significantly higher than in the control group. After 122 days of recovery, mussels previously exposed to ionic Cu showed significantly higher MN frequency than control and mussels exposed to CuO NPs.

Apart from MN, other cellular alterations were seldom seen in haemocyte preparations. Disseminated neoplasia was found in one individual exposed to CuO NPs sampled after 63 days recovery. No further details are presented here as the study is as yet unpublished.

Outputs from WP6

Outputs from WP6 are included in five deliverable reports (D6.1 - Report on standardized protocols for testing critical endpoints (cytotoxicity, genotoxicity, carcinogenic potential; D6.2 - Preliminary genotoxicity test results for CuO; D6.3 - Genotoxity and carcinogenesis test results with TiO2 and Au; D6.4 - Genotoxicity and carcinogenesis test results with remaining priority particles; D6.5 - Report on specific cellular mechanisms and functional processes involved in particle reactivity) as well as some publications and PhD theses; further publications are currently in preparation. Protocols for in vitro genotoxicity and carcinogenicity and and ecotoxicity experiments developed within NANORETOX are available upon request.

Results from WP7

Objective 7: To determine whether cellular responses between human cells, mammalian cells, cell lines and invertebrate cells or whole organisms are comparable or different with relevance to screening models

Why? To assimilate the data from the previous objectives to discover whether there is a hierarchy of nanoparticle reactivity and uptake. To determine which physicochemical characteristics of the nanoparticles confer toxicity and/or predict internalisation and in which models.

WP7 has been using data collected in all previous WPs, this WP compared species, particles of differing nature, as well as human and aquatic organism responses. All datasets were brought together into a unique 'global table'. The WP dedicated specific effort to finding commonalities among the different studies so as to maximize generalizations and applications to risk assessment. For example, many properties of cells are biologically conservative: that is, many similar mechanisms characterize the functioning of cells of all life forms. If there are commonalities in the way humans and other organisms react to nanoparticles then universal methods might be developed to both detect and better understand nanorisks.

Specific questions addressed were:

1) Do organisms differ from humans or among species in their stress responses and/or sensitivity?

2) Can we use abiotic reactivity to predict toxicity?

3) Is in vitro dose response to metal nanoparticles indicative of in vivo responses?

4) Is there a pattern of cellular reactivity and/or toxicity related to physicochemical properties, i.e. a hierarchy of activity?

As described above, the data are from studies conducted in 12 different laboratories (the European Union Framework 7 project NANORETOX) all focused on the same suite of Me-ENMs to control inconsistencies. The result is a 'macroarray' of bioavailability and toxicity testing results with physical, biological and ecological dimensions. From this array models are developed that link abiotic reactivity to biological effects and compare how differences in physicochemical properties influence the biological implications of Me-ENMs across different types of organisms (in vitro and in vivo) and mammalian (including human) cell types. An important goal is to define which attributes of ENMs play the most important role in toxicity and to evaluate if there is a common definition of 'relative toxicity' across an array of toxicity testing protocols.

Generalizations about environmental and health implications of Me-ENMs are impeded by the challenge of experimenting with a broad and complex spectrum of ENM properties, poor knowledge of the mechanisms by which adverse effects may occur, and interpreting a vast array of different testing approaches to evaluate toxicity. At the first level of complexity nano-size reactivity as distinct from reactivity of the ionic or bulk forms of an element may affect implications of a Me-ENM. But composition, size, shape, capping agent, crystal structure and surface properties of each Me-ENM, for example, can be broadly categorised as nano-specific properties that are also influential. The variety of synthesis methods may result in inconsistencies in these properties from ENM-to-ENM. Once released to the environment, the behaviour of the ENM in the exposure media can lead to surface modifications, structural/chemical transformations, redox reactions, dissolution and/or hetro- and homo-aggregation all of which could influence environmental implications. Finally, biological reactivity may vary from test-to-test and can depend on the species, cell type, exposure model, dose and endpoint. The greatest single knowledge gap is limited understanding of the importance of any one of these influences relative to others.

Outputs from WP7

Outputs from WP7 are included in one comprehensive deliverable report (D7.1 and D7.2 - A model linking NP physicochemical properties with biological effects across different types of organisms/humans / A model linking abiotic reactivity to biological effects) integrating all of the project findings, which is also being prepared for submission as a peer review

publication. The deliverable describes a model linking NP physicochemical properties with biological effects across different types of organisms/humans and a model linking abiotic reactivity to biological effects.

Results from WP8

Objective 8: To establish universal approaches to risk assessment model and risk communication

Why? To develop a universal set of criteria to define hazard and risk from nanomaterials and a similar set of standards for communicating risks. To maximise the practical application of study results and assist regulation/legislation. (WP8)

Risk assessment: Ultimately a formal assessment of nanoparticle risks is essential. NANORETOX, in one study, addressed multiple nanoparticle formulations, in multiple media, using multiple species (including humans) and employed in vitro and in vivo approaches. The goal of WP8 was to incorporate this broad set of data from a single study into a risk assessment. Though there is increasing attention toward studying human health risks from nanoparticles, a common framework for conducting risk assessments is lacking. Information on environmental risks associated with nanoparticles, and particularly metallic nanoparticles, is scarce. An important outcome of the project was the development of a conceptual model to guide evaluation of hazards and risks from nanoparticles. The model was developed to be applicable to the body of evidence that will surely grow quickly as knowledge of nano-materials grows.

Risk communication: The profile of nanotechnology and any associated risk is high in the media; so inadvertent mis-communication is possible. Another goal of NANORETOX was to develop a risk communication strategy that guided project communications, and contributed in advising recipients of the project's results (government, industry) communicate risks in a balanced, robust manner. It is essential to 'get the risks from nanoparticles right' because the technology offers many potential benefits. The costs of over-stating or under-stating risks could be high. Although general risk assessment procedures are well known, there are many unique attributes of nanoparticles that required new or adjusted methodologies. Communicating new results in an unbiased, balanced and value free way is critical to public credibility. Communicating risks appropriately also requires a holistic view of the issues, as well as a careful, rational and transparent approach.

Outputs from WP8

Outputs from WP8 are included in four deliverable reports (D8.1 - A conceptual model to guide both hazard evaluation, and risk assessment for human health and environment; D8.2 - Guidelines for nanotechnology workers; D8.3 - Formal risk communication strategy; D8.4 - Workshop series). At least one publication (risk assessment) is planned from this WP.

Key conclusions from NANORETOX

NANORETOX has created a large and significant set of (eco)toxicity findings. The importance of the produced work lies in the fact that the same sets of nanoparticles were studied in a variety of in vitro and in vivo models; nanoparticle properties (dry and/or in suspensions) were monitored to ensure no changes occurred during experimentation and thus to give confidence to the toxicity studies. This has created a very important internally consistent and reproducible set of data from which a number of important conclusions have been drawn, which are summarized as follows:

Conclusion 1. In general, NANORETOX data refute the hypothesis that elemental composition of an ENM does not determine its toxicity. A relatively consistent ranking of toxicity based upon composition was possible among 9 different testing approaches. Cu- and Cd-ENMs were consistently the most toxic (although testing of CdS was limited). Results from both in vitro and in vivo tests, human cells, mouse cells, mussel cells and zebra fish consistently ranked ENMs containing these two elements among the top three in terms of toxicity. ENMs composed of SiO_2 were the least frequently toxic and ranked the lowest in toxicity wherever tested. ENMs composed of TiO_2 also were usually of low toxicity except to mussels; similarly Au-ENMs were often ranked among the lowest toxicity and never were ranked the highest. ENMs composed of Zn and Ag were more difficult to generalize. Ag-ENMs were the most toxic of any in some tests (invertebrates, zebra fish, some mammalian cells) and the least toxic in other tests (human skin; human lung cells). The toxicity responses were similarly diverse with ZnO-ENMs. These were the most toxic products to human lung and skin cells, but only moderately toxic to animals. When responses to the different tests were compared systematically, it was clear that composition was the most important property of ENMs determining toxicity ranking.

Conclusion 2. The higher chemical reactivity of smaller particles and/or the larger number of particles per unit mass can have important influences on toxicity. Dramatically stronger effects at lower particle size do occur with some tests and with some ENMs. For example, the smallest sized maltose capped Ag-ENM, which fell in the range of exponentially

increasing chemical reactivity, was more than an order of magnitude more toxic than larger particles to zebra fish development. In mouse embryo fibroblasts, chromosomal damage and comet assay results correlated better with number of Ag-PVP particles than with Ag concentration but cell toxicity did not.

Conclusion 3. Importantly, in no case did particle size cause a change in the ranking of toxicity among ENMs, based upon composition. For example, Ag-ENMs were always more toxic than Au and SiO2 to mussel cells no matter what the particle size.

Conclusion 4. The particle size dependence of toxicity was not a universal result. For example, the relative toxicity of Ag-mal ENMs to mussel cells, zebra fish survival and zebra fish hatching increased with smaller particles, but particle size had no effect on Ag-mal toxicity in mouse embryo fibroblasts, mouse macrophage cells or in human lung and skin cells. Nor were instances observed where no toxicity occurred from particles larger than 30nm but particles smaller than that size were of high toxicity (e.g. mortality in polychaete worms or mussels in vivo; human lung cells; human skin cells).

Conclusion 5. Larger particles can be more toxic than smaller particles under some circumstances. The single dose, longer terms studies with clams and polychaete worms compared Au-ENMs of 5, 15 and 40 nm size at 100 µg/L in seawater. Bioaccumulation of 40 nm Au-ENMs was significantly greater than bioaccumulation of Au from 5nm particles in both clams and worms. Particles of larger initial size (15 and/or 40nm) also had greater effects on burrowing, metallothionein induction and oxidative stress (SOD, CAT) than did exposure to the 5 nm particles in clams and induced more CAT in worms. Dissolution was not a factor in these experiments but differences in aggregation in seawater were a possible influence in the high ionic strength seawater.

Conclusion 6. Biology can be important. Greater bioaccumulation of larger Au particles in the single dose experiments suggests larger particles were more accessible to the organisms than smaller particles. Greater oxidative stress in clams (Scrobicularia plan) than in polychaetes (Hediste diversicolor) suggested this effect was more important for molluscs. Similar results were observed in dose response tests comparing Au bioaccumulation in H. diversicolor and a different clam, Macoma balthica exposed to Au-ENM-spiked sediments. Higher bioaccumulation from larger particles was observed in the clam than the worm. Facultative filter feeding animals, especially, retain particles within set size ranges determined by species-specific factors such as morphology.

In some animals larger particles may be more easily retained and therefore elicit greater responses than the smallest of ENMs. This speaks to the challenge of relying upon simple single explanations (e.g. chemical reactivity) for risks that may be influenced by multiple processes, including processes specific to the organism being studied.

Conclusion 7. NANORETOX data demonstrates that a comparison of risks among Me-ENMs of different characteristics is feasible, but it requires coordinated study among multiple laboratories from multiple disciplines. This means the large multi-national, multidiscipline grants funded by institutions such as the EU Framework 7 process are the best way (and perhaps the only way) to address comparative risks from ENMs if appropriately coordinated. Although NANORETOX is just a beginning, it shows that an array of tests (in vitro and in vivo; mammalian cell lines and sublethal animal tests) is the most convincing for defining the most important factors driving relative risks or toxicity.

Conclusion 8. NANORETOX has shown that controlled synthesis of well characterized ENMs can reduce uncertainties about the cause of outcomes of the toxicity tests. Such multi-discipline projects studying the same well controlled particles can at least begin to point toward Me-ENM properties that raise or lower the potential for toxicity to both humans and animals and toward specific types of risk that might otherwise be missed.

Potential Impact:

The impact of NANORETOX will come from:

[1] The project's unique combination of fully characterized sets of metal ENMs whose physicochemical properties, reactivity and toxicity were assessed in detail with bioavailability and effects studies using a variety of biological receptors and methods of exposure and assessment. The overall work was anticipated to create an unparalleled set of data in magnitude and scope, which is directly contributing to a better understanding of the impact of increasing use of metal ENMs on health, safety and the environment.

[2] The project's critical mass of data (direct and indirect, i.e. through synergistic projects) that allow establishment of unambiguous toxicity information and thus directly support and form the basis of future risk assessments and potential regulations and legislation. Work for NANORETOX was carried out in the context of other national, international and EU-funded research. The data generated have been captured in a manner that (as far as possible) ensures that it is compatible with existing EU databases (from other running FP projects).

[3] The project's different methodologies of assessing exposure, bioavailability and toxicity, which allowed identification of the most effective methodologies for diagnostic use. NANORETOX identified best approaches, thus generating new improved in vitro and in vivo methodologies suitable for the regulatory needs for the safety assessment of nanotechnology products.

[4] NANORETOX's new approach in toxicity tests across species that generated more comprehensive and sensitive indicators of toxicity, particularly to environmental receptors, and created a useful tiered risk assessment tool that industry and regulators can use to assess current and future ENMs. This tool may make a significant contribution to the establishment of new guidelines for the sustainable and responsible development of nanotechnologies.

[5] The project's evaluation of health considerations for nanotechnology workers; guidance notes based on its findings with emphasis on applying the broad understanding emerging from the research to a revision of specific protective measures, where such are necessary. NANORETOX generated a series of guidelines to such workers (WP8) to ensure safe and cost-effective minimization of their exposure to ENMs, in collaboration with other relevant EU projects (NanoImpactNet).

[6] NANORETOX's datasets and methodologies developed by NANORETOX have been made extensively available in the scientific literature and at conferences in order to support other future research. A number of new projects have directly benefited from NANORETOX data and methodologies (notably ModNanoTox, NanoValid, QualityNano, NanoMILE).

[7] NANORETOX's risk communication strategy, which aimed to facilitate regulatory and legislatory application/enforcement of the work, and which could be directly incorporated into future definitions of appropriate measures should these become necessary.

[8] The project's contribution to the implementation of the European Commission's Action Plan for Nanotechnology which stated in section 1.3: 'It is essential that the aspects of risk are addressed upfront as an integral part of the development of these technologies from conception and Research and Development (R&D) through to commercial exploitation, in order to ensure the safe development, production, use and disposal of products from nanotechnology. Nanotechnologies present new challenges also for the assessment and the management of risks. It is therefore important that, in parallel with technological development, appropriate Research and Development (R&D) is undertaken to provide quantitative data on toxicology and ecotoxicology (including human and environmental dose response and exposure data) to perform risk assessments and, where necessary, to enable risk assessment procedures to be adjusted. '

[9] NANORETOX's involvement in the EU's Strategic Research Agenda, where the project contributed an ecotoxicological angle to the document.

[10] The project's involvement of industry (2 SMEs) in the partnership, which allowed a pragmatic and relevant focus throughout the work produced.

NANORETOX contribution to science

NANORETOX has resulted in over 20 high quality, high impact publications across the whole range of relevant subject areas, including nanoparticle synthesis and characterisation, behavior and transformation in environmental conditions, and interaction with and impact on a wide variety of aquatic and sediment species in a variety of acute and chronic tests. Many of the approaches and protocols developed have already been incorporated into other national (e.g. NERC FENAC, DEFRA, EPSRC) and international (e.g. PROSPeCT as part of the OECD sponsorship programme for nanomaterials), as well as other EU programmes such as the QualityNano research infrastructure. Further publications are currently in preparation.

H-index for 'NANORETOX' is already 5 (=5 of the NANORETOX publications have been cited 5 or more times), and this will continue to grow with time.

NANORETOX contribution to standardisation

NANORETOX has generated sets of standard reference nanoparticles, which will be offered to OECD/JRC for consideration as reference materials. Toxicological protocols developed for NANORETOX may also be appropriate for OECD consideration and have been placed in a

report that will be delivered to OECD. Finally a number of NANORETOX protocols have een delivered to project NanoValid and are currently tested through round robins for validation and standardization.

NANORETOX contribution to ongoing EU research activity

A key output from NANORETOX is the unique and extensive range of datasets that was produced, and as well as the dedicated and focussed effort to find commonalities among the different studies so as to maximize generalizations and applications to risk assessment. These datasets are a unique resource in Europe, and as such they are being fed into a number of subsequent projects, in order to extract additional value from the research effort, and to provide a firm foundation for the next generation of research studies. Among the projects that are utilising NANORETOX data, or building on approaches generated within NANORETOX are:

ModNanoTox

ModNanoTox (start date November 2011, duration 2 years) is a modelling-based project that aims to develop a number of well-documented and technically advanced models describing the behaviour of engineered nanoparticles in an environmental or biological context.

ModNanoTox is a relatively small project, with no experimental component to it, and therefore critically depends on data from other projects. ModNanoTox is coordinated by the NANORETOX coordinator, and as a result the data sharing is a natural process. Indeed, ModNanoTox was designed based on the potential to use outputs from NANORETOX, both in terms of expertise and data interpretation/availability, and this has been successfully implemented. The NANORETOX data is also being complemented with literature data, further strengthening the data base and the modelling and predictive potential of the NanReTox outputs.

NanoMILE

NanoMILE (start March 2013, duration 4 years) intends to establish a fundamental understanding of the mechanisms of nanomaterial interactions with living systems and the environment, across the entire life cycle of nanomaterials and in a wide range of target species. The project will identify critical properties (physico-chemical descriptors) that confer

the ability to induce harm in biological systems. This is key to allowing these features to be considered in nanomaterial production ('safety by design').

The overarching objective of NanoMILE is thus to formulate an intelligent and powerful paradigm for the mode(s) of interaction between manufactured nanomaterials and organisms or the environment to allow the development of a single framework for the classification of nanomaterial safety and the creation of a universally applicable framework for nanosafety.

NanoMILE is based on many of the same principles as NANORETOX, including the need for systematically varied sets of nanoparticles, and the need for assessment across a range of species and using multiple end-points. NanoMILE goes further however, focussing on mechanisms of interaction, and including also a systems biology approach to potentially identify biomarkers of nanoparticle exposure and/or impact.

NanMILE is coordinated by the NANORETOX coordinator, ensuring that the lessons learned from NANORETOX are carried seamlessly into NanoMILE, and allowing the NanoMILE project to get up to speed and full functionality rapidly and effectively.

Feeding into NanoValid, MARINA and QualityNano

NanoValid and MARINA are two large Seventh Framework Programme (FP7) projects, started in 2012 and dedicated to the development and implementation of reliable reference materials and methods for nanosafety assessment. There has been a direct uplift of NANORETOX knowledge and approaches for stable isotope-labelling of nanoparticles to facilitate tracking and detection of engineered nanoparticles, especially in complex environmental scenarios, where the large amount of background particles can make detection by conventional methods challenging. The NANORETOX coordinator, is a partner in NanoValid. Knowledge is also being fed into the MARINA project: JRC at ISPRA are the main linkage between NANORETOX and MARINA.

QualityNano is an infrastructure project, where expertise developed in NANORETOX, notably nanoparticle detection, characterisation and stable istope labelling are directly fed into all three project strands: access, networking and research.

Feeding into the NanoSafety Cluster

NANORETOX have been an active partner in the EU NanoSafety Cluster, the grouping of EU-funded projects addressing nanosafety topics, since its initiation in 2009, despite not having a formal budget for participation in NanoSafety Cluster events (as NANORETOX had started prior to the establishment of the NanoSafety Cluster). NANORETOX presented data at several NanoSafety Cluster brokerage events, including at Nanosafety Cluster Brokerage event / SIINN workshop "Safe implementation of nanotechnologies: common challenges" in Grenoble from 29-31st May 2012. In addition, NANORETOX has contributed to every edition of the NanoSafety Cluster compendium of projects to date, giving annual updates on progress and outputs.

Key resources developed within NANORETOX

Several key concepts and approaches were pioneered within NANORETOX, and highlights of these are provided below. The examples presented here provide a clear indication of the project's impact on the state of the art in nanosafety.

Stable isotope labelled nanoparticles and their applications

A key outcome from the NANORETOX project has been the application of stable isotope labelling to the tracing of engineered nanoparticles in an environmental context (e.g. in vivo exposures). An example includes the stable-isotope labelling of zinc oxide nanoparticles, among others. Zinc (Zn) was chosen as an exemplar particle, as due to the high levels of Zn present naturally in organisms and the environment (water and sediments), it is appropriate to use a labelling technique, such as stable isotope labelling, to separate Zn derived from ZnO NPs from pre-existing Zn. Thus, it is appropriate to work with isotope to separate Zn from NPs from natural Zn. ZnO NPs isotopically enriched (89.6%) with a rare isotope of Zn (67Zn, natural abundance of 4.1%) were synthesized by a NANORETOX partner, and tested by several other partners.

Indeed this approach is so valuable that it has also been picked up immediately after its inception for further trials in other projects. A variant of the ZnO nanoparticle labelling approach involved the use of a more abundant (therefore lower cost) Zn isotope (68Zn) using a more sensitive detection technique (multi-collector ICP-MS). In addition, in that work, novel bioimaging techniques were used to characterize parallel water-borne exposures of the common mudshrimp Corophium volutator to 68ZnO NPs, bulk 68ZnO, and soluble 68ZnCl2 in the presence of sediment. C. volutator is an important component of coastal ecosystems where river-borne NPs will accumulate and is used on a routine basis for toxicity assessments. The results demonstrate that ionic Zn from ZnO NPs is bioavailable to

C. volutator and that Zn uptake is active. Bioavailability appeared to be governed primarily by the dissolved Zn content of the water, whereby Zn uptake occurs via the aqueous phase and/or the ingestion of sediment particles with adsorbed Zn from dissolution of ZnO particles. The high sorption capacity of sediments for Zn thus enhances the potential for trophic transfer of Zn derived from readily soluble ZnO NPs 14.

A Global Reactivity Table

As part of the project deliverables, a global reactivity table was constructed that contains details on the source and key features of each ENM tested (including laboratory where ENMs were synthesized) as well as details on their abiotic reactivity. This was presented in detail in Deliverable 7.1.

The table shows that commercial ENMs were also purchased for most types as a comparison with the experimental ENMs. Special attributes that might affect toxicity were compared where relevant. For example, a variety of particle sizes were tested for each type of ENM, and some notable examples were described above. The significance of this table, in terms of impact, is that it provides a rigorous, self-contained and well established summary/database of the extensively researched NANORETOX data. Furthermore, it provides a model for data collection and management in nanosafety.

Leading to a Global Heat-Map of Toxicity

In order to compare the responsiveness of different toxicity tests, 13 categories of tests were employed to study the various ENMs. The details of every protocol are presented for external users in D1.3 (OECD guidance document).

In vitro, 10 different mammalian cell lines and two invertebrate cell lines were employed and stress was determined by dose response in 31 different tests, combining cell line, category of test and type of response.

In vivo 9 different aquatic species were tested; some by exposure via both water and food. In vivo stress was determined by dose response in 30 different tests combining species, category of test, route of exposure and type of stress. In order to test responses to ENMs under more realistic conditions longer-term (21 day) experiments were conducted at one environmentally realistic dose

The composition of Me-ENMs strongly affected toxicity in all NANORETOX tests. There were also many consistencies among tests in the ranking of toxicity, as demonstrated by the generation of a heat-map. The heat-map is a conceptual model based upon NANORETOX data, showing the relative toxicity of ENMs of different composition and the window of toxicity for each driven by factors such as test organism or cell line, particle size, shape, aggregation potential or dissolution potential. The heat-map was presented in deliverable 7.1 and is currently in preparation for publication in the peer-reviewed literature. The significance of this heat-map, in terms of impact, is that it presents a simple, coherent and easy-to-understand (and demonstrate/explain to a lay audience) visual model of toxicity, which could make a valuable contribution to the risk assessment efforts in nanosafety.

Ranking of toxicity among ENMs

In general, NANORETOX data demonstrate that elemental composition of an ENM determines its toxicity. A relatively consistent ranking of toxicity based upon composition was possible among 9 different testing approaches.

Cu- and Cd-ENMs were consistently the most toxic (although testing of CdS was limited). Results from both in vitro and in vivo tests, human cells, mouse cells, mussel cells and zebra fish consistently ranked ENMs containing these two elements among the top three in terms of toxicity.

ENMs composed of SiO_2 were the least frequently toxic and ranked the lowest in toxicity wherever tested. ENMs composed of TiO_2 also were usually of low toxicity except to mussels; similarly Au-ENMs were often ranked among the lowest toxicity and never were ranked the highest.

ENMs composed of Zn and Ag were more difficult to generalize. Ag-ENMs were the most toxic of any in some tests (invertebrates, zebra fish, some mammalian cells) and the least toxic in other tests (human skin; human lung cells). The toxicity responses were similarly diverse with ZnO-ENMs. These were the most toxic products to human lung and skin cells, but only moderately toxic to animals.

The significance of this ranking, in terms of impact is that it presents a generalised concept of nanotoxicity, based on compositional aspects. This could be valuable to both risk assessment and the development of further models (e.g. QSAR type models).

Use of positive and negative controls in all impact assessments

In vitro tests with lung cells in NANORETOX addressed this question by employing both a negative control (no exposure to the Me-ENM) and a positive control. In the positive control metal salt was injected into the cell at the given concentration under the implicit assumption this would generate the maximum possible response.

Where ENMs were toxic the results typically fell between the two controls. The concentration of Me-ENM at which an effect occurred was always greater than the concentration of metal salt that caused that response. In no instance was there greater toxicity from the injected ENM than from the injected metal salt; but the degree of effect seemed related to how much of the Me-ENM dissolved within the cell.

These comparisons are valid for the circumstance in which an equivalent concentration of metal reached the interior of the cell. This may not be reflective of the concentration of the two in the environment exterior to the cell because environmental transformations (e.g. geochemical metal speciation) will affect metal salt and perhaps ENM uptake.

The primary 'nano-effect' was delivery of the nano-packet of metal into the cell, and the maximum effect occurred when that packet released all of its metal.

An approach for normalising dose response

One of the difficulties in quantitatively comparing toxicity among different kinds of studies is the different range of doses employed. Within NANORETOX, we tested whether generalizations about comparative toxicity are possible based upon metal concentration.

A second issue is that differences in dose-response are an inherent function of legitimately different approaches to assessing toxicity. Different tests have different sensitivities and different pragmatic requirements. Well run toxicity assessments bracket concentrations expected in realistic circumstances. But both the circumstances and the demands of the testing environment also influence the range of concentrations tested.

For example, the range of concentrations employed in in vitro tests are justified on a physiological basis: what concentrations might be expected within the tissue of choice. On the other hand experiments conducted with aquatic animals in moderately hard freshwater, for example, bracket concentrations expected in contaminated circumstances in nature. These are orders of magnitude lower than physiological concentrations. In addition, toxicity testing sometimes requires trade-offs between time and dose; exposing the experimental subject to high doses for shortened time periods in order to elicit a response in a practical time span. Mandating one range of doses to achieve comparability between two entirely different types of tests will not improve comparability; it will simply eliminate some important pieces of information.

Comparability requires that methods are found to normalize the legitimate dosing differences. To assemble a comparable macroarray of results, the different dose-response regimes were addressed here by scaling each result to a similar standard. Each partner that assessed toxicity over a range of exposure concentrations completed a table that summarized their dose-response studies. In nearly all cases the concentrations ranged from below where effects occurred to those required to achieve at least a 50% effect. Each row in the table showed a specific particle, the test, the doses employed, and the dose at which a standardized effect was observed (usually the concentration at which a 50% response occurred). Results for aqueous metal (ionic) and bulk forms were included as well.

The goal of normalizing was not to identify concentration thresholds of toxicity but to achieve comparability by creating a common scale of results based upon the range not the absolute concentration. To accomplish this, the particular range of doses for each test was scaled into four bins, labeled very high (VH), high (H), moderate (M) and low (L) toxicity. For example, most in vitro studies tested for responses within the range 0.1 through 100 mg/L. The bins for these tests were 0.1 - 1 = VH; 1 - 10 = H; 10-40 = M; 40 - 100 = L. For environmental tests, which varied more widely among tests, a similar partitioning across exposures was employed.

The letter ranking system was constant, but the doses defining each letter differed with the test. Hypotheses were addressed across tests by simply counting the number of results in each bin from all tests that considered a given hypothesis or question. To address some of the subquestions that required greater sensitivity, bins were broken into sub-bins labelled 1, 2 and 3. To assess the severity of an effect, each measure of response was categorized as determining either: (A)ctivity = bioreactivity, bioaccumulation, defense mechanisms, mediators and cytokines; (B)iomarkers of sublethal damage and (T)oxicity defined as cell or organism mortality.

There appear to be important effects that are specific to the exposure being via a nanoparticle. There are also many instances in the NANORETOX data where simple dissolution of the metal toxin from the ENM cannot explain results. Relative toxicity does not follow tendency to dissolve. Where ENMs are ingested or where tests address sensitive endpoints, instances exist where ENM toxicity exceeds aqueous metal and certainly bulk metal toxicity. These instances suggest nano-specific effects are likely. The size of many ENMs is consistent with natural particles sizes that cells and organisms experience in nature (viruses) or even seek to accumulate (micelles). But the nature of ENMs is unprecedented in nature (a packet of potential toxins unaffected by geochemical speciation). It is not surprising that toxicity can occur via 'accidental' attachment to membranes or uptake of these potentially toxic packets. Across an array of NANORETOX tests there are enough instances of greater toxicity or unusual toxicity to suggest that these unique characteristics constitute a hazard, whether via delivery of toxins onto or into the cell/organism or otherwise inducing stress within cells (e.g. by generation of free oxygen radicals).

Together these approaches represent a systematic, and self-consistent approach which has never previously been applied in nanosafety research, and provide practical ways to compare nanomaterials, identify those physico-chemical parameters that are relevant for toxicity assessment, and form the basis for an implementable risk assessment framework.

List of Websites:

http://www.nanoretox.eu