National Institute for Public Health
and the Environment
Ministry of Health, Welfare and Sport

Grouping nanomaterials

A strategy towards grouping and read-across

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Groeperen van nanomaterialen

De risicobeoordeling van stoffen wordt gebaseerd op informatie over de effecten die ze hebben op mens en milieu. Het kost echter veel tijd, geld en proefdieren om elke stof volledig op de effecten te testen. Om toch de gewenste informatie te verkrijgen wordt daarom zo veel mogelijk gebruikgemaakt van data over vergelijkbare materialen (read-across). Deze werkwijze wordt ook voor nanomaterialen ingezet. Het RIVM heeft een teststrategie laten ontwikkelen om voor nanomaterialen te beoordelen of de data van vergelijkbare stoffen geschikt zijn om voor read-across te gebruiken. Op deze manier hoeven er minder nieuwe data te worden gegenereerd en worden er zo min mogelijk proefdieren gebruikt.

Voor de ontwikkeling van de teststrategie is een overzicht gemaakt van de fysisch-chemische eigenschappen die van belang zijn voor de manier waarop een stof zich in organismen gedraagt. Dit is gedaan met behulp van de huidige kennis over het gedrag en de schadelijkheid (toxiciteit) van nanomaterialen. Op basis van deze fysisch-chemische eigenschappen is vervolgens aangegeven welke informatie minimaal nodig is om nanomaterialen te kunnen karakteriseren. Hoe verplaatst de stof zich bijvoorbeeld in een organisme? Hoe reageert het op andere stoffen, zoals eiwitten en zouten? In welke mate wordt het onderweg afgebroken? De teststrategie geeft aan hoe per nanomateriaal op basis van deze fysisch-chemische eigenschappen kan worden beoordeeld onder welke voorwaarden data bruikbaar zijn voor read-across, en hoe dat is te verifiëren.

De ontwikkelde teststrategie is getoetst op twee fictieve voorbeelden (nanozilver en nanotitaniumdioxide) en is bruikbaar bevonden. Wel blijkt dat de gedetailleerde informatie die nodig is over de relevante fysisch-chemische eigenschappen en over de condities waaronder de data zijn verkregen, niet altijd voldoende is gedocumenteerd.

Kernwoorden: nanomaterialen, read-across, groeperen, milieu en gezondheid, volksgezondheid, strategie, toxiciteit, REACH-verordening

Synopsis

Grouping nanomaterials

Scientists evaluate the risks from exposure to chemical substances by testing the effects that chemicals have on humans and on other species, such as fish. However, testing substances for the full set of effects requires a lot of time, money and test animals. To minimize costs and animal use, the existing data for similar substances can be used to fill data gaps for a chemical substance via a process called read-across. This approach is also applied for nanomaterials. RIVM has commissioned the development of a strategy to evaluate the potential for read-across in cases of missing data for nanomaterials, with a focus on fulfilling data requirements in regulatory frameworks.

To develop this strategy, a literature review was compiled on physico-chemical parameters (such as the rate at which and amount to which a chemical dissolves) and their relevance for the behaviour, fate and toxicity of nanomaterials in organisms and the environment. This review was based on current knowledge on the behaviour and toxicity of nanomaterials. It resulted in a base set of physico-chemical parameters that are essential to characterise a nanomaterial and substantiate possibilities for read-across. The strategy provides a framework in which to evaluate each nanomaterial and decide on the applicability of read-across for nanomaterials.

The strategy has proven useful in two hypothetical case studies (nanosilver and nanotitanium dioxide). Nevertheless, it was concluded that improvement is needed for the documentation of the information from the laboratory testing of nanomaterials to support read-across. Particularly relevant physico-chemical properties of the nanomaterials and test conditions need more detailed descriptions. Furthermore, the scientific community needs to continue developing test methods that can characterize certain behaviours of nanomaterials to support read-across.

Keywords: nanomaterials, read-across, grouping, environmental health, human health, strategy, toxicity, REACH Regulation

Acknowledgement

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Samenvatting

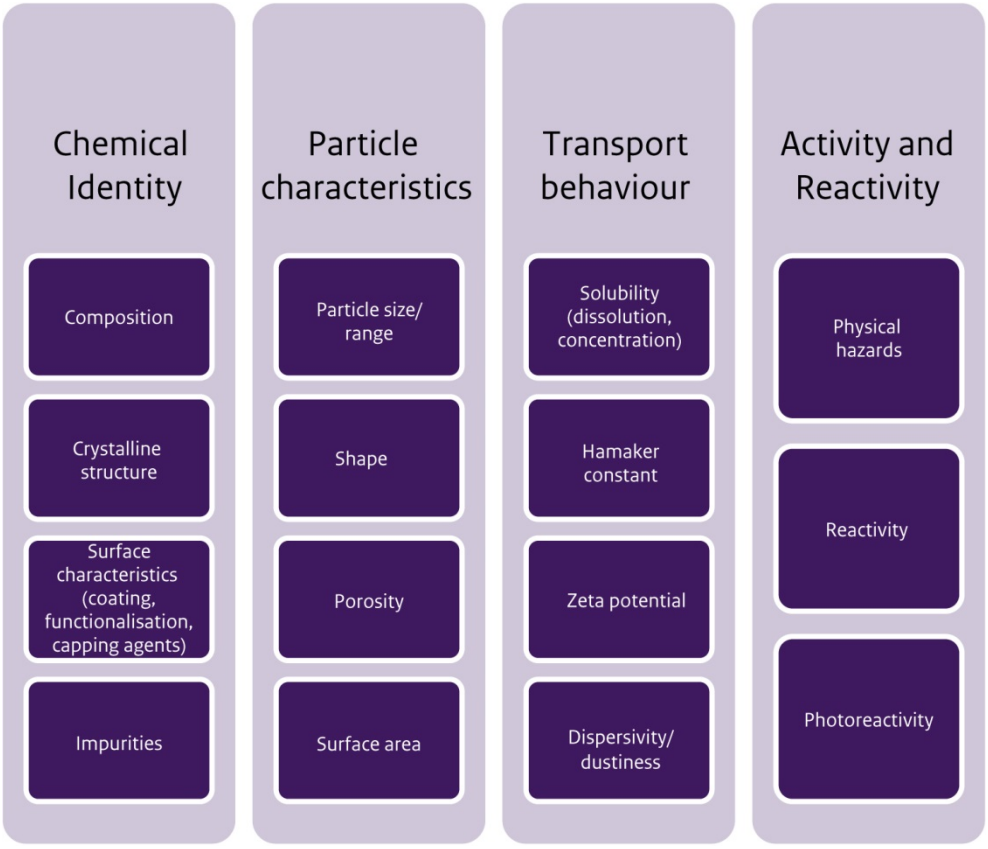
Nanomaterialen worden alom geprezen voor hun unieke eigenschappen. Zo lossen sommige nanomaterialen sneller op dan niet-nanovormen of ze zijn reactiever. Daarbij kunnen nanomaterialen zich niet alleen als stof, maar ook als deeltjes door het milieu bewegen en kunnen deze deeltjes in grootte en oppervlaktechemie veranderen in de tijd. Het specifieke gedrag van nanomaterialen en de toxiciteit van deze materialen voor mens en milieu worden in hoge mate bepaald door hun chemische samenstelling en fysisch-chemische eigenschappen. Begrip van dergelijke eigenschappen is daarom essentieel. Elk nanomateriaal testen op de volledige set van fysisch-chemische parameters en (eco)toxicologische eindpunten die onder de REACH-regelgeving vereist worden, zou echter enorme kosten met zich meebrengen en vele proefdieren vergen. Het is daarom van belang om een read-across-strategie te ontwikkelen waarmee de testresultaten van een stof of nanomateriaal gebruikt kunnen worden voor het beschrijven van het gedrag of de (eco)toxiciteit van andere nanovormen of nanomaterialen en zo de noodzaak van testen te verkleinen.

Dit rapport geeft een overzicht van de huidige kennis over fysisch-chemische eigenschappen van nanomaterialen en hun invloed op het gedrag, de toxicokinetiek van nanomaterialen en hun toxiciteit voor mens en milieu. Op basis van dit overzicht wordt een strategie voorgesteld waarmee op een systematische manier geïnventariseerd, geanalyseerd en onderbouwd kan worden of informatie over een stof, nanomateriaal of nanovorm gebruikt kan worden voor het beschrijven van het gedrag, de toxicokinetiek of de toxiciteit van andere stoffen, nanomaterialen of nanovormen. Doel van deze strategie is om voor nanomaterialen en nanovormen op de Europese markt met een zo minimaal mogelijk aantal testen te kunnen voldoen aan de datavereisten van de Europese REACH-Verordening[1]. Deze strategie kan mogelijk breder ingezet worden, bijvoorbeeld bij risicobeoordeling.

Het afgelopen decennium is binnen de wetenschap veel kennis ontwikkeld over nanomaterialen en de invloed van verschillende fysisch-chemische eigenschappen op gedrag, toxicokinetiek en toxiciteit. Binnen deze studie is gezocht naar de minimale set fysisch-chemische parameters die, op basis van de huidige kennis, het meest bepalend zijn voor gedrag, toxicokinetiek en toxiciteit van nanomaterialen. Deze minimale set staat samengevat in de onderstaande figuur. Per parameter is uiteengezet op welke wijze deze gedrag, toxicokinetiek en toxiciteit van een nanomateriaal beïnvloedt en op wat voor manier deze invloed afhankelijk is van omgevingsfactoren zoals de zuurgraad (pH), de zoutconcentratie en de aanwezigheid van eiwitten (in een organisme)

[1] REACH-verordening: De Europese wetgeving voor chemicaliën: Registratie, Evaluatie, Autorisatie en beperking van CHemische stoffen (REACH).

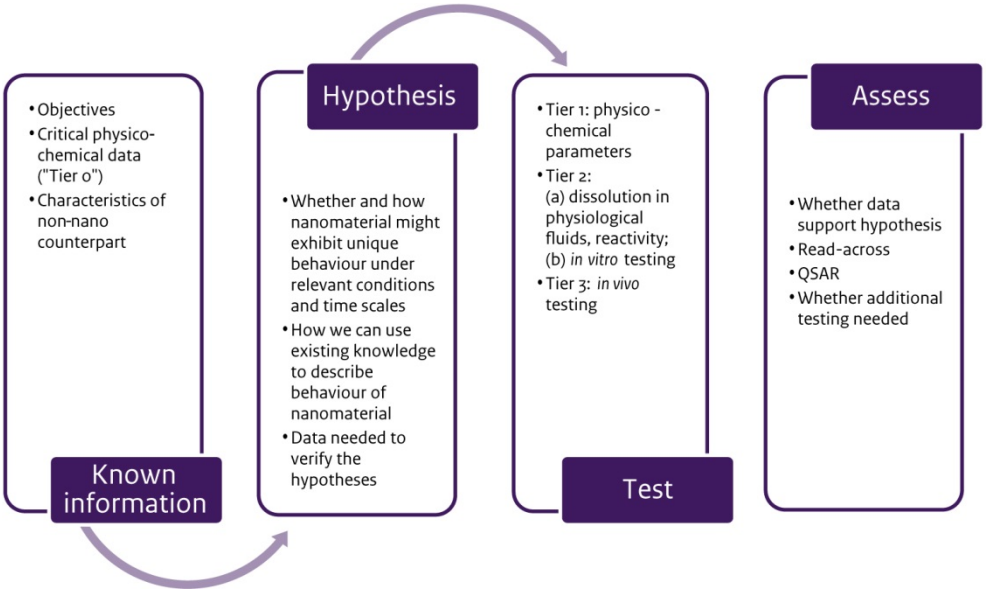
of organisch materiaal (in water). Deze analyses zijn gebaseerd op resultaten van honderden studies. Niettemin is het werk verre van compleet en worden de analyses gehinderd door een gebrek aan gestandaardiseerde testmethoden en een veelal beperkte karakterisering en beschrijving van het bestudeerde nanomateriaal en de gebruikte materialen en methode. De huidige kennis laat het definiëren van groepen van nanomaterialen op grond van goed gedefinieerde algoritmes nog niet toe. Wel kunnen op basis van weight-of-evidence enkele read-across-conclusies getrokken worden.



Onderstaand schema beschrijft de strategie die gevolgd kan worden voor het onderzoeken en onderbouwen van read-across. De wettelijke informatievereisten onder REACH zijn hiervoor een uitgangspunt. Hierbij is het mogelijk dat er voor elk missend informatievereiste een aparte hypothese met een eigen teststrategie zal moeten worden opgesteld. Voor het verzamelen van beschikbare informatie over een specifiek nanomateriaal kunnen de volgende vragen gesteld worden (zowel kwalitatief als kwantitatief):

- Wat is het productie-/importvolume en aan welke informatievereisten moet daardoor onder REACH voldaan worden?

- Welke fysisch-chemische gegevens zijn beschikbaar voor dit materiaal?
- Welke informatie is er beschikbaar over de toxiciteit van dit materiaal?
- Zijn er (eco)toxicologische data beschikbaar voor dit materiaal?
- Is er informatie beschikbaar over hoe het nanomateriaal en zijn eigenschappen veranderen in de tijd?
- Is het materiaal homogeen of heterogeen van aard?
- Is het nanomateriaal organisch of anorganisch?
- Heeft het nanomateriaal een coating (of andere oppervlaktebehandeling)?
- Wat is het beoogde effect van het nanomateriaal?
- Geeft de producent informatie over speciale eigenschappen van het materiaal die nader inzicht kunnen geven in het gedrag, de toxicokinetiek en toxiciteit (bijvoorbeeld of het materiaal transparant, reactief, antibacterieel is)?
- Hoe ziet het productieproces er uit?
- Zijn er andere nanovormen bekend die sterk lijken op het nanomateriaal?
- Bestaat er een niet-nanovorm van het materiaal?
- Welk type blootstelling kun je verwachten tijdens productie en gebruik?
- Bevat het nanomateriaal mogelijke onzuiverheden die leiden tot zorg voor mens of milieu?



Een aantal fysisch-chemische eigenschappen zijn zo essentieel dat ze verzameld/gegenereerd moeten worden om een nanomateriaal te kunnen karakteriseren. Dit geldt onder andere voor de chemische samenstelling, oppervlakte-eigenschappen, onzuiverheden, deeltjesvorm en -grootte en oppervlaktegrootte. Met deze (bekende) informatie kan

een eerste hypothese worden opgesteld over het gedrag, de toxicokinetiek en de toxiciteit. Aan de hand van deze eerste hypothese kunnen mogelijke referentiematerialen geïdentificeerd worden waarvan testgegevens gebruikt kunnen worden voor het vullen van ontbrekende informatie volgens de informatievereisten onder REACH. Vervolgens wordt de oorspronkelijke hypothese verder uitgewerkt met voorwaarden waarop de testgegevens van het referentiemateriaal gebruikt kunnen worden voor het nanomateriaal waarvoor informatie ontbreekt. De hypothese gaat hierbij ook in op de informatie die verzameld moet worden om de hypothese te onderbouwen (of te weerleggen). Wanneer testen moeten worden uitgevoerd om de validiteit van de hypothese te bevestigen zal daarvoor een teststrategie moeten worden opgesteld. De informatie die binnen de teststrategie gegenereerd wordt, kan gebruikt worden om read-across te onderbouwen, het gebruik van kwantitatieve structuur-activiteitsrelaties (QSAR) te ondersteunen en/of richting te geven aan additionele testen die wellicht nodig zijn om in een iteratieve aanpak tot verdere karakterisering van het nanomateriaal/de nanomaterialen te komen. Zoals weergegeven in de figuur wordt een teststrategie bij voorkeur stapsgewijs opgebouwd waarbij na elke stap opnieuw geevalueerd wordt:

- of verder testen noodzakelijk is voor het onderbouwen van de hypothese (of dat read-across kan worden toegepast);
- of de hypothese (en de teststrategie) op basis van eerste resultaten dient te worden aangepast;
- of resultaten uit de uitgevoerde testen de hypothese weerleggen en de informatie van het referentiemateriaal daarom niet als zodanig gebruikt kan worden voor het nanomateriaal.

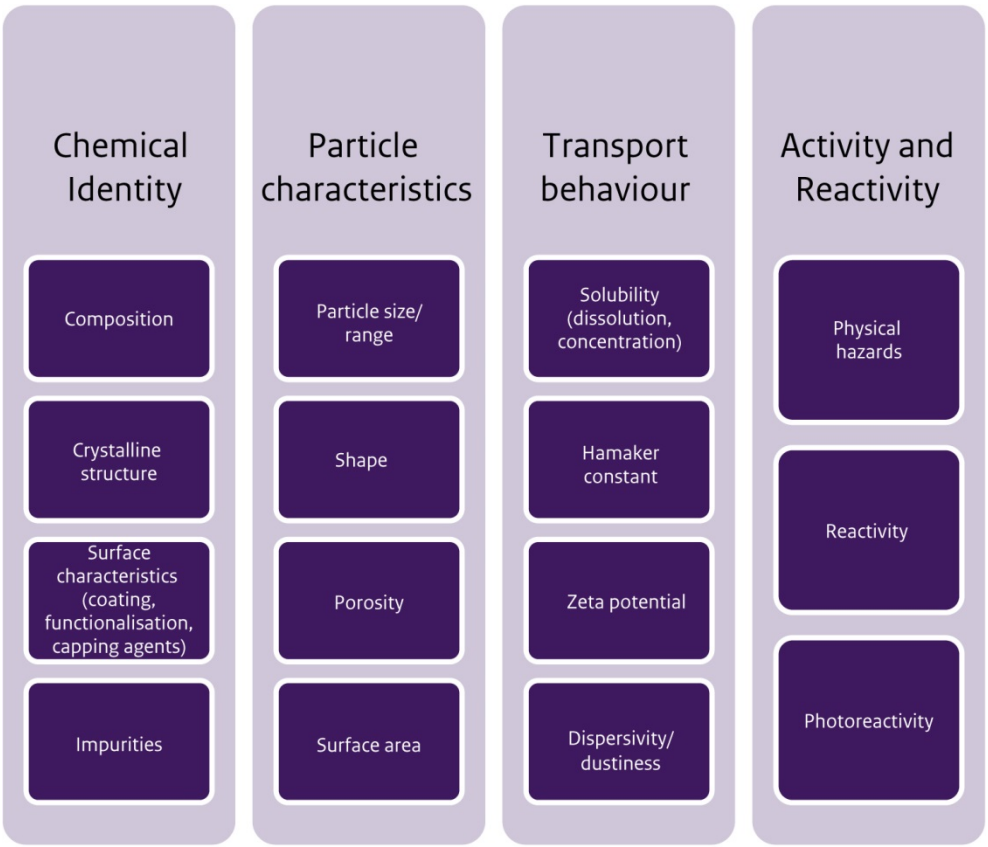
De stapsgewijze aanpak is ingericht op zo min mogelijk proefdiergebruik en wordt als volgt voorgesteld:

- Stap 1: Genereer additionele fysisch-chemische gegevens om aan REACH-eindpunten te voldoen en/of verschillende nanomaterialen of nanovormen te kunnen groeperen, of read-across te onderbouwen.
- Stap 2: Verzamel gegevens die het gedrag van het nanomateriaal beschrijven, waaronder één of meer van de volgende typen van informatie: (snelheid van) oplossen in media die relevant zijn voor milieu of fysiologie, reactiviteit of fotoreactiviteit, of *in vitro*-toxiciteitstesten om zonder dierproeven mogelijke blootstellingseffecten te schatten. Ondanks hun relevantie zijn *in vitro*-testen momenteel mogelijk lastig uit te voeren door een gebrek aan gestandaardiseerde testmethoden. Idealiter zouden deze gegevens een basis moeten geven voor het groeperen van nanomaterialen of nanovormen en voor het uitvoeren van read-across.
- Stap 3: Indien nodig, voer *in vivo*-(eco)toxiciteitstesten uit om aan de testvereisten (voor het betreffend tonnage) van REACH te voldoen.

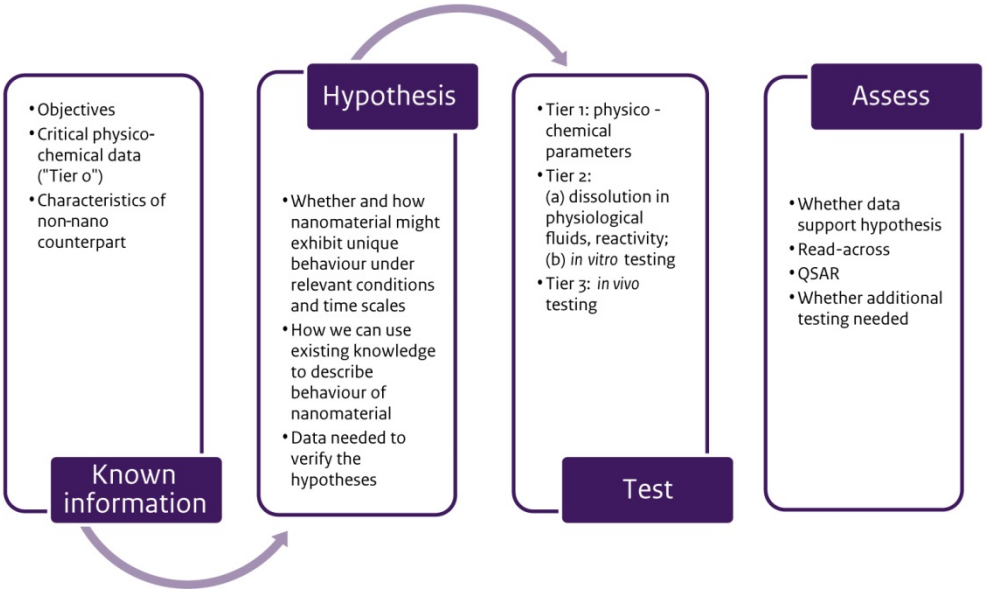
Deze strategie is uitgewerkt aan de hand van een tweetal illustratieve voorbeeldstudies, nanozilver en nanotitaniumdioxide.

Summary

This report describes a project intended to develop testing strategies for nanomaterials with respect to characterising the potential risks to human health and the environment posed by exposure to nano-materials. It was written from the perspective of compliance with the European Union Regulation on Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH) but may find broader applicability. Materials scientists prize many nanomaterials for their unique properties. Some nanomaterials may dissolve more quickly than their non-nano counterparts or be more reactive. Nanoparticles move through the environment in ways that differ from most conventional chemicals. They are transported as particles that can change in size or in surface chemistry over time. Such characteristics must be understood within the context of the potential for toxicity or ecotoxicity. Testing each nanomaterial for the full suite of physico-chemical parameters and (eco)toxicity endpoints would, however, incur tremendous costs and require animal testing. It is therefore vitally important to develop means to extrapolate test results from one nanomaterial to another or from nanomaterials to non-nanoforms in order to limit the need for testing.



Scientists have determined many of the ways in which changing the size of a particle can change the properties of a material and have identified many of the other important variables that influence the behaviour of a nanomaterial, as illustrated in the graphic above. One can map the significance of these parameters from the point where a nanomaterial may be released into the environment to the point of exposure and then, considering the mode of action, within an organism to the point at which an effect may occur. This report contains such maps based on the results of hundreds of research studies. But the work is by no means complete or straightforward and is complicated by the lack of standardised testing methods for the many properties or effects of nanomaterials. The results of research to date do not allow for tightly defined algorithms for grouping nanomaterials. They do allow, as described in this report, for drawing some "read-across" conclusions based on the weight of evidence.
The testing strategy developed in this project consists of the four steps illustrated in the graphic below.



The process begins by compiling available information, both qualitative and quantitative. One would ask:

- What is the purpose of the nanomaterial?
- How was the nanomaterial designed to give it special properties?
- Is the material tightly specified or relatively heterogeneous (to the extent that could lead to variability in its properties)?
- Is a single nanomaterial or are multiple modifications of the same nanomaterial under consideration?
- Is the nanomaterial organic or inorganic?
- Does the nanomaterial have a coating?

- Based on knowledge of the manufacturing process or based on analysis, does the nanomaterial potentially have impurities that are of (eco)toxicological concern?
- Is there a non-nanoform of the material?
- Does the manufacturer make any claims regarding the special properties of this material that are related to its purpose that may be relevant to this inquiry? (e.g. transparency, reactivity, antibacterial)
- What is the tonnage to be manufactured or imported under REACH or other pertinent regulations?
- How might the manufacture and use of this substance result in exposures?
- What physico-chemical data are available for this substance?
- Is any information available about how this nanomaterial or its properties change as it ages?
- Are (eco)toxicological data available for this substance?

Some physico-chemical data are so essential to characterising a nanomaterial that they should be compiled during the initial step in the process. These data include chemical composition, surface characteristics, impurities and surface area. The analyst can use this information to form a hypothesis regarding whether and how a nanomaterial might exhibit a unique behaviour under relevant conditions and time scales, or whether it might behave similarly to one or more other well-tested materials that serve as a reference. In this step, one would also consider what data might be necessary to verify the hypothesis.
The next step in the framework would be to perform appropriate laboratory tests. Regulatory requirements provide the basis for determining the need for testing. A testing programme might also reflect the need to collect data that would support read-across to another substance or to the non-nanoform of a material. As illustrated in the graphic depiction of the testing strategy, testing might occur in three tiers, depending on the project needs:

- Tier 1: Obtain additional physico-chemical data to fulfil REACH endpoints and/or support grouping or read-across.
- Tier 2: Collect data that characterise the behaviour of the nanomaterial, which might include one or more of the following types of information: dissolution in environmentally or physiologically relevant media; reactivity/photoreactivity; or *in vitro* toxicity testing to gauge the possible effects of exposure without animal testing. Such testing, while conceptually relevant, may be difficult to execute now due to the lack of standardised testing methods. The data would ideally provide a basis for grouping or read-across.
- Tier 3: As necessary, *in vivo* (eco)toxicity testing to meet the testing requirements appropriate to the tonnage band under REACH.

As data were collected under this framework, one would assess whether the data supported the initial hypothesis or suggested alternate conclusions about the behaviour of the nanomaterial. The data might be used to add to the weight of evidence for read-across; support the use of quantitative structural activity relationships (QSAR), and/or suggest that additional testing might be needed in an iterative approach to characterise the nanomaterial(s).
This report concludes with an illustration of this testing strategy for two case studies, nanosilver and nanotitanium dioxide.

1 Introduction

This report describes RIVM project C6, Grouping Nanomaterials. It records the results of three phases of work to develop testing strategies with respect to characterising the potential risks to human health and the environment. Such testing strategies may include grouping. To introduce this report, the following subsections describe the objectives of the project and provide a synopsis of each phase of the project.

1.1 Objectives and scope of project

The objective of this project was to come to a motivated strategy for the development of concepts and criteria for the grouping of nanomaterials in order to test for hazard and risks. The proposed strategy would ideally:

- Explicitly but not exclusively involve the range of ideas of the RIVM Working Group on Nano.
- Reflect the current state of knowledge on nanomaterials.
- Be future-proof, i.e. able to address foreseen or anticipated next-generation nanomaterials, including complex assemblies.
- Be lean in terms of costs, time and materials.
- Be as easy to apply as possible for the envisaged stakeholders.

This work was accomplished in three phases:

1. Make an inventory of existing data.
2. Identify nanomaterials that have the highest priority for exploring grouping and developing read-across concepts.
3. Develop testing strategies.

1.2 Synopsis of Phase 1

In Phase 1, the project team inventoried the nanospecific characteristics that may be essential in the development of read-across concepts and grouping criteria as these may affect the kinetics and fate of nanomaterials and their hazard and risk assessment for humans and the environment. This list of characteristics, together with the scientific data to substantiate their listing, served as the basis for Phase 2 of the project.
The project team considered the following factors:

- Work by others on read-across and grouping of nanomaterials;
- The ways in which the physico-chemical properties of nanomaterials may differ from the properties of their non-nano counterparts, and the implications for environmental fate and transport and (eco)toxicity;
- Mechanisms of toxicity in nanomaterials, either to ecological or human (mammalian) receptors, and the factors that affect toxicity; and

- As relevant to this project, mechanistic insights that derive from the pharmaceutical industry.

After reviewing literature and indexing the publications reviewed, the project team identified the characteristics that may be relevant to defining nanomaterial read-across and grouping.

1.3 Synopsis of Phase 2

The objective of Phase 2 of the project was to identify and scientifically justify which nanomaterial characteristics have (or may have) the highest priority to explore the feasibility of grouping and developing read-across concepts and grouping criteria in the assessment of the kinetics, fate, hazard and risk of nanomaterials for humans and the environment. Their relation to characteristics required for substance identification (SID) was addressed. For the high-priority characteristics, minimal data and measurement requirements were established to the extent possible, relevant to existing Organisation for Economic Cooperation and Development (OECD) test guidelines. The work provided an overview of the available information relevant to the high-priority characteristics.
To refine the conclusions of Phase 1 and develop a justifiable basis for grouping and read-across criteria, the project team considered the following factors:

- Substance identification.
- Testing methods and limitations.
- Trends observed for the most common nanomaterials.
- Practicality.
- Future developments in nanotechnology.

Each is discussed below.

1.3.1 Substance identification

Clear substance identification is a cornerstone of testing strategies and read-across approaches; unfortunately, it has also been a weakness in early dossiers submitted under the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH) regulation for nanomaterials. The characterisation of a nanomaterial may reflect its chemical identity, size, shape, surface coating or other factors. Impurities in some nanomaterial preparations have affected the results of some toxicity testing. Particle size, clearly one of the defining features of nanomaterials, can change over time as particles agglomerate. The kinetics of dissolution and the partitioning of a nanomaterial may differ from that of its non-nano counterpart, as RIVM has observed for nanosilver (Pronk et al., 2009). So substance identification is both challenging for nanomaterials and critically important to this project, and may require some form of matrix approach.

1.3.2 Testing methods and limitations

OECD (2012a) has famously noted that:

> *…The approaches for the testing and assessment of traditional chemicals are in general appropriate for assessing the safety of nanomaterials, but may have to be adapted to the specificities of nanomaterials.*

However, the reality of characterising the toxicity and ecotoxicity of nanomaterials is more nuanced than simply adapting a standardised test. For example, the Group Assessing Already Registered Nanomaterials (GAARN) has observed that (ECHA, 2013a):

> *The half-life of nanoforms in suspension is often dependent on the initial loading concentration, with higher concentrations leading to faster precipitation rates…. High concentrations of nanoforms may impair the swimming ability of small invertebrates (e.g. daphnids). Testing at these high concentrations should be avoided as this type of physical impairment would not reflect the hazardous properties of the substance. For ecotoxicological endpoints, long-term studies are highly recommended for substances that show low toxicity in acute studies, as the experimental design and lower initial loading rates for sub-chronic studies will help to overcome problems of high agglomeration and sedimentation. …. Thus, given that the mode of action of nanoforms is yet to be properly characterised, carefully designed long-term studies might be of more relevance for an appropriate hazard identification.*

In recognition of such effects, ARCADIS considered in Phase 2 the limitations of standard testing guidelines for nanomaterials and the potential implications for read-across. In addition to considering current OECD test guidelines, it may also be prudent to consider next-generation testing methods such as high throughput and/or high content screening assays.

1.3.3 Trends observed for the most common nanomaterials

No single grouping or read-across scheme will perfectly fit the broad range of organic and inorganic materials currently available in nanoform, never mind the complexity added by future developments anticipated in complex nano-assemblies. As of 2010, RIVM had identified approximately 860 commercial products available in the European Union that contained nanomaterials (Wijnhoven et al., 2010).
Figure 1 lists some of the most commonly used substances in nanoform as identified by RIVM (undated) or as targeted in the OECD Sponsorship Programme for the Testing of Manufactured Nanomaterials (OECD, 2012a). It also provides a third indicator of use in the European Union, which is whether the nanoform had been notified under REACH as of 2012. As shown in Figure 1, the global trade in nanomaterials is substantial (European Commission, 2012).

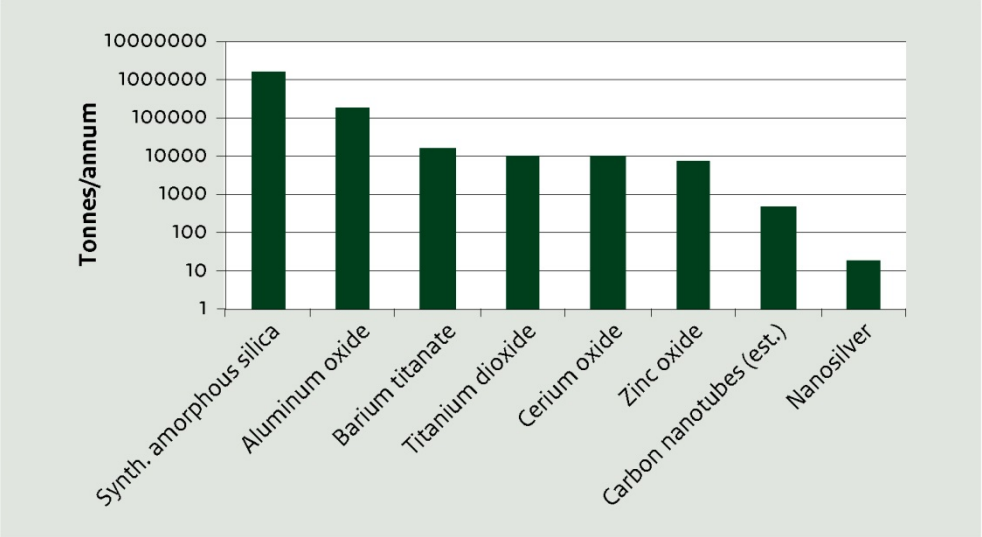


Figure 1 Common Nanomaterials in Commerce

1.3.4 Practicality

In order to be usable, a grouping and read-across approach must be readily understandable and generally easy to apply. That depends on both the complexity of the read-across logic (e.g. number of steps in the process, amount of data needed) and the commercial availability of the test data. This factor relates to two of the specific requirements of Phase 3: that the proposed test strategies should be lean in terms of costs, time and materials, and should be as easy to apply as possible for the envisaged stakeholders.

1.3.5 Future developments in nanotechnology

As much as is possible, this project must consider the next developments in nanotechnology and the need to assess the possible hazards and risks by grouping and read-across.
The development of the testing strategy incorporated key concepts for grouping, categorisation, and read-across, all of which were explored in Phase 1, including data on physico-chemical properties, mode of action, biokinetics and the overall toxicological profile of the nanomaterials. Those properties that best allow for justification of read-across were proposed for use in a tiered approach for Phase 3.

1.4 Synopsis of Phase 3

In Phase 3 of the project, the team developed testing strategies in further detail, with the objective of obtaining the knowledge needed to develop read-across concepts and criteria for grouping. The strategies incorporated concrete recommendations for test guidelines, considering:
The mode of action of nanomaterials;

- The physico-chemical characteristics of nanomaterials, which can change over time; and
- The kinetic profile of nanomaterials.

The recommendations were evaluated using the nanoforms of titanium dioxide and silver as illustrative cases.

Table 1 Commonly-Used Nanomaterials

Nanomaterial	RIVM Identified	OECD Sponsorship Programme	Nanoform Registered under REACH circa 2012
Fullerenes (C60)	✓	✓	No
Single-walled carbon nanotubes (SWCNTs)	✓	✓	No
Multi-walled carbon nanotubes (MWCNTs)	✓	✓	Yes
Silver nanoparticles	✓	✓	No[1]
Iron nanoparticles	✓	✓	Yes[2]
Titanium dioxide	✓	✓	Yes[3]
Aluminium oxide	✓	✓	Yes[4]
Cerium oxide	✓	✓	Yes[5]
Zinc oxide	✓	✓	Yes[6]
Silicon dioxide	✓	✓	Yes[7]
Dendrimers		✓	---[8]

[1] While the substance silver has been registered under REACH, the dossier states that the nanoform is not covered.
[2] The substances diiron trioxide and triiron tetraoxide have been registered under REACH. The registrations are not specific to the nanoform (although certain references could be interpreted as referring to the nanoform).
[3] The registration covers all forms of titanium dioxide, including the bulk and the nanoform, but does not differentiate between them.
[4] Aluminium oxide has been registered under REACH, but the registration is not specific to the nanoform. Certain references could be interpreted as referring to the nanoform.
[5] The substance cerium dioxide has been registered and the registrant has indicated that the substance has a nanoform and has provided separate information on the nanoform.
[6] The registration is not specific to the nanoform (although certain references could be interpreted as referring to the nanoform).
[7] Synthetic amorphous silica has been registered under REACH. The explanations in the registration dossier imply that the dossier mostly, if not exclusively, relates to the nanoform.
[8] Dendrimers are tree-shaped molecular structures similar to polymers. Polymers are not subject to REACH registration.

2 Basis for Study: Available Information

This study was based on two aspects of the published literature. Studies that assessed the mode of action and the effects of exposure to nanomaterials, whether by humans or in the aquatic environment, provided the scientific basis for identifying critical parameters and developing testing strategies. Characterisation of the properties of nanomaterials, as collected in publicly available databases, provided the basis for evaluating those strategies.

2.1 Literature Search

Figure 2 illustrates the line of inquiry used on this project.

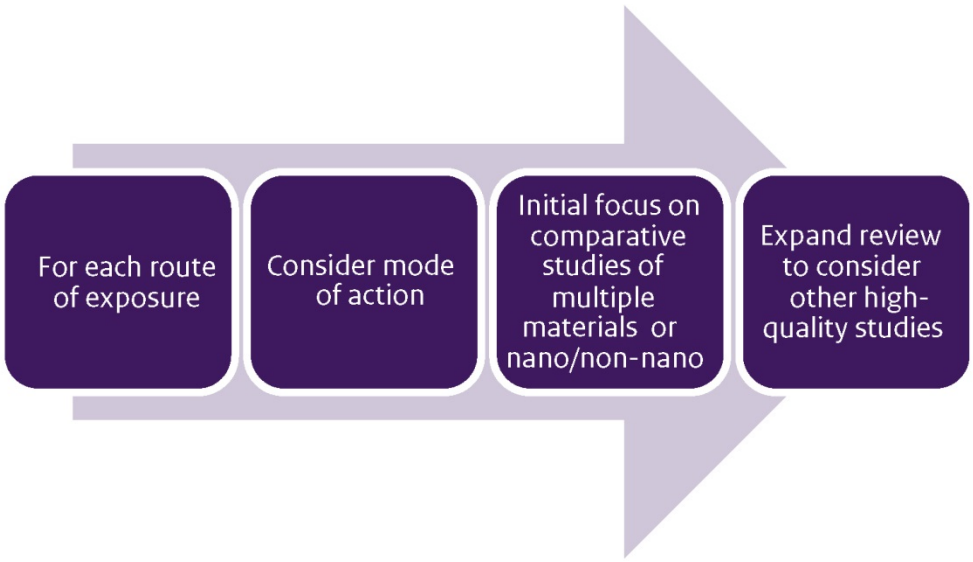


Figure 2 Line of Inquiry

ARCADIS began by identifying review papers. Such literature compilations allow ready access to the kind of comparative data that support the development of read-across or grouping approaches and are an efficient way to access the broader literature. These review papers were identified through a search of the Virtual Journal of Nanotechnology, Environment, Health & Safety maintained by the International Council on Nanotechnology, or ICON™. This Virtual Journal, which is available at http://icon.rice.edu/virtualjournal.cfm, is a searchable database of abstracts of papers published in the peer-reviewed literature on nanotechnology. ICON provided the following information about the database content (Dr David R. Johnson, Personal Communication, 29 November 2011):

> *ICON develops a candidate list of published journal articles for the Virtual Journal database by querying the following databases: Web*

of Science, Medline and Toxline, using keywords intended to identify a majority fraction of all potential papers published on environmental health and safety issues related to nanomaterials. Papers identified through these database queries are screened, based on the paper title and abstract, before inclusion in the Virtual Journal. ICON also tracks a few key journals (e.g. Nanotoxicology) to add papers published in those journals that may not be identified by the original database query.

ARCADIS searched the ICON database for review papers in November 2013 and supplemented the search in December 2013. A Dialog database search identified primary sources of information that could be available to supplement information in review papers. Table 2 summarises the results of the literature searches.

Table 2 Summary of Literature Search

Category	Intent	Relevant Papers[1]
Baseline knowledge	Fundamental information captured in tender and RIVM publications	21+
Review papers	Reviews identified through ICON database provide efficient view into literature	95 initially identified; upon evaluation, 59 indexed
DIALOG® search	Comprehensive search: DIALOG captures 58 databases of scientific literature	Identified around 930 papers, approximately half of which may be relevant; selected papers referenced in this report

[1] Some papers duplicated in different searches.

This literature search strategy was designed to provide an efficient snapshot of the state of the science and not to provide a comprehensive review of the ever-evolving literature on ecotoxicity and toxicity. Figure 3 illustrates the challenge of tracking critical developments in the field: as a result of increased attention in research laboratories given to the potential toxicity and ecotoxicity of nanomaterials, approximately one thousand papers are currently published each year (ICON, 2014). While this report reflects the team's best efforts to reflect the current state of the science, it must be acknowledged that the science is advancing daily. Consequently, this initial look at review papers was supplemented throughout the project with papers on specific research projects. Those papers are referenced in this report. As the work progressed, however, the project team did not perform iterative comprehensive reviews of the literature.

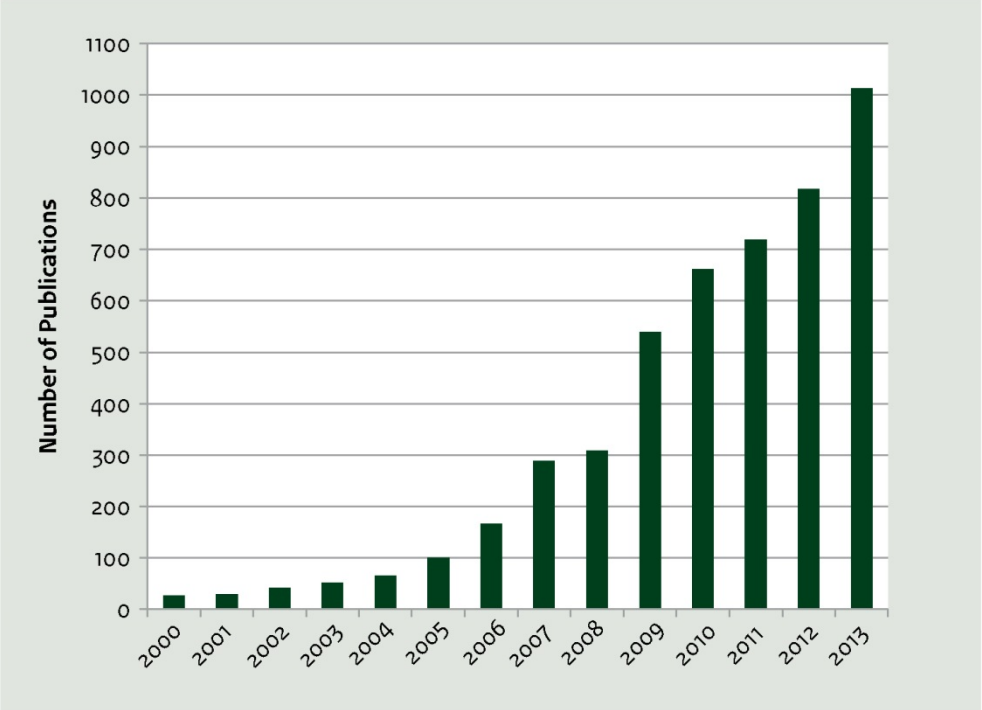


Figure 3 Number of Papers Published Annually on the Hazards of Nanotechnology

2.2 Nanomaterial Characterisation

Some data for nanomaterial characterisation are available from REACH dossiers, although the use of such data may be restricted. As of February 2012, seven registrations and eighteen notifications had voluntarily indicated "nanomaterial" as the form of the substance (European Commission, 2012). Figure 1 indicated the identity of some of those substances.
OECD's Sponsorship Programme is producing base-set data on specific nanomaterials listed in Figure 1. The available information includes physico-chemical data on nanoforms of titanium dioxide, silicon dioxide, zinc oxide and multiwall carbon nanotubes. Some publicly available databases and data compilations provide relevant information. These include the nanoINFO Knowledgebase, the Nanomaterial Registry and the Nano-Bio Interactions Knowledgebase.
The nanoINFO Knowledgebase is a product of the project *Data and knowledge on nanomaterials* (DaNa/DaNa2.0). An interdisciplinary team of scientists from the German Federal Ministry of Education and Research and a team of universities have worked to compile a non-biased and quality-approved knowledge base on nanomaterials. The database includes both physico-chemical data and information on human and environmental toxicology gleaned from the literature for 25 types of nanomaterials.

The Nanomaterial Registry (NIH, 2014) currently holds records on nearly 2,000 substances. Figure 4 shows the data available on physico-chemical properties and their compliance level, or relative reliability. The database also holds 14 environmental studies and 608 biological studies (82 % of which are *in vitro* and 18 % are *in vivo*). If the information in the database is representative of the state of nanomaterial characterisation in general, Figure 4 suggests that relatively little is known regarding many nanomaterials beyond their composition and particle size.
The originators of the Nano-Bio Interactions Knowledgebase intend to define "the relationships between nanomaterial physico-chemical properties and the biological responses to their exposure". As of November 2014, the database contains 222 entries (ONAMI & Oregon State University, 2014).

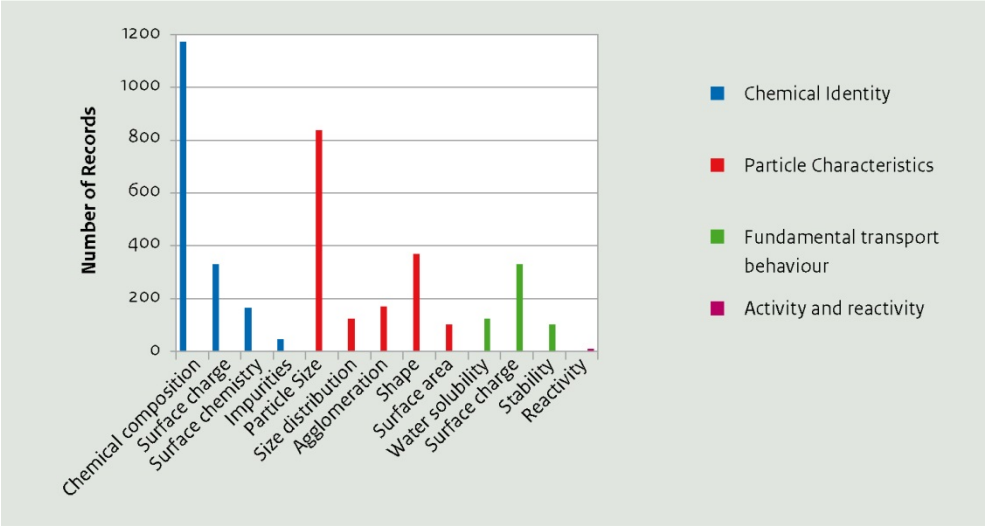


Figure 4 Data available in the Nanomaterial Registry

3 Physico-chemical properties critical to the behaviour of nanomaterials

"Nanosizing" a substance can change its characteristics in sometimes startling ways, affecting such fundamental behaviours as solubility, reactivity, environmental transport, and toxicokinetics. These changes in behaviour can affect the fate of a nanomaterial released to the environment and the effects of an organism's exposure to it. As a consequence, the Group Assessing Already Registered Nanomaterials (GAARN) has noted that (ECHA, 2013a):

> *When considering reading across to another nanoform or a counterpart bulk material, a solid scientific justification should be provided in the IUCLID dossier of the registered substance. It is insufficient to justify the use of data for read-across based only on the chemical composition of a nanomaterial, and further physicochemical parameters such as aspect ratio, shape, form, solubility, surface area, charge, surface treatment etc. should provide a reliable dataset to support a sound scientific interpretation of the similarities or differences among (nano)forms.*

The description of the physico-chemical parameters of nanomaterials that follows, therefore, provides the context for subsequent discussions of the mechanisms of fate and transport, ecotoxicity and human health toxicity in this report. Information on physico-chemical properties is based largely on the RIVM project *A1: What Defines Nanomaterials?* (Sellers and Hassinger, 2012). The information is organised according to a logical grouping of properties that is meant to illustrate scientific principles rather than correspond precisely to regulatory substance characterisation requirements.

3.1 Chemical Properties

Table 3 summarises the particle characteristics and chemical properties relevant to this study. Each is described below. (Later sections of this report provide further perspective on the relevance of these parameters to testing strategies and read-across.)

3.1.1 Chemical Identity

Chemical identity, within the context of this report, includes chemical composition, crystalline structure, surface chemistry (which affects surface charge) and the presence of impurities.

Table 3 Important Physico-chemical Properties and Characteristics

Category	**Characteristic or Property**	
Chemical identity	Chemical composition	
	Crystalline structure	
	Surface characteristics/ surface charge	Coating
		Functionalisation
		Capping agents
	Impurities	
Particle characteristics	Particle size/range	
	Shape	
	Porosity	
	Surface area	
Fundamental transport behaviour	Water solubility	Rate of dissolution
		Equilibrium solubility concentration
	Hamaker constant	
	Zeta potential	
	Dispersiveness	
	Dustiness	
Activity and reactivity	Physical hazards	Flammability
		Autoflammability
		Explosiveness
	Reactivity	
	Photoreactivity	

3.1.1.1 Chemical composition

The chemical composition of a substance fundamentally determines its fate and transport, ecotoxicity and human health toxicity. As defined in Article 3 of the Regulation on Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH), a "substance" is:

> *A chemical element and its compounds in the natural state or obtained by any manufacturing process, including any additive*

necessary to preserve its stability and any impurity deriving from the process used, but excluding any solvent which may be separated without affecting the stability of the substance or changing its composition.

3.1.1.2 Crystalline structure

Crystalline structure describes how the molecules of an inorganic substance are physically arranged in space. Many materials with the same chemical composition can have different lattice structures and consequently exhibit different physico-chemical properties. Several aspects of the crystallinity of metals and metal oxides may vary with particle size. With changes in particle size, the unit cell can contract or expand, as represented by changes in lattice parameters. Particles of two different sizes can also assume different crystalline phases. The synthesis of the literature in the A1 project found the following size dependence of this property: at 11.7-200 nm, distortion of the crystal lattice structure occurred, in some cases leading to different crystalline forms. In short, decreasing particle size to the nanoscale can affect the crystalline structure. Changing the crystalline structure can affect a particle's reactivity and, in some cases, its toxicity.

3.1.1.3 Surface characteristics: coating, functionalisation, and capping agents

The surface coating on a particle may affect the behaviour of a nanoparticle and its (eco)toxicity. Surface coating is not necessarily uniform; the degree of coating can vary from particle to particle within a batch of manufactured nanomaterials or between batches of nanomaterials. Some examples of the effect of coatings follow. While these examples do not represent a comprehensive view of the effects of surface coatings, they do illustrate their potential importance.
Fauss et al. (2011) investigated the effect of various capping or functionalising agents on the dissolution rate of nanosilver (nAg) and the generation of reactive oxygen species (ROS). They tested three types of particles: 20 nm diameter citrate-capped nAg, 30 nm diameter starch (maltose)-capped nAg, and silver proteinate functionalised particles approximately 15 nm in diameter. The results were normalised to surface area so that the effect of capping or functionalising agents could be examined without particle size being a variable. Nanoparticles released dissolved silver at a rate of 0.02 to 13 micromoles per square meter per hour (μmol/m^2 hr), depending on the functionalisation and total silver concentration. ROS generation ranged from 0.01 to 400 μmol/m^2 hr; the rate was proportional to surface area and depended on the capping agent.
In another study, Zhao and Wang (2012) examined the effects of exposure to nAg particles coated with lactate (AgNanoparticles-L), polyvinylpyrrolidone (AgNanoparticles-P), and sodium dodecylbenzene sulfonate (AgNanoparticles-S) by *Daphnia magna*. In addition to differing by coating, the nanoparticles differed by particle size. The dominant particle sizes (number-weighted) were in the range of 550 nm for all three nanoparticles. The effective diameters (intensity-weighted)

were 123.9 ± 1.3 nm for AgNanoparticles-L, 79.7 ± 0.4 nm for AgNanoparticles-P, and 65.3 ± 1.1 nm for AgNanoparticles-S. Zhao and Wang found that toxicity was mainly caused by the release of soluble silver and attributed the significant difference in the toxicity of the three differently coated particles to the effect of coating on the solubility of silver.

Surface functionalisation can also affect the behaviour of nanomaterials and influence the effects of exposure to nanomaterials. For example, multiwall carbon nanotubes (MWCNT) can be functionalised with –OH or –COOH moieties. In a study pertaining to the transport of MWCNT in the environment, Kennedy et al. (2008) evaluated the half-life of MWCNT suspended in moderately hard reconstituted water (that is, the time at which only half the particles remained in suspension after the remainder settled). MWCNT with no functionalisation had a half-life of 7 minutes; MWCNT-COOH had a half-life of 21 minutes, and MWCNT-OH, 51 minutes. The research team also found that the functionalisation of MWCNT affected the survival of *Ceriodaphnia dubia*.

Finally, manufacturers may use stabilising or capping agents, e.g. to prevent agglomeration of aqueous nanosilver suspensions (Tolaymat et al., 2010). Such agents function by two essential mechanisms (Kvitek et al., 2008; Hotze et al., 2010): steric stabilisation and electrostatic repulsion, sometimes combined and referred to as electrosteric stabilisation. Steric stabilisation occurs, for example, when a polymer or surfactant with a hydrophilic tail sorbs onto a nanoparticle. Stabilisation agents used with nanosilver commonly include polyethylene glycols (PEG), polyvinyl alcohols (PVA), polyvinylpyrrolidone (PVP), and polyoxyethylene-sorbitan monooleat (Tween 80). Electrostatic repulsion occurs when a charged layer is formed around the nanoparticle; ions with the opposite charge within the solution will then surround the colloidal particles and create a double layer around each nanoparticle. The mutual repulsion of these double layers provides stability. Sodium dodecyl sulphate is one anionic surfactant used to stabilise nanosilver particles. Neither steric stabilisation nor electrostatic repulsion is permanent or unchangeable; when a suspension of stabilised nanoparticles is released to the environment, the stabilising agent may desorb from the particles or be displaced by natural organic matter.

As this discussion implies, the surface chemistry of the nanoparticle – whether it reflects the molecular composition of the particle itself, functional moieties, a coating, or sorbed capping agents – influences the particle's surface charge. The surface charge of a particle in colloidal suspension can be determined by measuring the zeta potential as described further below. Surface charge can also be represented by the isoelectric point (i.e. the pH at which a particular molecule or surface carries no net electrical charge). Each of those measurands, zeta potential and the isoelectric point, reflect both the characteristics of the nanoparticle itself and the solution in which it is suspended.

3.1.1.4 Impurities

Some nanomaterial samples have contained substantial amounts of impurities. Such impurities can influence the effects of exposure. Tests of low purity, as-produced fullerenes, illustrate the potential effects (Hull et al., 2009). Analysis of the material showed that it contained impurities such as barium and boron. Scientists leached impurities from the C60 samples and then tested the toxicity of the leachate. They found that the leachate was toxic to *Pimephales promelas* and *Ceriodaphnia dubia*. Adding the chelating agent ethylenediaminetetraacetic acid (EDTA) to the leachates decreased toxicity, which implied that divalent transition metals were the source of toxicity.

3.1.2 Particle characteristics

Relevant particle characteristics include the particle size, shape, porosity and surface area.

3.1.2.1 Particle size

OECD (2010) has defined particle size as follows:

> *The physical dimensions of the smallest discrete form of a substance under specified measurement conditions. If a group of particles are of differing sizes they may be described by a Particle Size Distribution.*

As the size of a particle decreases, the proportion of atoms on the surface of the particle increase and, consequently, the relative reactivity of the particle can increase. At very small particle sizes (e.g. below 15 to 20 nm for some materials), the decreased particle size affects the surface free energy of a particle, thereby increasing the catalytic activity of surface atoms. (The effect of nanosizing on particle reactivity is discussed further below.)
In summary, changing the particle size can change certain physico-chemical properties and also the toxicokinetics of the material. Particle size and the particle size distribution, or granulometry, may be critical to read-across.

3.1.2.2 Shape

Nanoparticles may take different shapes: spherical, triangular, dendritic, or needle-like, for example. The term "aspect ratio" refers to the ratio between a particle's length and width. This parameter can be relevant to the toxicity of carbon nanotubes, nanowires, and other "needle shaped" particles. Its relevance is perhaps best understood by analogy to the inhalation toxicity of asbestos and is discussed further in later sections of this report.

3.1.2.3 Porosity

Porosity measures the fraction of the particle that is devoid of material. A material's porosity affects its fate in the environment by affecting

particle density and colloidal stability and may permit a nanomaterial to act as a vector for other constituents of a solution. While this parameter may relate to the degree of agglomeration, it does not depend on primary particle size. Increasing porosity can increase the effective surface area of a particle and, thereby, its reactivity.

3.1.2.4 Surface area

Relative surface area is related to particle size, shape and porosity. As the size of a particle decreases, the ratio of surface area to volume increases or, in other words, the proportion of the atoms on the surface of the particle increases. This characteristic is important with respect to the rate of reaction, dissolution and adsorption. Specific surface area appears to be relevant for a number of parameters for toxicological and ecological risk assessment. It will dictate the surface charge density in cases in which nanomaterials are surface functionalised, which has direct consequences on: (a) nanomaterial interaction (i.e. agglomeration) with other naturally occurring particulate matter (i.e. contaminant vectors); (b) route of exposure as a function of surface ligand-biological interface (i.e. bioaccumulation pathway, bioavailability); and (c) mechanisms of toxicity.
Particle surface area can be an important parameter to consider when comparing the results of studies with differently sized particles. In some cases in which different behaviours were observed for different sized particles, the apparent difference disappeared when the results were normalised to surface area. Auffan et al. (2009) cite the following two examples. The apparent difference in toxicity of 20 and 250 nm anatase TiO_2 particles (where the 20 nm particles appeared to be more toxic per unit mass) was eliminated when the results were compared based on the specific surface area of the particles. Similarly, while it appeared that 7 nm CeO_2 nanoparticles induced stronger oxidative stress and damage to DNA *in vitro* than did 300 nm CeO_2 particles, no significant difference existed once the data were normalised to surface area.

3.1.3 Fundamental transport behaviour

The movement of a substance throughout the environment or within an organism can depend upon a nanomaterial's water solubility, dustiness, dispersiveness and tendency to agglomerate or resist agglomeration. These characteristics can reflect not only the nature of the nanomaterial itself, but also the nature of the medium in which the nanomaterial is suspended.

3.1.3.1 Water solubility

OECD (2010) offers the following definition and distinction:

> *Water Solubility/Dispersibility refers to the mass proportion of a given sample of nanomaterial which is held in water solution or as a colloidal suspension in water as a function of time or where the sample of nanomaterial loses its particulate character as it changes from a particle form to a molecular form. It must be recognised*

> *that Solubility and Dispersibility are not identical though the distinction can be difficult to recognise with [manufactured nanomaterials].*

Water solubility can depend upon particle size, with obvious implications for ecotoxicity and toxicity. Two considerations may be relevant. Briefly,

- The rate of dissolution of soluble materials increases with decreasing particle size.
- The Ostwald-Freundlich equation predicts that equilibrium solubility should increase with decreasing particle size. (Experimentally, this is often not the case due to non-ideal behaviour.)

Water solubility also depends upon the solution characteristics and can depend on the particle coating.

3.1.3.2 Dispersibility

Dispersibility refers to the relative number (or mass) of primary particles in a suspending medium. This property characterises the way in which nanoparticles can form colloidal suspensions, which might be formed by the dispersion of nanoparticles in a liquid, that differ from solutions of dissolved substances. The pH and ionic strength of the aqueous phase can affect a nanomaterial's dispersibility (OECD, 2010) OECD (2012a) has discussed the distinction between solubility and dispersibility as follows:

> *Most dosing techniques require the test material to be in a liquid phase (generally aqueous) for delivery and (eco)toxicologists sometimes use the terms "in solution" or "solubility" to infer this. However, in particle chemistry these terms are inappropriate. The introduction of an insoluble or very sparingly soluble nanomaterial to a liquid or other aqueous medium with the intention of making a stock "solution" will involve dispersion. A stable dispersion of a nanomaterial in a liquid is referred to as a colloidal dispersion. […] Some metal nanoparticles may release ions from the surface into the surrounding water (corrosion/degradation) and it is therefore possible that these nanomaterials will eventually degrade completely […] Because of the particle size of many nanomaterials, it can be difficult to distinguish between when a nanomaterial is dispersed and when it is dissolved.*

No OECD test guideline exists to measure the dispersion of primary or agglomerated nanoparticles. Some methods are available or are under development (OECD, 2014b).

3.1.3.3 Dustiness

Dustiness refers to the propensity to generate airborne dust during handling. Data regarding dustiness provide a basis for estimating the potential health risk due to inhalation exposure. The ability to generate dust depends on particle size and density (thereby buoyancy) (OECD, 2010).

3.1.3.4 Tendency to agglomerate: Van der Waals energy/Hamaker constant

Van der Waals energy, as represented by the Hamaker constant, can be an important characteristic of a nanomaterial with respect to its behaviour. Van der Waals force, a weak attractive force resulting from transient polarity related to shifts in electron density, can cause nanoparticles to agglomerate[2] when Brownian motion induces collisions between particles. Agglomeration increases the net particle size, thereby changing the behaviour of the original nanomaterial. The Hamaker constant represents the net Van der Waals attraction. This parameter is often not considered in the risk assessment of nanoparticles (e.g. SCENIHR, 2009; OECD, 2010).

3.1.3.5 Counter to agglomeration: Zeta potential

Surface charge, as represented by zeta potential, influences the fate and transport of nanoparticles. Any surface charge on nanoparticles causes electrostatic repulsion between particles of like charge, which can counter the tendency to agglomerate.
Zeta potential is an abbreviation for the electrokinetic potential in colloidal systems. From a theoretical viewpoint, zeta potential is the electric potential in the interfacial double layer (DL) at the location of the slipping plane versus a point in the bulk fluid away from the interface. In other words, zeta potential is the potential difference between the dispersion medium and the stationary layer of fluid attached to the dispersed particle.
The zeta potential can be related to the stability of colloidal dispersions. The zeta potential indicates the degree of repulsion between adjacent, similarly charged particles in dispersion. For molecules and particles that are small enough, a high zeta potential will confer stability, i.e. the solution or dispersion will resist agglomeration. When the potential is low, attraction exceeds repulsion and the dispersion will break and flocculate.
In nanotoxicology, zeta potential (surface charge) plays a key role in determining (1) the degree of colloidal interaction, which is itself a function of the pH and ionic strength of the bulk solution, and (2) the bioavailability of a compound when considering mass transport through charged membranes as related to exposure.
Zeta potential is not measurable directly, but it can be calculated using theoretical models and an experimentally determined electrophoretic mobility or dynamic electrophoretic mobility. It depends on the nature of the nanomaterial and on the solution in which the nanomaterial is suspended.

[2] ASTM International (2006) distinguishes between agglomeration and aggregation of nanoparticles as follows. An agglomerate is a group of particles held together by relatively weak forces (such as van der Waals force) that can be broken apart. An aggregate is a discrete group of particles composed of individual components that are tightly bonded together and not easily broken apart. (These definitions are consistent with those used under REACH, as described in Section 4.1.1.) This report adheres to these definitions with one exception. The definitions of agglomerate and aggregate are not consistently reflected in the scientific literature. The authors of this report have, when citing the literature, used the terminology as used in the publications cited. Otherwise this report adheres to the definitions provided by ASTM International.

3.1.4 Activity and Reactivity

"Nanosizing" can affect the flammability and explosivity of a particle, and can also influence its reactivity and photoreactivity.

3.1.4.1 Physical hazards: flammability, autoflammability and explosiveness

Physical hazards from flammability, autoflammability and explosiveness can increase at small particle sizes. These terms are defined as follows (ECHA, 2008):

- Flammability refers to two phenomena. A substance is considered pyrophoric if it spontaneously ignites upon exposure to air. A substance may also be classified as flammable if it becomes spontaneously flammable or emits flammable gases in dangerous quantities upon contact with water.
- Autoflammability is determined by assessing the auto-ignition temperature, which is the lowest temperature at which a substance will ignite under defined test conditions.
- Explosivity is the tendency of a substance to undergo violent and rapid decomposition, under appropriate conditions, to produce heat and gas.

Published information on these phenomena relative to nanomaterials is relatively limited and tends to focus on autoflammability and explosivity, as well as the assessment of combustion time and temperature.
The combustion rate increases with smaller particle sizes with an optimal combustion at particle diameters of approximately 10 to 15 µm, according to one authority (Eckhoff, 2003, as cited in Pronk et al., 2009).
The increase in combustibility with a decrease in particle size is illustrated by a study of the ignition of aluminium particles of 100 nm and 6.5 µm in diameter. The experiments determined ignition temperatures of 1,350 and 2,100 K, respectively (Huang et al., 2007).
For solids, the self-ignition temperature will also depend on the particle size (ECHA, 2008).
In general, dust explosions may occur when the particle diameter is smaller than 1 to 0.1 mm. One recent study (Worsfold et al., 2012) found that the relationship between particle size and explosivity is not straightforward. As the particle size decreases and the specific surface area increases, the degree of explosiveness tends to increase. However, if the nanoparticles do not disperse readily in air (the property of dustiness) or tend to agglomerate rapidly, these phenomena tend to counter the increase in explosivity with decreasing primary particle size. Worsfold et al. made three observations regarding the explosivity of nanoparticles:

- The minimum explosion concentration does not seem to change significantly with decreasing particle size;
- The minimum ignition energy decreases with decreasing particle size; and
- The minimum ignition temperature decreases with decreasing particle size.

Worsfold et al. illustrated their points with data for aluminium particles. Micron-sized powders ignited in air at 610 °C, while the nanopowders ignited at 100 °C. The increase in ignitability/explosivity becomes more significant at a particle size of less than 10 nm. For organic materials, explosivity may become more pronounced for particle sizes of less than approximately 10 µm.

3.1.4.2 Reactivity

Reactivity (including redox-activity and the ability to generate reactive oxygen species, or ROS) can increase with decreasing particle size. Decreasing the particle size affects the surface free energy. Reactivity is further increased due to surface atoms being less stable and the ability to form bonds increases with decreasing size, due to the higher surface free energy (JRC, 2011). "Nanosizing" can markedly affect reactivity. For example, gold, which is inert at the non-nanoscale, becomes an effective oxidation catalyst when the particle size is reduced to a few nanometres (Auffan et al., 2009). The A1 synthesis of the literature found the following size dependence of this property: maximum catalytic activity generally occurs at 15 - 20 nm.

The effect of particle size on reactivity can be particularly important for certain metals and metal oxides that act as semiconductors. A review of the nomenclature for the energetic structure of atoms provides a context for this discussion. The band gap is defined as the difference in energy between the top of the valence band (E_v) of electrons and the bottom of the conduction band (E_c) and is measured in electron volts (eV). The valence band is defined as the highest range of energies containing electrons at absolute zero. The conduction band is defined as the range of energies required to free an electron from its atomic orbital so that it is free to move within the atomic lattice. E_c represents the lowest unoccupied molecular orbital that participates in electron transfers to and from a metal oxide surface (Zhang et al., 2013). Band gap values for metal oxides can be calculated or measured by ultraviolet-visible spectroscopy.

Semiconductor compounds such as metal oxides can transfer electrons to redox-active substances in solution or within a cell, depending on the similarities in the energetic states of the nanomaterials and the redox-active substances. If the band gap energy of the redox-active nanomaterial is exceeded, excited electrons are generated in the conduction bands and electron holes will occur in the valence bands. The excited electrons and electron holes can readily engage in redox reactions. Burello and Worth (2011) hypothesised that nanoparticles larger than 20 to 30 nm do not have surface states in the band gap and behave like non-nanomaterials.

3.1.4.3 Photoreactivity

Photocatalytic activity can also increase with decreasing particle size. As described by one authority (U.S. EPA, 2011), "photoactivity refers to the generation of electron-hole pairs by nanomaterials exposed to light. These electron-hole pairs can produce free-oxygen radicals, which

results in oxidation or reduction of molecules in contact with their surfaces." Experimental data have indicated that some nanoparticles may, by virtue of their relatively large surface area and reactive potential, become activated by light (SCENIHR, 2009). Photocatalytic activity is highly material-dependent. Within materials, it is size-dependent (SCENIHR, 2010). Based on the literature reviewed for the A1 project, data from studies of TiO_2, CdS, and Au and various Au composites generally showed that photoreactivity increased with decreasing particle size. In some cases, the behaviour of the material changed at a particle size of approximately 5 to 10 nm. For example, some studies of TiO_2 showed that photoreactivity reached a maximum at a particle size of approximately 7 to 11 nm and decreased at smaller sizes. A study of CdS showed that particles above 6 nm in size were not photoreactive at all, but that smaller particles effectively catalysed the dehydrogenation of methanol. In summary, the A1 synthesis of the literature found that maximum photocatalytic activity generally occurred at 5 – 10 nm.

3.1.4.4 Other parameters

Other parameters may be used to characterise nanomaterials but were not judged to be crucial to a testing strategy for most nanomaterials. The following parameters are not discussed in detail in this report because their influence on environmental fate and the effects of exposure are not clear or do not appear to be of primary importance.

- Crystallite size. A crystallite is a part of a larger piece of material that has the same crystal structure and orientation (OECD, 2010). While the crystallite size may influence the behaviour of a nanomaterial, its relationship to the environmental fate and (eco)toxicity effects discussed in this report is unclear.
- Octanol water partition coefficient. OECD (2010) initially proposed that this parameter should be used to characterise the potential for a nanomaterial to partition into lipids and therefore bioaccumulate. More recently (OECD, 2014), an expert group has concluded that this parameter is not suitable for predicting bioaccumulation.
- Pour density. OECD (2010) has identified pour density, which is the apparent density of a bed of material formed in a container of standard dimensions when a specified amount of the material is introduced without settling, as a critical parameter to characterise nanomaterials. However, the relationship between this parameter and the environmental fate and (eco)toxicity effects discussed in this report is unclear.
- Magnetism. A magnetic attraction between certain materials, such as zerovalent nano-iron, can contribute to agglomeration (U.S. EPA, 2011) and thus influence net particle size and behaviour. Magnetism may be related to size (Park et al., 2007). This property is mentioned for completeness but not discussed in detail in this report, as it is a secondary consideration for most substances.

3.1.5 Summary

The following physico-chemical properties can affect the behaviour of a nanomaterial:

- Substance identity, including chemical composition, crystalline structure, surface coating, functionalisation and capping agents, all of which influence surface charge and reactivity;
- Particle characteristics, including size, porosity, surface area (which depends on particle size and porosity) and aspect ratio (shape), all of which generally influence mobility and transport;
- Fundamental transport behaviour, which reflects characteristics of the nanoparticle and of the medium, and depends on water solubility (rate of dissolution and equilibrium concentration, both size-related), tendency to agglomerate, zeta potential, dispersiveness and dustiness; and
- Activity and reactivity.

It is important to note that these parameters can influence not only the toxicity and ecotoxicity of a nanomaterial, but also the interactions between the nanoparticle and the environment, whether external or within an organism.

3.2 Data and Measurement Requirements

In keeping with the objectives and scope of this study, this section of the report discusses minimal data and measurement requirements relevant for existing (OECD) test guidelines and provides an overview of the available information for the high-priority characteristics. Table 4 summarises information on the availability of standardised test methods for critical parameters.

"Nanosizing" can alter the relative reactivity of certain materials, as discussed previously in this report. Unlike many of the parameters listed in Table 4, reactivity does not readily lend itself to a standard test. But that general property, which underlies the commercial development of some nanomaterials, may well be known early in the characterisation of a nanomaterial. More specifically, the ability to generate reactive oxygen species (ROS) or induce an organism to generate ROS is a critical determinant of the effect of exposure to nanomaterials. Braakhuis et al. (2014) stated that surface reactivity might be the most important nanoparticle characteristic determining their effect; while this statement pertained specifically to the effects of inhalation, particle reactivity is also vitally important to the mode of action via other routes of exposure. Braakhuis et al. (2014) identified several techniques that can be used to characterise chemical reactivity "cell-free" and biochemical reactivity. In cell-free conditions, one can measure the oxidation potential of nanoparticles by electron spin resonance (ESR) techniques. "These techniques use a spin-trapping agent to detect the nanoparticle-elicited generation of hydroxyl radicals in the presence of hydrogen peroxide." Such testing does not perfectly predict the reactions within a cell. *In vitro* assays can provide information about the reactivity of nanoparticles within a cell, although no single validated assay is

appropriate for all types of nanomaterials. Testing options to determine the intracellular induction of ROS include the following (Braakhuis et al., 2014):

- ESR techniques in combination with *in vitro* cellular exposure;
- 2'-7'-dichlorodihydrofluorecein diacetate (DCFH-DA) assay, which uses a fluorescent probe to visualise the induction of ROS in cells exposed to nanoparticles;
- Free radical analytical system (FRAS) assay, which measures the formation of reactive oxygen metabolites (ROM) after exposure to nanoparticles;
- Erythrocyte haemolysis assay, which measures the amount of haemoglobin released after exposure of red blood cells to nanoparticles;
- Vitamin C yellowing assay, which measures the chemical reactivity of nanoparticles toward an anti-oxidant.

Table 4 Data and Measurement Guidelines

Characteristics identified as likely to be important to grouping/ read-across	**OECD (2010) Recommendations for nanomaterial characterisation**	**Available testing method(s) per**		
		OECD (2010)	**ECHA (2012a)**	**OECD (2014b)**
Particle size: Agglomeration/ aggregation	Agglomeration/ aggregation	• Transmission electron microscopy (TEM), mean particle size for powder (solids) • PCS/DLS, for mean particle size (liquid dispersions) • BET Surface area, mean particle size for solids • Small-angle X-ray scattering (SAXS), mean particle size • Scanning mobility particle size spectrometry (SMPS), mean particle size for aerosols	A number of parameters have been proposed for assessing agglomeration or aggregation state, including: • effective cross-section, determined by measuring aerodynamic/Light scattering properties or by electron microscopy; • average agglomeration number (AAN), which is derived from the ratio of the volume-based median particle size to the average equivalent spherical volume derived from BET gas adsorption.	New test guideline needed for nanomaterials.
---	Crystallite phase	• X-Ray Diffraction (XRD) • Electron diffraction	No information provided	

Characteristics identified as likely to be important to grouping/ read-across	OECD (2010) Recommendations for nanomaterial characterisation	Available testing method(s) per		
		OECD (2010)	ECHA (2012a)	OECD (2014b)
---	Crystallite size[1]	• Atomic force microscopy • Transmission electron microscopy (TEM) • Scanning electron microscopy (SEM) • X-Ray diffraction (XRD)	No information provided	
---	Dustiness	• EN 15051:2006 • DIN 33897-2 • Vortex shaker method	• Rotating drum method (prEN 15051-2)	

Characteristics identified as likely to be important to grouping/ read-across	OECD (2010) Recommendations for nanomaterial characterisation	Available testing method(s) per		
		OECD (2010)	**ECHA (2012a)**	**OECD (2014b)**
---	Octanol-water partition coefficient (P_{OW})	• OECD TG 107 (Partition Coefficient (n-octanol/water): Shake Flask Method • OECD TG 123 (Partition Coefficient (1-Octanol/Water): Slow-Stirring Method	OECD concluded that the following test guidelines might be applicable for nanomaterials under certain circumstances or to some classes of manufactured nanomaterials, although further work is required to make this determination and to modify these guidelines, if necessary: • OECD TG 107 (Partition Coefficient (n-octanol/water): Shake Flask Method • OECD TG 117 (Partition Coefficient (n-octanol/water): HPLC Method • OECD TG 123 (Partition Coefficient (1-Octanol/Water): Slow-Stirring Method Results might be impacted upon by the presence of a colloidal suspension, which could be present if the manufactured nanomaterial does not completely dissolve.	The expert group concluded that K_{OW} (P_{OW}) is not suitable for predicting bioaccumulation and is not an appropriate endpoint for the physico-chemical characterisation of nanoparticles.

Characteristics identified as likely to be important to grouping/ read-across	OECD (2010) Recommendations for nanomaterial characterisation	Available testing method(s) per OECD (2010)	ECHA (2012a)	OECD (2014b)
Particle size (distribution)	Particle size distribution (PSD) or granulometry (dry and in relevant media)	No standard methods available	• Optical microscopic examination • Sieving • Sedimentation (gravitational settling) • Electrical Sensing Zone (e.g. Coulter) method • Phase Doppler Anemometry • Transmission Electron Microscopy (TEM) • Scanning Electron Microscopy (SEM) • Centrifugal Sedimentation (ISO 13318-1:2001; ISO 13318-2:2007; ISO 13318-3:2004) • Ultrasonic spectroscopy (ISO/20998-1:2006) • Small Angle X-ray Scattering (SAXS) (ISO/TS 13762:2001) • X-ray diffraction (XRD) (BS EN 13925-1, BS EN 13925-2 and BS EN 13925-3) • Dynamic Light Scattering (DLS)/Photon Correlation Spectroscopy (PCS) (ISO/22412:2008; ISO/13321:1996; ASTM E2490 – 09)	

Characteristics identified as likely to be important to grouping/ read-across	OECD (2010) Recommendations for nanomaterial characterisation	Available testing method(s) per OECD (2010)	ECHA (2012a)	OECD (2014b)
Reactivity (Photocatalytic activity)	Photocatalytic activity	No standard methods available	No information provided	
Particle size/shape: Porosity	Porosity	• ISO 15901 Part 1 (mercury porosimetry) • ISO 15901 Part 2 (mesopore analysis by gas adsorption) • ISO 15901 Part 3 (micropore analysis by gas adsorption) • Dye absorption	<u>Shape/morphology:</u> • Transmission Electron Microscopy (TEM) (ISO/TR 27628:2007. ISO 13322-1:2004 and ISO 13322-2:2006) • Scanning Electron Microscopy (SEM) (ISO/TR 27628:2007. ISO 13322-1:2004 and ISO 13322-2:2006) • Scanning Probe Microscopy (SPM) (ISO TR/27628:2007) • Optical microscopic examination No information on porosity is provided	

Characteristics identified as likely to be important to grouping/ read-across	OECD (2010) Recommendations for nanomaterial characterisation	Available testing method(s) per OECD (2010)	ECHA (2012a)	OECD (2014b)
---	Pour density	• CEN Technical Committee (CEN/TC) 184; and • ISO Technical Committee (ISO/TC) 206 may publish relevant methods including nanoparticle 0702: Fine ceramics (advanced ceramics, advanced technical ceramics) Determination of bulk density of ceramic powders: Part. 2 Untapped density • ASTM D1513-05 to determine the Pour Density of Carbon Black may also be informative.	No information provided	
Reactivity (Radical formation potential)	Radical formation potential	No standard methods available	No information provided	
Reactivity (Redox potential)	Reduction/ oxidation potential (redox)	• Electrochemical experiments with electrode and potentiometer	No information provided	

Characteristics identified as likely to be important to grouping/ read-across	OECD (2010) Recommendations for nanomaterial characterisation	Available testing method(s) per OECD (2010)	ECHA (2012a)	OECD (2014b)
Specific surface area (SSA)	Specific surface area (SSA)	• ISO 9277:1995 (Brunauer, Emmett, and Teller method)	• ISO 9277:1995 (Brunauer, Emmett, and Teller method) – (applicable to adsorption isotherms of type II [disperse, nonporous or macroporous solids] and type IV [mesoporous solids, pore diameter between 2-50 nm]) • ISO 18757:2005 (applicable for determination of the total specific external and internal surface area of disperse or porous [pore diameter > 2 nm] fine ceramic materials.)	

Characteristics identified as likely to be important to grouping/ read-across	OECD (2010) Recommendations for nanomaterial characterisation	Available testing method(s) per OECD (2010)	ECHA (2012a)	OECD (2014b)
Substance identity (surface coating/ functionalisation)	Surface chemistry	• Chemical methods that compare the un-functionalised material with the functionalised material. • Physical methods such as electron energy loss spectroscopy (EELS) and, depending on the dimensions of the particles, possibly x-ray photoelectron spectroscopy (XPS) or Auger electron spectroscopy (AES). • Functionalised probe atomic force microscopy (AFM) might also be possible and could allow the location of the functionalisations to be determined. • More particle methods are available for ash content, TGA, TG-MS, total carbon content.	No information provided	

Characteristics identified as likely to be important to grouping/ read-across	OECD (2010) Recommendations for nanomaterial characterisation	Available testing method(s) per OECD (2010)	ECHA (2012a)	OECD (2014b)
---	Transmission Electron Microscopy (TEM) picture(s)	• Transmission Electron Microscopy (TEM) • Scanning Electron Microscopy (SEM)	• Transmission Electron Microscopy (TEM) (ISO/TR 27628:2007; ISO/13322-1:2004; ISO/13322-2:2006) • Scanning Electron Microscopy (SEM) (ISO/TR 27628:2007; ISO/13322-1:2004; ISO/13322-2:2006)	

Characteristics identified as likely to be important to grouping/ read-across	OECD (2010) Recommendations for nanomaterial characterisation	Available testing method(s) per OECD (2010)	ECHA (2012a)	OECD (2014b)
Solubility (rate of dissolution or equilibrium water solubility)	Water solubility/ Dispersibility	• OECD TG105 (water solubility)	**For nanomaterials with hydrolysis half-lives <48 hours[2]:** • Measure solubility at one loading rate and short equilibration time using shake flask method **For nanomaterials with hydrolysis half-lives ≥48 hours:** <u>For solids nanomaterials:</u> • Column elution method to determine water solubility <u>For non-solid nanomaterials:</u> • First, use shake flask method to determine equilibration time • Second, measure water solubility (methods depend on result of equilibration test)	New test guideline needed for nanomaterials. Dissolution tests must be conducted under different conditions (pH 4-7-9, with and without organisms, with or without natural organic matter/proteins, filtration.
Zeta potential (surface charge)	Zeta potential (surface charge)	• Measure electrophoretic mobility and calculate zeta potential	No information available	

[1] Note that crystallite size is not synonymous with particle size (ECHA, 2012a, p. 16).
[2] This summary is adapted from a flow chart that ECHA (2012a) presented as Figure R.7-1.1.

4 Background on Read-Across and Grouping

The physico-chemical characteristics of nanomaterials can change their behaviour, affecting such fundamental properties as solubility, reactivity, environmental transport and toxicokinetics. As scientists from RIVM have observed, this phenomenon has raised concerns that some of these materials may introduce new risks for humans or the environment (Bleeker et al., 2013). In order to fully characterise these risks, a significant amount of new data will need to be generated, which could involve a large number of test animals and be cost-prohibitive (Choi et al., 2009).
Society has faced a similar challenge in characterising conventional chemical substances and has developed scientific bases for maximising the use of available data. As described by the OECD (2007),

> *For reasons of resources and animal welfare, it is important to limit the number of tests to be conducted, where this is scientifically justifiable. One approach is to consider closely related chemicals as a group, or chemical category, rather than as individual chemicals. In the category approach, not every chemical needs to be tested for every required endpoint. Rather, the data for chemicals and endpoints that have been tested are used to estimate the corresponding properties for the untested chemicals and endpoints.*

The European Commission (EC) reflected on this approach in Annex XI of REACH, noting that:

> *Substances whose physicochemical, toxicological and ecotoxicological properties are likely to be similar or follow a regular pattern as a result of structural similarity may be considered as a group, or 'category' of substances. Application of the group concept requires that physicochemical properties, human health effects and environmental effects or environmental fate may be predicted from data for reference substance(s) within the group by interpolation to other substances in the group (read-across approach). This avoids the need to test every substance for every endpoint.*

More information on the approach to read-across under REACH and initial published opinions on read-across and grouping for nanomaterials are described briefly below. In order to read-across between substances, one must start by being clear about the identity of each substance.

4.1 Substance Identification

Regulatory definitions provide the foundation for substance identification and are summarised briefly below, followed by a comparison of the parameters identified in this project and the characteristics required for substance identification.

4.1.1 Regulatory Guidelines

REACH specifies the parameters used to identify a substance in Annex VI section 2:

> *2. IDENTIFICATION OF THE SUBSTANCE*
>
> *For each substance, the information given in this section shall be sufficient to enable each substance to be identified. If it is not technically possible or if it does not appear scientifically necessary to give information on one or more of the items below, the reasons shall be clearly stated.*
>
> *2.1. Name or other identifier of each substance*
>
> *2.1.1. Name(s) in the IUPAC nomenclature or other international chemical name(s)*
>
> *2.1.2. Other names (usual name, trade name, abbreviation)*
>
> *2.1.3. EINECS or ELINCs number (if available and appropriate)*
>
> *2.1.4. CAS name and CAS number (if available)*
>
> *2.1.5. Other identity code (if available)*
>
> *2.2. Information related to molecular and structural formula of each substance*
>
> *2.2.1. Molecular and structural formula (including SMILES notation, if available)*
>
> *2.2.2. Information on optical activity and typical ratio of (stereo) isomers (if applicable and appropriate)*
>
> *2.2.3. Molecular weight or molecular weight range*
>
> *2.3. Composition of each substance*
>
> *2.3.1. Degree of purity (%)*
>
> *2.3.2. Nature of impurities, including isomers and by-products*
>
> *2.3.3. Percentage of (significant) main impurities*
>
> *2.3.4. Nature and order of magnitude (… ppm, … %) of any additives (e.g. stabilising agents or inhibitors)*
>
> *2.3.5. Spectral data (ultraviolet, infrared, nuclear magnetic resonance or mass spectrum)*
>
> *2.3.6. High-pressure liquid chromatogram, gas chromatogram*
>
> *2.3.7. Description of the analytical methods or the appropriate bibliographical references for the identification of the substance and, where appropriate, for the identification of impurities and additives.*

Adaptation of this paradigm to nanomaterials begins with the definition of a nanomaterial. Commission Recommendation 2011/696/EU defines a nanomaterial as follows (European Commission, 2011):

2. *'Nanomaterial' means a natural, incidental or manufactured material containing particles, in an unbound state or as an aggregate or as an agglomerate and where, for 50 % or more of the particles in the number size distribution, one or more external dimensions is in the size range 1 nm-100 nm.*

   In specific cases and where warranted by concerns for the environment, health, safety or competitiveness the number size distribution threshold of 50 % may be replaced by a threshold between 1 and 50 %.

3. *By derogation from point 2, fullerenes, graphene flakes and single wall carbon nanotubes with one or more external dimensions below 1 nm should be considered as nanomaterials.*

4. *For the purposes of point 2, 'particle', 'agglomerate' and 'aggregate' are defined as follows:*

   (a) 'particle' means a minute piece of matter with defined physical boundaries;

   (b) 'agglomerate' means a collection of weakly bound particles or aggregates where the resulting external surface area is similar to the sum of the surface areas of the individual components;

   (c) 'aggregate' means a particle comprising of strongly bound or fused particles.

5. *Where technically feasible and requested in specific legislation, compliance with the definition in point 2 may be determined on the basis of the specific surface area by volume. A material should be considered as falling under the definition in point 2 where the specific surface area by volume of the material is greater than 60 m^2/cm^3. However, a material which, based on its number size distribution, is a nanomaterial should be considered as complying with the definition in point 2 even if the material has a specific surface area lower than 60 m^2/cm^3.*

In short, this definition specifies that a nanomaterial falls within a certain particle size range and may be characterised by a surface area that corresponds to the specified particle size range.
The European Commission's Joint Research Centre's first REACH Implementation Project on Nanomaterials (RIP-oN 1) pertained to "Substance identification of nanomaterials". The objectives of the project were to evaluate the applicability of existing guidance and, if warranted based on that review, to develop specific advice on how to establish the substance identity of nanomaterials. (European Commission Joint Research Centre Institute for Health and Consumer Protection, 2011)
The project evaluated various parameters that might be used to identify nanomaterials, including:

- Size;
- Surface treatment;

- Physico-chemical characteristics (i.e. solubility/dispersibility, photocatalytic/optical properties, surface energy/redox radical formation, density); and
- Geometrical characteristics (i.e. agglomeration/aggregation, specific surface area (SSA), and shape, including aspect ratio).

The European Commission has described the results of RIP-oN 1 as follows (European Commission, 2012):

> *The opinions of the participating experts … diverged on several key issues, including whether size or surface treatment/functionalisation should affect substance identification or the characterisation of physicochemical properties. It was not possible to reconcile these opinions. Therefore, the report mainly describes options/approaches rather than providing explicit recommendations. ECHA has been asked to develop such recommendations as it starts gaining practical experience through the evaluation of relevant registration dossiers.*

ECHA has subsequently published endpoint-specific guidance on testing for nanomaterial characterisation, including endpoints relevant to substance identification (e.g. ECHA, 2012a). GAARN (ECHA, 2012b) has observed that data may need to be generated using non-standard methods in order to characterise nanomaterials, particularly with respect to particle size. Relevant information is reflected in Table 4.
The European Commission reportedly plans to provide new rules on the registration requirements for nanomaterials under REACH in 2015. The following points may be introduced into REACH annexes, according to an early report on the actions under consideration (Paun, 2014):

- A legally-binding definition of nanomaterials;
- An explanation of what is meant by the "form" of a nanomaterial;
- Requirements for explaining the relevance of information in the dossier to the nanoforms of the substance; and
- Requirements to submit data regarding the nanomaterial name, particle distribution, surface treatment, shape, morphology, surface area and test conditions.

4.1.2 Comparison

Table 5 below provides a succinct comparison between the requirements for substance identification under REACH as of this writing and the properties or characteristics of nanomaterials identified in this project as potentially being critical to read-across or grouping strategies. It includes only promulgated regulatory requirements and therefore does not reflect the work of RIP-oN 1 or proposed information requirements for nanomaterials. As noted above, modifications to REACH annexes currently under discussion may require the submittal of information about a nanomaterial's particle size distribution, surface treatment, shape, morphology, and surface area (Paun, 2014).

Table 5 Comparison between Characteristics Relevant to Read-across or Grouping and SID

REACH Substance Identification per Annex VI section 2	**Primary Characteristics Identified in Current Project[1]**
Name or other identifier of each substance	Name or other identifier of each substance is of fundamental importance
Information related to molecular and structural formula of each substance	Information related to molecular and structural formula of each substance is of fundamental importance
Composition of each substance • Degree of purity (%) • Nature of impurities • % of main impurities • Nature and order of magnitude of any additives (e.g. stabilising agents or inhibitors) • Spectral data • High-pressure liquid chromatogram, gas chromatogram • Description of the analytical methods or appropriate bibliographical references	Composition of the nanoparticle and its surface functionalisation if any, is of fundamental importance. In addition to information about impurities, the composition of surface coatings or stabilising or capping agents can also be critical. Spectral or chromatography data may or may not be relevant to a particular nanomaterial, and may not apply to solid particles without extensive sample preparation. Documentation of analytical methods is particularly important when standard guidelines are not available.
---	Particle size (primary and agglomerated)
---	Particle shape (diameter, length, porosity)
---	Surface charge/zeta potential

[1] As discussed in this report, solubility (in particular, whether 'nanosizing' has influenced the rate of dissolution) and reactivity are also critical physico-chemical parameters. However, they do not pertain to substance identification per se.

4.2 Read-Across and Grouping under REACH

The following is an overview of read-across and grouping principles, followed by a summary of published views on applying these principles to nanomaterials.

4.2.1 Overview

REACH Annex XI defines the basic precepts for defining the similarities between substances in order to support read-across and grouping:

> *The similarities may be based on:*
>
> *(1) a common functional group;*

(2) the common precursors and/or the likelihood of common breakdown products via physical and biological processes, which result in structurally similar chemicals; or

(3) a constant pattern in the changing of the potency of the properties across the category. …

In all cases results should:

- *be adequate for the purpose of classification and labelling and/or risk assessment,*
- *have adequate and reliable coverage of the key parameters addressed in the corresponding test method referred to in Article 13(3),*
- *cover an exposure duration comparable to or longer than the corresponding test method referred to in Article 13(3) if exposure duration is a relevant parameter, and*
- *adequate and reliable documentation of the applied method shall be provided.*

ECHA (2009) has developed practical guidelines for read-across, which are summarised in Figure 5.

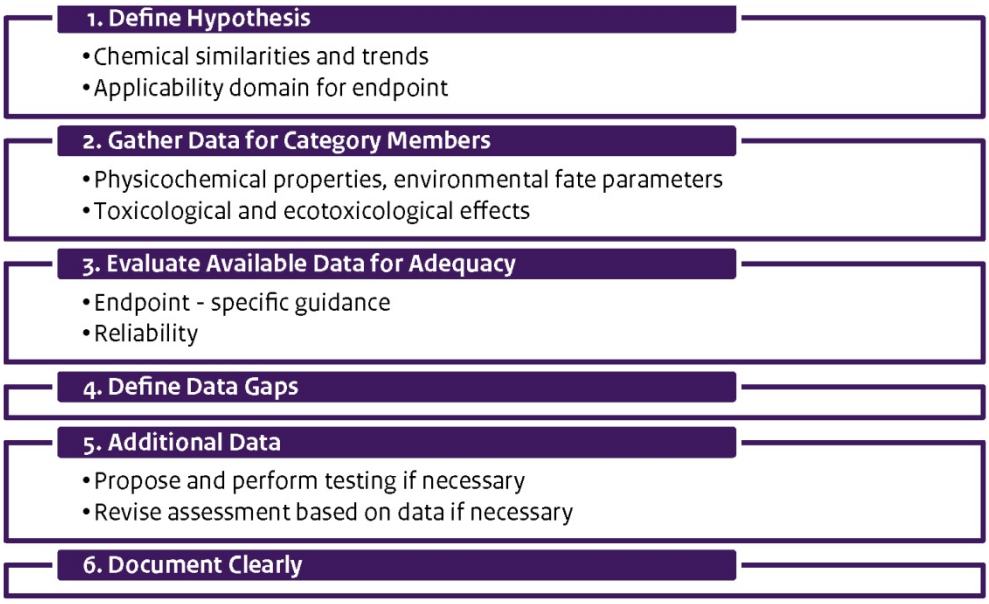


Figure 5 ECHA Guidelines for Read-across and Grouping

A 2012 workshop on the 'Use of Read-Across for Chemical Safety Assessment under REACH', organised by ECHA with the active support of Cefic LRI, discussed practical experiences and lessons learned regarding read-across under REACH (Patlewicz et al., 2013; CEFIC, 2013?; Stone et al., 2013). Among the important concepts that emerged from that meeting were the following:

- The primary factors that affect the uncertainty of a read-across prediction (or, conversely, confidence in that prediction) were the experimental data used, the chemical similarity on which the grouping was based and the weight of evidence supporting the categorisation scheme.
- Factors that indicate whether or not substances are similar to each other include: the presence of similar functional groups, biomodification, a constant pattern of changes in a particular property across a category, common chemical reactions, and two-dimensional molecular similarity. (Note that this discussion pertained to "conventional" chemicals and not to nanomaterials.)
- Knowledge of how a chemical interacts with a biological system or, more specifically, the mode of action for a particular endpoint is fundamentally important.
- Toxicokinetic data help to substantiate read-across.

4.2.2 Considerations on Read-Across and Grouping Related to Nanomaterials

The Competent Authority Subgroup on Nanomaterials (CASG-nano) has discussed read-across and grouping as one of the key issues to amending the REACH annexes to ensure clearer registration of nanomaterials (Jones, 2013). GAARN has offered the following initial conclusions relevant to read-across and grouping for nanomaterials (ECHA 2013a; ECHA 2014):

- Dossiers should reflect thorough characterisation of the physico-chemical properties of nanomaterials.
- Read-across cannot be based solely on the chemical composition of a nanomaterial. Physico-chemical parameters such as aspect ratio, shape, form, solubility, surface area, surface charge (zeta potential), surface treatment, etc. "should provide a reliable dataset to support a sound scientific interpretation of the similarities or differences among (nano)forms" (ECHA, 2013a).
- The similarity rules specified in Annex XI of REACH should be used as a basis for grouping nanomaterials.
- Toxicokinetics data might also need to be considered in relation to read-across approaches or when extrapolating from *in vitro* to *in vivo* situations. Where evidence indicates the systemic translocation of nanoparticles, further investigation of absorption, distribution, metabolism and excretion should be considered. Toxicokinetic data, if available, should also be considered in the testing strategies for environmental endpoints to the extent that data from mammalian studies are relevant to non-mammalian species.
- The hypothesis, or basis for the grouping, should be used to define what nanomaterial characteristics distinguish a category. Similarity rules might be used individually and are case-dependent, but a category (and similarity) may be justified on more than one basis. "The hypothesis will help to show if the grouping applies to the category members for either environmental or toxicological endpoints or both, and whether it

> is adequate for all routes of exposure and duration of effects" (ECHA, 2013a).

The first substance evaluation decision for a nanomaterial under the Community Rolling Action Plan (CoRAP) provisions of REACH was adopted in December 2014 (Davies, 2014). This precedential decision on silicon dioxide focused on grouping different nanoforms in order to minimise testing for physicochemical properties. The decision was reached to focus on two key properties: surface area and surface chemistry, i.e. the level of hydroxylation (number of –OH groups).

4.3 Testing strategies and grouping of nanomaterials

Various authorities have developed opinions regarding testing strategies and the grouping of nanomaterials, as described briefly below.

4.3.1 OECD Guidance on Grouping of Chemicals: Initial Considerations Applicable to Manufactured Nanomaterials

OECD (2014a) recently offered the following opinions regarding the grouping of nanomaterials. Five factors complicate efforts to characterise the behaviour of nanomaterials within the context of grouping nanomaterials:

- Nanomaterials share properties associated with both solutes and separate particle phases;
- Challenges of measuring and characterising nanoparticles in environmental and biological matrices;
- Challenges of preparation and testing procedures for bioavailability and (eco)toxic effects;
- The number and variety of relevant physico-chemical characteristics (which include structure, size, shape, surface area, surface modification, surface reactivity and electronic properties, agglomeration state and water solubility); and
- Potential changes to physico-chemical characteristics during the material life cycle.

OECD has further noted the many initiatives to resolve such uncertainties and stated that:

> *At present, it seems premature to develop guidance on grouping specifically for nanomaterials. Nevertheless, research efforts will pave the way for common approaches and frameworks to grouping nanomaterials for purpose of hazard assessment in the future. In addition, expand further on why certain properties tend to elicit certain effects in vitro or in vivo and where opportunities may exist to group nanomaterials together to rationalise testing [sic].*

4.3.2 ITS Nano

The objective of the project Intelligent Testing Strategy for Engineered Nanomaterials (ITS Nano) was to deliver a responsive, flexible, agreed and intelligent testing strategy for engineered nanomaterials relevant to both human health and the environment (Heriot-Watt University,

undated). In the course of that work, the project team addressed the grouping and ranking of nanomaterials for risk assessment purposes (Stone et al., 2013; 2014). ITS Nano offered the following definitions (Stone et al., 2013):

> *… grouping simply refers to the arrangement of nanomaterials into groups based on common attributes. For risk assessment purposes, these groups must be based on an attribute that is relevant to risk such as a common hazardous physicochemical property, or an exposure potential that infers a greater harm or risk of exposure.*

ITS Nano identified the components required for the development of a grouping/ranking approach for nanomaterials and speculated on the time scale for developing these components as shown in Table 6.
In the view of ITS Nano, the following factors are of particular importance to grouping nanomaterials:

- The influence of particular physico-chemical parameters on exposure and dose, considering how those parameters may change throughout the life cycle;
- The mode of action, which may not currently be fully understood or detectable depending on the test methods used, must be considered so that grouping appropriately reflects conventional toxicology or, if necessary, new paradigms. The relationship between mode of action and physico-chemical characteristics is crucial;
- Linking hazard to physico-chemical properties will support the goal of limiting toxicity testing; and
- The weight of evidence and uncertainties must be carefully considered.

In light of these considerations, ITS Nano hypothesised that grouping may eventually reflect a functional approach, allowing for the ways in which nanomaterials can change throughout their life cycle. Three essential functions are:

- What they are (chemical composition, size, size distribution, specific surface area, crystalline phase, porosity);
- What they do (electron transfer, photoreactivity, catalytic activity, ROS production, ion release, mechanical resistance/fibres, dustiness); and
- Where they go (hydrophobicity/hydrophilicity, aggregation/agglomeration, surface charge, biodegradability, zeta potential, composition of the protein corona).

The latter two factors pertain to relevant environmental and biological compartments (i.e. where (eco)toxic effects would be exerted).
ITS Nano has noted that the future development of a functional and rational grouping approach is an area of key focus for the NanoSolutions project (Stone, et al., 2013).

Table 6 Components Needed to Develop a Grouping Approach Identified by ITS Nano[1]

Component Category	**Short Term (< 5 years)**	**Mid Term (5-10 years)**	**Long Term (10-15 years)**	**Distant Future (> 15 years)**
Physico-chemical identification	Determine base set of particle characteristics; *In situ* characterisation; Standard reference materials	Identify physico-chemical properties influencing internal dose		
Exposure identification	Standard protocols different matrices; Fate and behaviour	Multi-metric detection methods		
Hazard identification	Bioavailability/toxicokinetics; Mode of action; Dosimetry and dose metrics; Hazard models; Relevant *in vitro* and *in vivo* biomarkers	Short/long term, reversible/irreversible effects; Direct or indirect effects; Cohort/population relevant effects; Standard protocols		
Cross-cutting issues		Dosimetry; Interpretation of data; Minimum testing requirements	Life cycle/transformation; Integration of data; Integration into legal framework	

Component Category	Short Term (< 5 years)	Mid Term (5-10 years)	Long Term (10-15 years)	Distant Future (> 15 years)
Implementation into risk assessment framework			Prototype grouping/ranking procedures; Data quality; Weight of evidence	Dealing with uncertainty; Decision tree(s) for monitoring/testing; Use of human data
Final goal of integrated testing strategy			*In vitro-in vivo* extrapolation (IVIVE); Quantitative nanostructure activity relationship (QNAR); Grouping/ranking procedures	Risk assessment

[1] For a graphical depiction of this information, please see Figure 4-1 in Stone et al. (2013) at: http://www.nano.hw.ac.uk/images/ITS %20Nano %20FINAL %20VERSION %20website.pdf.

4.3.3 *NanoSafety Cluster Working Group 10*

The NanoSafety Cluster Working Group 10 has discussed a three-tiered toxicity testing scheme which is summarised in Figure 6 (Oomen et al., 2013; Byrne et al., 2014).

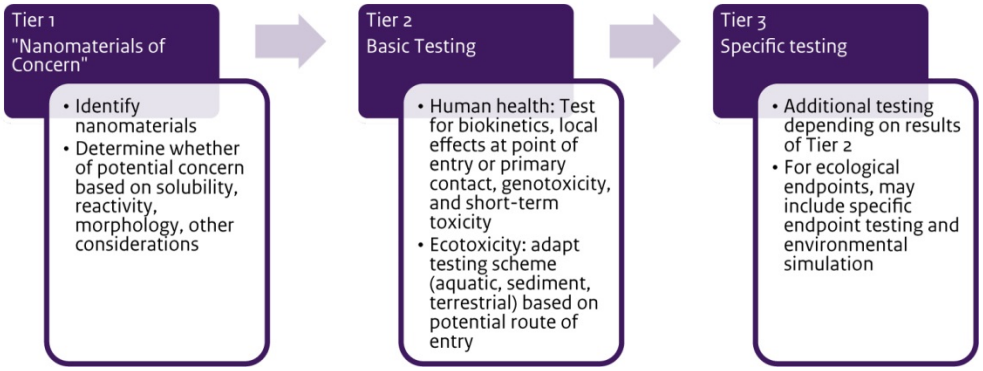


Figure 6 (Eco)Toxicity Testing Scheme Discussed by the NanoSafety Cluster Working Group 10

The NanoSafety Cluster Working Group 10 has noted both the importance and the challenges of developing read-across and grouping approaches to limit the need for testing (Byrne, 2014), noting that grouping could be based on similar biopersistence and biokinetic characteristics and/or similar or common biological effects. The grouping of nanomaterials by concern was also considered a potential route. The NanoSafety Cluster Working Group 10 offered the following recommendations regarding the grouping of nanomaterials:

- Define and validate scientifically sound grouping criteria based on:
  - Available data and material properties;
  - Biopersistence;
  - Fate;
  - Absorption, distribution, metabolism and elimination (ADME) or absorption, distribution, corona formation and elimination/deposition (ADCE); and
  - Toxic effects.
- Use quantitative structure activity relationships (QSAR), if applicable.

4.3.4 *Regulatory Cooperation Council's Nanotechnology Initiative*

The Regulatory Cooperation Council (RCC), comprising members from the U.S. and Canada, has formed a Nanotechnology Initiative that is developing a classification scheme to achieve two goals. Firstly, the work group seeks to identify which classes of nanomaterials typically require nanospecific considerations in risk assessments. Secondly, this work is intended to support the selection of appropriate analogue and/or read-across information to be used in substance-specific risk

assessments for nanomaterials. The summary which follows is based on reports of the draft output of that work (NIA, 2014; Chemical Watch, 2014; RCC, 2014).
RCC considered classification schemes based on exposures, use profiles, toxicological mode of action and physico-chemical properties, yet "acknowledge that sufficient comprehensive scientific knowledge does not yet exist to develop a validated classification scheme of nanomaterials…[but] nonetheless believe that a classification scheme for nanomaterials based on similarities in chemical composition is suitable, given the existing regulatory frameworks, and provides a good starting point from which to move forward" (RCC, 2014).
The proposed classification scheme is based on similarities in chemical composition and includes seven categories of nanomaterials.

Table 7 RCC Classification Scheme

Properties	**Carbon nanotubes**	**Inorganic carbon**	**Metal oxides and metalloid oxides**	**Metals, metal salts, and metalloids**	**Semi-conductor quantum dots**	**Organics**	**Other[1]**
Size		✓	✓	✓	✓	✓	
Shape		✓	✓	✓	✓	✓	
Length	✓						
Diameter	✓						
Number of Walls/ Number of Layers	✓	✓					
Chirality	✓						
Capped/Uncapped	✓						
Crystal Structure/Crystallinity			✓			✓	
Composition			✓	✓			
Surface Chemistry						✓	
Surface Reactivity			✓				
Surface Functionalisation	✓	✓	✓	✓	✓		
Chemical Modifications		✓					
Core Shell Composition					✓		
Oxidation States				✓			
Solubility			✓	✓	✓		
Nano-property Being Exploited						✓	

[1] "Other" includes emerging nanomaterials, such as metal alloys like tungsten carbide, nanoclays, and tubular structures of metals/metal salts/metalloids.

Table 7 indicates those categories and the physico-chemical parameters which RCC has noted "may be important in identifying whether two

nanomaterials share sufficient similarities to utilise read-across and/or analogue information" (RCC, 2014).
RCC noted that this scheme has the following limitations:

- Substances such as organic polymers and pigments do not have unique nanoscale properties/phenomena and so are not included in this scheme; and
- Hybrid nanomaterials, such as a carbon nanotube with a metal oxide surface modification, are not part of this classification scheme as they fall into multiple classes and must be evaluated on a case-by-case basis.

4.3.5 Nanomaterial Registry

The Nanomaterial Registry maintained by the U.S. National Institutes of Health (NIH) archives curated nanomaterial data on the physico-chemical characteristics, environmental interactions, and biological interactions of nanomaterials. Those data include information on several nanomaterials investigated by the National Institute of Standards and Technology (NIST).
The Nanomaterial Registry includes a software module to identify nanomaterials that are similar with respect to their properties and behaviour. The ultimate goal is to be able to predict the characteristics of new materials based on knowledge of existing materials. Similarity matching, in this model, depends on particle size, shape, surface chemistry and surface charge, as represented by the isoelectric point (i.e. the pH at which a particular molecule or surface carries no net electrical charge), according to the rules outlined below, which enable the software to score a match of between 10 % and 85 % (NIH, 2014).
If shape is defined for both nanomaterials, it must be equal, regardless of all other data.
If the nanomaterials have the same instance of characterisation for size and the instance of characterisation is not "as processed," the nanomaterials are a 30 % match.
If there is no size information, look for aggregation/agglomeration state.
If the nanomaterials have the instance of characterisation "as processed" for size and the techniques for characterisation are the same, the nanomaterials are only 22.5 % similar.
If the size values are within 10 %, those two nanomaterials are an additional 15 % match.
But if the size values are within 25 %, those two nanomaterials are only an additional 10 % match.
If both nanomaterials have the same material type for their most outward chemistry, they are an additional 25 % similar.
If isoelectric point value is within 10 % and the nanomaterials were characterised in the same way, another 15 % similarity can be added.
But if isoelectric point value is only within 25 % and the nanomaterials were characterised in the same way, only another 10 % similarity can be added.
The criterion that the database considered the material "as processed" reflects the recognition that the properties of nanomaterials can change

over time and when they are put into solution for testing. These rules determine similarity, in part, on the form of the nanomaterial that was actually tested.

4.3.6 SolNanoTox

The goal of the project SolNanoTox, which began in March 2014, is to group nanomaterials on the basis of specific properties and to allocate toxicological properties to these groups. The project will evaluate the role of solubility in determining the accumulation and potential toxic properties of nanomaterials. The work will also include *in vitro* tests and analysis of the accumulation of nanomaterials in biological samples, focusing at a cellular level on whether nanoparticles alter the structure of biomolecules. The project is scheduled to conclude in 2018 (Federal Institute for Risk Assessment, 2014).

4.3.7 Related Efforts with RIVM Participation

RIVM is also participating in the following work groups that are performing work related to this current project:

- MARINA, operating between 2011 and 2015, will address the four central themes in the risk management paradigm for engineered nanomaterials: Materials, Exposure, Hazard and Risk. MARINA looks at the grouping and categorisation of Nanomaterials in a more conceptual format. (RIVM scientists participated in the NanoSafety Cluster Working Group 10 on the work described above through engagement with MARINA.)
- NanoMILE intends to formulate a paradigm for the mode(s) of interaction between manufactured nanomaterials and organisms or the environment to create a single framework for the classification of manufactured nanomaterials based on potential toxicity. This will reflect the physico-chemical and biological properties required for safety evaluation. Work began in 2013 and will continue until 2017.
- NANoREG has four goals: (i) Provide technical information to regulators, (ii) Provide tools for risk assessment, characterisation, toxicity testing and exposure measurements, (iii) develop new testing strategies, (iv) establish collaboration amongst authorities, industry and science.
- GUIDEnano is an ongoing project for developing innovative methodologies to evaluate and manage the human and environmental health risks of nano-enabled products, considering the whole product life cycle.
- SUN focuses on Smart design of Nanomaterials, looking at health and safety parameters, including costs-benefits, following a life-cycle approach.

As these workgroups progress, their thinking may help to refine concepts presented in this report.

4.3.8 Summary of Work by Others

Adapting existing paradigms for read-across and grouping to nanomaterials presents several challenges. As discussed further in this report, scientists are still working to standardise test protocols for nanomaterials. Even when standardised tests are available to characterise a nanomaterial property, analysts recognise that the properties of a nanomaterial change over time. Certain nanoparticles dissolve; others sorb organic matter; and particle size can change as agglomeration occurs. These changes over the course of an experiment, which depend both on the nanomaterial itself and on the characteristics of the test solution, complicate efforts to test nanomaterials and to accurately group them or read-across between them.
Such challenges notwithstanding, scientists working in this field have devised several preliminary schemes for read-across and grouping. These schemes represent different concepts about how to group and test nanomaterials:

- ITS Nano focused on the functionality of nanomaterials (i.e. what they are, what they do and where they go);
- The Nano Safety Cluster Working Group 10 focused their testing scheme on effects, indicating that nanomaterials could be grouped based on (a) similar biopersistence, biokinetics and/or bioeffects or (b) by concern.
- RCC focused on chemical identity, specifically similarities in chemical composition.
- The Nanomaterial Registry determines similarity based on four specific physico-chemical properties: size, shape, material type and surface charge as represented by the isoelectric point.
- SolNanoTox will be focusing on solubility and certain biological parameters as a possible basis for grouping.

Notably, authorities such as the OECD and ITS Nano have recently stated that current scientific knowledge may not allow for the development of read-across and grouping concepts for nanomaterials in the short term.

5 Ecological effects and characteristics that may be critical to read-across

5.1 Evaluation

One of the main differences between human toxicity research on nanomaterials and research into ecological effects is that, in the latter case, the nanomaterial being tested interacts both with the environment (exposure medium) and with the organisms. Furthermore, a variety of types of organisms (bacteria, plants, invertebrates, vertebrates) in different types of environment (aquatic (freshwater/marine), sediment, terrestrial) are to be covered. Consequently, a multitude of interactions needs to be unravelled and understood before firm conclusions can be drawn.
For these reasons, it is not surprising that the evaluation presented below will show that many nanomaterial characteristics can have different, even opposite effects on toxicity, depending on the type of environment and organism under consideration. Firstly, an overview is given of the most important processes a nanomaterial may be subject to in the environment and the known interactions between nanomaterial characteristics and environmental factors that determine the material's behaviour. Secondly, the focus will be on toxic effects and modes of action (distinguishing between types of organisms, where relevant), with specific attention given to nanomaterial characteristics that have been reported to affect ecotoxicity in one way or another.

5.1.1 Environmental fate

The environmental fates to which nanomaterials may be subject are illustrated using the aquatic compartment (water column/sediment) as the main example. The interactions with living organisms (adsorption, internalisation, excretion, etc.) are also discussed from an environmental fate point of view (e.g. in view of the evaluation of bioconcentration, bioaccumulation and biomagnification).
The literature review that follows focuses on the aquatic compartment. Less information is available for sediment and soil specifically. The distribution between solid and aqueous components of these environmental compartments, as well as the speciation in the sediment/soil pore water, will largely determine how sediment/terrestrial organisms will most likely be exposed. This is also affected by the type of organism and its feeding behaviour.

5.1.1.1 Adsorption/desorption to suspended matter

Chemical substances (both organic and inorganic) can adsorb to suspended matter. Adsorption can be due to interactions with the organic parts of suspended matter, which is the predominant interaction for organic compounds. Nanomaterials may also interact with

(predominantly negative) binding sites on the inorganic parts of suspended matter (for inorganic compounds, e.g. interaction between cationic metal types and negative binding sites). Adsorption can result from van der Waals attraction, but is expected to be mainly affected by the ***interaction between pH and the surface charge*** of both suspended matter and nanomaterial. For organic nanomaterials or nanomaterials with organic coatings or functionalisation, the interaction, especially with the organic part of suspended matter, will strongly depend on the ***surface chemistry***. The process is considered to be reversible and prone to competition with other cations/substances in the environment.

5.1.1.2 Aggregation/agglomeration

Nanomaterials can agglomerate in the environment (Handy et al., 2011) to increase the net particle size. The tendency to agglomerate reflects the net van der Waals forces and may be countered by electrostatic repulsion between particles of like charge. The environmental factors affecting this behaviour include ***pH, ionic strength (of divalent ions, in particular) and conductivity, presence and type of natural organic matter (NOM), and temperature*** (Handy et al., 2011). ***Surface charge, surface reactivity, the presence of coatings or functionalisation*** (and structural features resulting in steric hindrance) are factors from the nanomaterial point of view that play an important role.

In the end, it is the interaction between the environmental factors and the nanomaterial characteristics that will determine whether or not aggregation/agglomeration will occur. For instance, the presence of hardness (cations) can interact with the surface chemistry of nanoparticles, favouring agglomeration. Counter-ions can depress the electric double-layer and lower the absolute zeta potential of colloidal nanoparticles and thereby affect the electrostatic interaction between nanoparticle and organisms. This will facilitate the aggregation of nanoparticles (Ma and Lin, 2013). Another example is when nanoparticles become coated with natural organic matter and form stable dispersions (hence not forming aggregates/agglomerates). The colloidal behaviour and the factors affecting aggregation are described in literature, e.g. by Handy et al. (2008a, 2011) and Shaw and Handy (2011) and others. Overall, nanoparticles tend to agglomerate more easily in hard water with high ionic strength and conductivity and low dissolved organic matter (DOC) concentrations. Therefore, agglomeration will occur more rapidly in marine water versus brackish water versus fresh water. In freshwater systems, high-DOC soft waters more easily give rise to stable dispersions of nanoparticles. However, as the surface charge and reactivity of nanoparticles are also important, caution should be taken about generalising the effects of water chemistry on nanoparticle behaviour (Handy et al., 2011).

Note that agglomerates may also capture organisms with them, e.g. interlinking bacterial or algal cells, or even through attachment to the

exterior of planktonic invertebrates, hampering normal swimming behaviour (Baun et al., 2008).

5.1.1.3 Sedimentation/re-suspension

The sediment could act as a sink for certain nanomaterials, either due to the direct sedimentation of nanoparticles (Baun et al., 2008) or the sedimentation of adsorbed or aggregated/agglomerated nanoparticles (Handy et al., 2011, Ma and Lin, 2013). The nanoparticles in the sediment can then have effects on zoobenthos (e.g. oysters) or on the embryos of fish (e.g. salmonids) (Handy et al., 2013; Ma and Lin, 2013).

5.1.1.4 Dissolution

For many nanomaterials, especially those containing metals, dissolution is considered to be one of the predominant processes determining their potential toxicity in the environment. In their review on the mechanisms of toxic action of Ag, ZnO and CuO nanoparticles in bacteria, algae, daphnids, fish, and mammalian cells *in vitro*, Ivask et al. (2013) mentioned that dissolution plays an important role in determining toxicity, but that the observed toxicity cannot be explained by dissolution alone in all cases.
Dissolution is a surface-area-dependent process that, in engineered nanoparticles < 25 nm, is additionally influenced by surface curvature/roughness, crystalline structure, structural defects or size-dependent surface tension and activation energies (von Moos and Slaverykova, 2013). Small particles with high specific surface area (SSA) display higher solubility and faster dissolution than their non-nano counterparts. Solubility will also depend on the medium properties (such as pH), particle properties (SSA, surface characteristics) and by the concentration gradient between the particle and the medium. The dissolution of engineered nanomaterials can be altered with surface coatings. Proteins and organic substances are capable of increasing the dissolution rates of inorganic engineered nanoparticles.
Next to this, for nanosilver it is also known that dissolution mainly takes place in aerobic environments, because oxidation is needed (Ivask et al., 2013, Zhang et al., 2011a, Liu and Hurt, 2010). In summary, various nanomaterial characteristics, as well as environmental characteristics, affect dissolution behaviour. The former include ***chemical identity/composition, size, the presence of coatings or functionalisation, surface charge, reactivity, shape and surface area***. Regarding the latter, ***aerobic conditions*** are particularly important.

5.1.1.5 Dispersion

Nanomaterials can interact with ***natural organic matter or dispersants*** to form stable dispersions. This is generally assumed to result in an increased possibility for contact with living organisms and therefore potentially increased toxicity. However, the interaction with natural organic matter may also decrease the nanomaterial's

bioavailability (and hence toxicity) due to a change in the surface properties of the nanomaterial, resulting in decreased dissolution (e.g. observed for several metallic nanomaterials such as Ag, ZnO and CuO – although contrasting results are available on this) (Bondarenko et al., 2013; Ivask et al., 2013). The use of organic test media (e.g. for bacteria, yeast) may partly explain observed differences in toxicity between standard test organisms. For instance, Bondarenko et al. (2013) mentioned that CuO nanoparticles are typically less toxic than copper ions, but that in yeast the presence of proteins in the test medium results in a protein coating of the nanoparticles, enhanced sorption to the cell wall and an increased dissolution of copper at the nano-bio-interface. For CuO, similar examples of higher-than-expected toxicity are available for mammalian cells and bacteria (see Bondarenko et al., 2013).

5.1.1.6 Biodegradation

Biodegradation may be relevant for some organic nanoparticles or for inorganic particles with an organic coating. For metal nanoparticles, it is generally accepted that the degree to which the metal is able to dissolve from the particle (as well as dissolution kinetics) will determine or at least contribute to the toxicity of the nanomaterial under consideration. The presence of organic ***coatings*** may slow down the process of dissolution and hence reduce or postpone the toxic effects caused by the dissolved metal. The ease and speed by which an organic coating can be biodegraded in the environment, therefore, will affect both the environmental fate and the toxicity of the metal nanomaterial. Additionally, biodegradation requires the bioavailability of the organic material to microorganisms. Bioavailability can be expected to be affected by environmental factors, as well as by nanomaterial characteristics. Furthermore, biodegradation also requires that the material is not toxic to the microorganisms capable of biodegradation. Although the organic material or coating may not be toxic and may be easily biodegradable, the presence of toxic co-contaminants adsorbed to the surface may hamper biodegradation by affecting the microorganism population in an adverse way.

5.1.1.7 Interaction with organic biomolecules at the nano-bio-interface

At the nano-bio-interface, nanomaterials may react with ***biomolecules*** (proteins, exudates, etc.) that are excreted/secreted by the organism under consideration. One process may be protein corona formation (outside of the organism), which may enhance uptake (Ma and Lin, 2013).

5.1.1.8 Interaction with other contaminants

Nanomaterials can interact with ***other contaminants*** in the environment. The nanomaterial under consideration may, for instance, have a high capacity for adsorbing certain other contaminants. In this way, it can act either as a Trojan horse, increasing the bioavailability of the co-contaminant(s), or vice versa, by decreasing the co-

contaminants' bioavailability. Another way of interaction is competition with other pollutants for certain environmental fate processes such as adsorption to suspended matter or reaction with dissolved natural organic matter (e.g. competition of carbon nanotubes with metals for sorption to natural organic matter – Jackson et al., 2013).
Both ***surface chemistry*** and ***aqueous solution chemistry*** (e.g. solution pH in relation to pK_a of nanomaterial, ionic strength, presence of organic matter, etc.) influence the adsorption of co-contaminants. Furthermore, the ***specific surface area*** of the nanoparticles may largely affect the amount of co-contaminants that can be absorbed (Jackson et al., 2013).

5.1.1.9 Interaction with living organisms

Several interactions have been identified:

- Adsorption to the nano-bio-interface (particles or agglomerates/aggregates).
- Internalisation/uptake at the nano-bio-interface (external nano-bio-interface).
- Internalisation/uptake via the dietary pathway (internal nano-bio-interface).
- Distribution.
- Transformation.
- Excretion.
- Bioconcentration, bioaccumulation.
- Transfer via the food chain (adsorbed or internalised nanomaterials).

Each of these interactions is discussed below.

Adsorption

The first contact between a nanoparticle in the aquatic environment and an organism occurs at the cell wall/membrane. Then nanoparticles can be adsorbed on the cell walls or membranes by multiple forces (Ma and Lin, 2013). Those main adsorption mechanisms are: Van der Waal forces, hydrophobic forces (nanoparticles with hydrophobic surfaces would be adsorbed on the hydrophobic surface zones of the cells), electrostatic attraction and specific interactions (e.g. receptor-ligand interactions). Through electrostatic attractions, the charged nanoparticles can become adsorbed on the cell surfaces with opposite charges. For example, positively charged CeO_2 nanoparticles and alumina coated SiO_2 were absorbed on the algal surface. In the experiment with SiO_2, the toxicity was caused by surface interactions. Absorption on the microorganisms can then lead to physical damage and biochemical injury. The absorbed nanoparticles can also cause pitting in the cell walls (leading to intracellular leakage), block nutrient uptake and influence cell metabolism (Ma and Lin, 2013).
In addition to the interactions between nanoparticles and cell wall/membrane, the interaction between nanoparticles and biomolecules should be taken into consideration (Ma and Lin, 2013). Proteins, lipids

and polysaccharides are common on the inner and outer interfaces of the organisms. Proteins can associate with nanoparticles as a corona and bind the nanoparticles and cells together. For polysaccharides, hydrogen bonding, ionic interactions and the dehydration of polar groups may be the main binding factors. Those interactions may facilitate a further step, i.e. the internalisation of the nanoparticles into the cell.
The charge of a nanoparticle can be influenced by the medium. This has, for instance, been reviewed by Bondarenko et al. (2013) for CuO, ZnO and Ag. For CuO and ZnO nanoparticles, the negative surface charge is due to oxygen atoms. In Ag nanoparticles, the surface first needs to be oxidised under aerobic conditions. Negative hydroxo-groups and oxo-groups then cause the negative surface charge of the particles. Clearly, dissolved oxygen concentration of the test medium can also play a role in determining the potential for interactions at the nano-bio-interface. Note that adsorption (e.g. to the gills of fish) may result in a locally increased delivery of metal ions available for uptake. As a result, the nanoparticle itself is not necessarily taken up (Handy et al., 2011).

Internalisation at the external nano-bio-interface

Although there are not too many studies available in which specific methodologies have been applied to identify the actual uptake of nanomaterials by living organisms (in the environment or environmental test media), uptake directly from the environmental medium under consideration seems to be quite different for different types of organisms. In general, bacteria and algae seem to be quite resistant to uptake, whereas multicellular aquatic organisms (such as daphnids and fish) seem to internalise nanoparticles more easily (Ivask et al., 2013, Bondarenko et al., 2013).
The potential for nanoparticles to be internalised depends, firstly, on the type of organism under consideration (Ma and Lin, 2013). The rigid cell wall of unicellular organisms such as bacteria and unicellular algae does not typically allow internalisation. Uptake in this kind of organism is mostly due to an increased permeability due to previous injury (Ivask et al., 2013). Uptake may also occur via non-specific diffusion, non-specific membrane damage or specific uptake through porins (which depends on particle ***size***). Von Moos and Slaveykova (2014) mention that the cell wall pores of bacteria/algae, which typically have diameters between 5 and 20 nm, are potential ports of entry for small nanoparticles. Due to the interference of nanoparticles with such pores, larger pores may be generated, affecting cell wall permeability.
Coatings also determine whether or not internalisation is likely to occur. For instance, the presence of polyvinyl alcohol coatings can increase membrane permeability in bacteria, as alkaline compounds dissolve the external part of the cell membrane, which is the major cellular protective barrier (Ivask et al., 2013).
Clearly, organisms with a cell wall, such as bacteria and algae, are quite resistant to internalisation, whereas *direct* uptake (i.e. at the external nano-bio-interface) is much more likely in animal organisms (no rigid

cell wall) owing to the occurrence of endocytosis. Internalisation via endocytosis is known to occur in mammalian cells, with the internalisation efficiency dependent on particle ***size*** (highest efficiency for particles with a diameter of ca. 40-50 nm). Endocytosis also occurs in other animal organisms (including protozoa), but other intake mechanisms (e.g. dietary) may be more important in organisms such as aquatic invertebrates and fish (Ivask et al., 2013, Ma and Lin, 2013)). In any case, *direct* internalisation pathways other than endocytosis, such as via ion transporters, paracellular diffusion and pinocytosis, are less likely than endocytosis, mainly due to size limitations.
Internalisation processes are extensively discussed by Ma and Lin (2013). These authors mention the following nanoparticle characteristics as factors affecting the likeliness for and the outcome of interactions at the nano-bio-interface: ***size, shape, surface charge (zeta potential), functionalisation and surface chemistry (the presence of functional groups) and the presence of coatings***. Environmental conditions that affect the nano-bio interactions are reported to be ***pH, ionic strength, the presence of other toxicants, the presence of natural organic matter, temperature and light***.
In their review on the toxicity of carbon nanotubes in the environment, Du et al. (2013), citing Powers et al. (2006), mentioned that ***smaller sized particles*** have more opportunity to get into cells and be translocated through different cellular barriers. But neither Du nor Powers et al. cite experimental data or quantify that conclusion.
Shape is also mentioned as an important factor that determines internalisation in non-mammalian organisms. Shape may enhance internalisation through perforation (e.g. in fish embryos, Handy et al., 2011).

Dietary pathway and internalisation at the internal nano-bio-interface

Hou et al. (2013) reported that daphnids can filter particles with sizes ranging from 0.4 to 40 µm, implying that most of the materials retained for dietary intake in daphnids are agglomerates. They also mentioned that nanoparticles in daphnid guts mainly remain in the gut, with limited absorption. Overall, these authors did not find substantial evidence for a relationship between bioaccumulation and nanomaterial composition, aspect ratio, primary particle size and the presence of surface coatings. They indicated that further research is needed.
Handy et al. (2011) reviewed the effects of manufactured nanomaterials on fish. They reported that, when internalised via the dietary pathway, uptake through the gut epithelium may occur (affected by the same nanoparticle characteristics as uptake at the external nano-bio-interface). In the gut, the nanoparticles may also form aggregates/agglomerates. A review by Baun et al. (2008) indicated that the agglomeration of nanoparticle depends on surface charge and pH. As the pH in the gut of, for example, a daphnid is between 6.8 and 7.2, the change in pH and ionic strength can lead to a change in nanoparticle agglomeration. This will affect the particle uptake in the organism. Furthermore, they can interact with the gut content (e.g. adsorption of

nutrients, interaction with digestive enzymes, microbial communities, etc.). The presence of aggregates/agglomerates may also affect gut transit time, motility and blood flow.

Distribution, transformation, excretion

Handy et al. (2011) mentioned the different processes that may occur after translocation to or injection in the circulatory system by, say, fish. Because nanomaterials easily aggregate/agglomerate under saline conditions, generally speaking, this may also be the case in the circulatory system. It may be assumed therefore that uptake in and translocation via the circulatory system requires a change of surface chemistry, e.g. through the adsorption of macromolecules such as proteins. Protein corona formation may allow the existence of stable dispersions in the circulatory system. With respect to the behaviour of nanomaterials in the circulatory system, many other gaps in data exist, such as the interaction with nutrients, blood cells, immune components, etc.
Not much information is present on transformation, but it can reasonably be assumed that, for both inorganic and organic nanoparticles, transformation processes (physical and/or chemical) will occur and will contribute to observed toxicity.
Similarly, not much information is available on excretion. Yet it is known that daphnids are able to purge engineered nanomaterials from their bodies/guts (Hou et al., 2013). Feeding with non-contaminated algae and/or translation in a medium without nanoparticles would help to eliminate the nanoparticles. Nevertheless, it seems that elimination is not complete (Hou et al., 2013). It is important to note that the excretion of nanoparticles after ingestion and/or actual uptake may result in the release of nanoparticles or aggregates with a different environmental fate, due to the removal of coatings, aggregation, a change of surface properties.

Bioconcentration, bioaccumulation

A review of the bioaccumulation of nanomaterials (with a focus on TiO_2, Fe_2O_3, Ag, Au, fullerenes, carbon nanotubes, CdSe/ZnS quantum dots, Al_2O_3, CuO, ZnO and CeO_2) in protozoa, aquatic and terrestrial invertebrates, and aquatic vertebrates has been performed by Hou et al. (2013). These authors present an overview of the available studies and the bioconcentration factors (BCF)/bioaccumulation factors (BAF) calculated from them (dry weight based). Although it is mentioned that nominal concentrations were used (which may have resulted in an underestimation of BCFs or BAFs) and not all studies analytically confirmed the presence of nanoparticles in the body of the organisms studied, this review provides a first indication of the order of magnitude of bioconcentration/bioaccumulation.
For daphnids, mean log BCFs of 3.16 to 5.64 were reported. As described above, it is generally known that daphnids can filter particles with sizes ranging from 0.4 to 40 µm; hence most of the retained materials are likely aggregates. Nanoparticles were reported to remain

mainly in the guts, with limited absorption. Daphnids can purge nanoparticles from their bodies, which is improved by feeding; however elimination is probably never complete. The purged nanoparticles may have different properties when they return to the environment. The results of the available studies suggest a lack of dependence by bioconcentration on nanomaterial characteristics such as composition, aspect ratio, primary particle size and surface coatings. But aggregation or stabilisation by coating with organic matter in the exposure media can diminish the impact of nanoparticle properties (Hou et al., 2013). In fish, mean log BCFs were 1 to 2 orders of magnitude smaller than in daphnids (1.27 to 2.87) (Hou et al., 2013). The greater nanomaterial accumulation in daphnids is considered to be due to their feeding behaviour (filter feeders). Some fluorescence studies indicate the presence of nanomaterials mainly in the gills and intestines of fish exposed to nanomaterials. Some results are available on the presence in other organs and the circulatory system, but it was not entirely clear whether this may also have been due to a leaching out of the fluorescent tag. Current literature on studies with fish indicates that the major route of uptake is via direct ingestion or dietary exposure and suggests relatively low bioaccumulation potential (Hou et al., 2013). Low BSAFs (biota-to-soil-accumulation factors) were also reported for earthworms (-1.68 to -0.34). Here, too, the available reports (Petersen et al., 2011, Hou et al., 2013) suggest that nanomaterial surface properties have a minimal impact on accumulation behaviour. There is some confirmation available on nanoparticle uptake and accumulation for gold nanoparticles. Results from a study with gold nanoparticles and ionic gold indicated that ionic gold was five times more bioaccumulative than particulate gold (Unrine et al., 2010, Hou et al., 2013).
Overall, the methodologies for the analytical measurement of actual nanoparticle concentrations in environmental or test media, as well as the methodologies for distinguishing between, for example, metal ion uptake and metal nanoparticle uptake, have not yet been optimised and therefore the results from most of the studies considered in this review should not be considered as conclusive (Hou et al., 2013). Further research in this area is needed. Nevertheless, it seems that most results indicate a low bioconcentration/bioaccumulation potential, with limited uptake of nanomaterials by organisms, the main pathway most likely being via the oral/dietary route.

5.1.1.10 Transfer via the food chain (adsorbed or internalised nanomaterials)

On biomagnification, Hou et al. (2013) reported a discrepancy between the available study results, depending on which food chain was considered. Some studies demonstrated a potential for biomagnification (e.g. BMF (biomagnification factor) of ca. 5 for the transfer of CdSe quantum dots from bacteria to ciliated protozoa; BMFs of 6.2 to 11 for the transfer of gold nanoparticles from tobacco leaves to tobacco hornworms), whereas others did not (e.g. BMFs < 1 in a study with CdSe-ZnS core-shell quantum dots for the transfer from bacteria to ciliates to rotifers; and for the trophic transfer of quantum dots from

algae to daphnids and the transfer of quantum dots or TiO_2 nanoparticles from daphnids to fish). The discrepancy between the studies depends on the types/strains of organisms under consideration, as well as on the potential for uptake/internalisation in the lower trophic levels, and was also influenced by the properties of the nanomaterials. Note that nanomaterials adsorbed to the outer surface of living organisms can also contribute to a transfer via the food chain.

5.1.2 Toxicity to organisms in the environment

This section discusses the information gathered from recent review papers, with the focus on potential mechanisms of toxicity as well as on the level of toxicity in various test organisms and the reported toxicity mediating factors (from the nanomaterial's point of view).
Several review papers made an extensive effort in summarising toxicity data for various types of living organisms. Certain reviews give specific attention to toxicity mitigating factors from the nanomaterial's perspective.
Annex A provides a summary of that information, which was distilled to provide the information below on the mechanisms of toxicity.
The following mechanisms of toxicity are each discussed below:

- Oxidative stress,
- Catalytic activity and interference with biological redox systems,
- Change in gene expression and genotoxicity,
- Interference with respiratory or osmoregulatory functions,
- Physical interference,
- Chemical interference,
- Dissolution effects,
- Trojan horse effects and other indirect effects caused by adsorbed co-contaminants.

5.1.2.1 Oxidative stress

Extracellular or internalised nanoparticles can cause oxidative damage and has been considered one of the main causes of nanoparticle toxicity (Ivask et al., 2013). Reactive oxygen, species-induced oxidative stress has been demonstrated at almost all the levels of biological organisation (from bacteria to fish and in mammalian cell lines *in vivo*).
Nanoparticles can induce the generation of non-radical reactive oxygen species (ROS, e.g. H_2O_2, singlet oxygen, ozone, etc.) and radical reactive oxygen forms (e.g. oxygen and hydroxyl radicals). Excessive ROS can result in peroxidation of polyunsaturated fatty acids in cell and mitochondrial membranes, change cell membrane permeability and damage cells.
For an overview of oxidative stress induced by inorganic nanoparticles in bacteria and aquatic microalgae, reference can be made to von Moos and Slaveykova (2014). These authors mention that ROS generation can be divided into direct or indirect effects. Indirect effects are mediated by the dissolved ion form of the engineered nanomaterials that trigger ROS generation. Direct effects are mediated through the increased production of ROS and can lead to oxidative damage in cellular

compounds. ROS generation by engineered nanomaterials can be explained by the chemical reactivity of nanomaterials and impurities and by direct physical interactions of engineered nanomaterials with biological redox-catalysing subcellular structures. The factors which are possibly linked to generating ROS are: chemical composition and purity, size and surface area, surface coatings and functionalisation, surface charge and band gap energy. (See the related discussion of catalytic and photocatalytic effects below.)
ROS-related effects are reported to be due to extracellular or intracellular ROS formation, the regulation of ROS responsive genes, the inhibition of ROS quenching enzymes, the depletion of glutathione (GSH), lipid peroxidation, DNA damage, and interference with cell signalling. At present, the causal link between particle properties and biological effects or ROS generation has not yet been well established (von Moos and Slaveykova, 2014).
Ivask et al. (2013) shows a detailed overview of oxidative stress mechanisms in mammalian cell cultures. The oxidative stress mechanisms have been demonstrated in various non-mammalian types of organisms, too, but the exact mechanisms may differ between organisms and even between tissues and organelles. For assessing the potential of nanoparticles to exert oxidative stress, several biomarkers, such as the increased activities of superoxide dismutase (SOD), catalase (CAT), glutathione-S-transferase, glutathione peroxidase (GPx) and increased levels of metallothionein-like proteins, have been measured. However, studies that monitor those biomarkers have not reported on a common mechanism of action of, for example, zinc oxide nanoparticles since the patterns are different between species and even in different organs of the same individual. For example, SOD activity showed (1) a decreasing tendency in zebrafish liver, (2) an increase in zebrafish gut tissue and in the gill, liver, brain and intestine of carp and (3) no change in SOD activity in a marine crustacean (invertebrate).

5.1.2.2 Catalytic activity, interference with biological redox systems

Metal oxides can be of particular concern with respect to causing oxidative stress due to their potential catalytic activity. It is important to note that interference with biological redox systems does not necessarily require internalisation, but it may also be caused by nanoparticles adsorbed to the cell wall or membrane.
Von Moos and Slaveykova (2014) reviewed the catalytic activity of inorganic nanomaterials in bacteria and aquatic microalgae. Zhang et al. (2013) described the use of information about the band gap of 24 metal oxide nanoparticles to predict oxidative stress. Puzyn et al. (2011) assessed the correlation between certain molecular parameters and bacterial cytotoxicity for the nanoparticles of 17 metal oxides. A summary of the information in these publications follows.
Particle size and crystal phase composition may affect a nanoparticle's catalytic or photocatalytic activity. If the ***band gap energy*** of the redox-active nanomaterial is exceeded, excited electrons are generated in the conduction bands and electron holes will occur in the valence

bands. The excited electrons and electron holes can readily engage in redox reactions. If the energy structure of the nanomaterial is close to that of biological redox couples, redox reactions or catalysis can occur. The standard redox potential of biologically active redox pairs is estimated to lie between -4.12 and -4.84 eV. Burello and Worth (2011) hypothesised that nanoparticles larger than 20 – 30 nm do not have surface states in the band gap and behave like non-nanomaterials. However, the literature review for this project did not identify any formal tests of this hypothesis with respect to biological redox reactions. The work done by Zhang et al. (2012), which is described briefly below, tested particles of larger size.

Modelling of relative positions of energy band levels showed that a considerable number of inorganic oxide nanomaterials are capable of unbalancing the redox state in the cells of living organisms (i.e. titanium, copper, zinc, iron oxides). (Burello and Worth, 2011)

Zhang et al. (2012) found that overlap of the conduction band energy levels with the cellular redox potential was strongly correlated with the ability of Co_3O_4, Cr_2O_3, Ni_2O_3, Mn_2O_3, and CoO nanoparticles to induce ROS production, oxidative stress and pro-inflammatory effects. But a comparison of E_c to the redox potential of biologically important redox pairs is not an absolute predictor of effects. While E_c for TiO_2 fell within the biologically important range of -4.12 and -4.84 eV, exposure to TiO_2 did not elicit significant pro-oxidative and oxidative stress effects in the tests conducted by Zhang et al. (As reported by others, illumination of nano-TiO_2 by ultraviolet light can promote an electron from the valence to the conduction band and create an electron-hole pair on the oxide surface, thus resulting in oxidative stress reactions (Burello and Worth, 2011; Puzyn et al., 2011).) Conversely, CuO and ZnO generated oxidative stress that was not predicted by E_c; but Zhang et al. attributed that effect to metal ions released from the nanoparticles.

The work by Zhang et al. (2011) indicated that the E_c values for oxides of Al, Si, Y, La, Gd, Yb, Hf, Zr, In, Ni, Sb, Ce, Zn, Sn, Fe, Cu, and W were outside the biologically important range of -4.12 and -4.84 eV and therefore did not predict toxicity based on the band gap energy. Burello and Worth (2011) calculated that the E_c values for oxides of Al, Ni, Ga, Sn, Hg and Ge were also outside that critical range and, citing work by others, noted that those oxides did not display *in vitro* toxicity by an oxidative stress mechanism.

Work by Puzyn et al. (2011) may be relevant to this discussion. Their research assessed the correlation between certain molecular parameters and bacterial cytotoxicity for nanoparticles of 17 metal oxides. They performed this work within the context of developing Quantitative Structure Activity Relationships (QSAR). Data from tests of particles in the nominal size range 10 – 90 nm indicated that ZnO, CuO, NiO and CoO exhibited the highest cytotoxicity and TiO_2 nanoparticles was the least toxic. The research team attempted to correlate the toxicity testing results with the following parameters.

- Standard heat of formation of the oxide cluster.
- Total energy of the oxide cluster.

- Electronic energy of the oxide cluster.
- Core-core repulsion energy of the oxide cluster.
- Calculated area of the oxide cluster.
- Calculated volume of the oxide cluster.
- Energy of the highest occupied molecular orbital (HOMO) of the oxide cluster.
- Energy of the lowest unoccupied molecular orbital (LUMO) of the oxide cluster.
- Energy difference between HOMO and LUMO energies.
- Enthalpy of detachment of metal cations (Me^{n+}) from the cluster surface.
- Enthalpy of formation of a gaseous cation.
- Lattice energy of the oxide III.

Puzyn et al. found that they could successfully predict cytotoxicity based on one descriptor, the enthalpy of formation of a gaseous cation (ΔH_{Me+}) having the same oxidation state as that in the metal oxide structure. In essence, this variable describes the chemical stability of metal oxides. Pyzyn et al. reached several additional conclusions. Firstly, ΔH_{Me+} is not related to the size of the nanoparticle. Therefore, for a series of metal oxide nanoparticles of similar size, the particle size does not determine variations in toxicity. Secondly, two parameters that might logically have been related to toxicity did not correlate with cytotoxicity. Those parameters were lattice energy (which describes the dissolution of nanoparticles without oxidation or reduction of the cation) and the electronic properties that describe redox potential (HOMO, LUMO, and the band gap between the two). The latter finding appears to contradict the conclusions drawn by Zhang et al. (2011) that E_c (LUMO) within the range of biologically important redox pairs could predict toxic effects, although the two research teams reported different values of HOMO/E_v, LUMO/E_c and band gap for each of the metal oxides common to the two research efforts. That suggests a fundamental difference in the research that cannot be explained without probing deeply into the calculations.

5.1.2.3 Change in gene expression and genotoxicity

Changes in gene expression and genotoxicity may take the following primary forms (e.g. Du et al., 2013, Ivask et al., 2013):

- Up and down regulation of genes.
- Inhibition of DNA (deoxyribonucleic acid) repair and activation of inflammatory cells.
- Changes in mRNA (messenger ribonucleic acid) expression, protein expression, protein activity changes, etc.
- Direct DNA damage (which may also be the result of oxidative stress).

The papers reviewed did not describe the mechanisms or critical characteristics of nanoparticles that influence these effects.

5.1.2.4 Physical interference

Physical interference may take many forms; these effects cannot yet be linked to specific nanoparticle characteristics based upon research to date. Particle size (primary and secondary) and shape are likely to be relevant, as are the parameters that affect sorption (surface charge, coating).

- Damaged function of ion channels by changing proteins through direct interference, assembly of actin filaments, etc. (e.g. Du et al., 2013).
- Perforation of cell membranes, cell walls or damage to epithelia (e.g. gut or gill epithelium) (e.g. Handy et al., 2011; Ivask et al., 2013; Jackson et al., 2013; Ma and Lin, 2013).
- Physiological effect on organisms of the microbial communities in the gastro-intestinal tract (e.g. Handy et al., 2011).
- Effect on gut function (transit time, motility, blood flow around the gut, etc.) (e.g. Handy et al., 2011; Jackson et al., 2013) or gill function (physical blockage, clogging, e.g. Shaw and Handy, 2011; for carbon nanotubes: Jackson et al., 2013).
- Physical effects in the circulatory system (e.g. adherence to blood cells) may result in adverse effects on blood function/functioning of the circulatory system (e.g. Handy et al., 2011).
- Attachment to the outer side of organisms and interference with movement, food intake, normal functioning, etc. For instance, the formation of agglomerates of unicellular organisms such as bacteria or algae can hamper the ability of these organisms to function or move in an efficient way and hence affect bacterial/algal populations in an adverse way (e.g. Jackson et al., 2013; Ma and Lin, 2013). For photosynthetic organisms such as algae, the formation of cell agglomerates through interlinking with adsorbed nanoparticles may result in shading, hampering efficient photosynthesis and cell growth. The attachment of nanoparticles and/or nanoparticle agglomerates to the setae, mantle and labial palps of aquatic invertebrates may impair normal movement or feeding behaviour, thereby affecting these organisms' populations in an adverse way.
- Adsorption to gills may affect respiratory function (e.g. increased ventilation rate) or inhibit branchial N/K ATPases (e.g. Handy et al., 2011; Shaw and Handy, 2011) by fish.

5.1.2.5 Chemical interference

Chemical interference can take several forms, depending on the reactivity of the nanoparticle (which is influenced by particle size, surface coating and functional groups):

- Interaction with nutrients in the gastro-intestinal or circulatory system (e.g. Handy et al., 2011), causing nutrient deficiency.
- Interaction with digestive enzymes in the gastro-intestinal tract (e.g. Handy et al., 2011).

- Toxic interaction with microbial communities in the gastro-intestinal tract (e.g. Handy et al., 2011) – may also be a co-contaminant effect.

5.1.2.6 Dissolution effects

For several nanoparticles, the observed toxicity can be – at least partly – explained by dissolution, i.e. release of toxic forms (e.g. metal ions). Many factors can affect dissolution. The dissolution of nanoparticles can have a localised effect: adsorption at the nano-bio-interface may result in locally increased delivery of dissolved toxic forms available for uptake. In other words, while the nanoparticle is not necessarily taken up in this case, the released toxic form may be (e.g. Handy et al., 2011; Ivask et al., 2013).
Note that some of the mechanisms mentioned above, such as oxidative stress or interference with genes, can be *shared* mechanisms of toxicity for the nanoparticles and the dissolved toxic form, and that it may be extremely difficult to distinguish between the portion of the observed effect that is caused by the nanoparticle and the portion that is caused by the dissolved toxic form.
Examples of nanoparticles for which toxicity is at least partly driven by dissolution are ZnO, CuO and Ag nanoparticles (the latter only in aerobic environments, as explained above in the environmental fate section) (Ivask et al., 2013). Not necessarily all observed toxicity can be explained by dissolution for these nanoparticles. For instance, for silver nanoparticles, different gene expression patterns are observed than are for silver ions (affecting protein metabolism and signal transduction instead of developmental processes). Furthermore, for zinc oxide nanoparticles, shape effects have been observed in diatoms (higher toxicity of needles versus spherical particles) (Ivask et al., 2013).

5.1.2.7 Trojan horse effects and other indirect effects caused by adsorbed co-contaminants

Indirect adverse effects by exposing organisms to adsorbed co-contaminants have been described for various types of nanomaterials (Baun et al., 2008, Jackson et al., 2013). For instance, Jackson et al. (2013) report, in their review on the typically great sorption capacity of carbon nanotubes, that these types of nanomaterials often affect the bioavailability of other (hydrophobic) organic contaminants.
Bioavailability can be increased as a result of Trojan horse effects resulting from co-uptake of the adsorbed co-contaminants or, such as in plant roots, increased internalisation of co-contaminants due to perforation and the increasing permeability of plant root cell walls, etc., but in certain cases also decreased internalisation. For instance, sorption may reduce the availability of the organic contaminant to microorganisms for biodegradation.

5.2 Summary of Characteristics Critical to Ecotoxicity Endpoints

The most critical parameters in determining nanoparticle ecotoxicity are 1) parameters of the test medium itself and 2) the following nanoparticle characteristics: size/surface area, shape (especially for algae and fish embryo), reactivity, photoactivity, the presence of functional groups/coatings and surface charge. Each set of parameters, related to the medium or to the nanoparticle, is discussed below.

5.2.1 Test Medium

When applying a read-across strategy or grouping of nanomaterials, in a first step, these key nanoparticle characteristics should be determined (physico-chemical properties). As stressed in the OECD (2014a) expert meeting report on ecotoxicology and environmental fate of manufactured nanomaterials test guidelines, the physico-chemical properties of a nanoparticle are not steady and those properties can change with sample preparation, choice of testing media, dispersant use, the presence of environmental ligands and other factors. The OECD Guidance on sample preparation and dosimetry for the safety testing of manufactured nanomaterials (2012a) provides tentative guidance. The way in which nanoparticles are added/dispersed in the aqueous test medium will also influence the fate/behaviour of the nanoparticle and influence the ecotoxicity effects. OECD (2014a) has stated that dispersion methods can lead to a change in nanomaterial properties and therefore the nature of the nanomaterial in the dispersion must be characterised in order to quantify exposure. In summary, the dispersion methods and the composition of the aqueous media could all determine ecotoxicity endpoints. Therefore, when one wants to read-across between nanoparticles, the dispersion method and the composition of the aqueous media have to be fully described.

5.2.1.1 Dispersion of nanomaterials into aqueous medium

The OECD guidance on the sample preparation of nanoparticles (2012a) describes the possible methods of suspension (e.g. stirring, sonication, grinding, use of solvents). But there is no consensus on the best approaches for preparing nanomaterial samples. Handy et al. (2012) give an overview of the advantages and disadvantages of dispersion methods: sonication, use of (natural) dispersants, stirring/mixing and no dispersion. The use of natural dispersants such as humic acids could influence the toxicity of metal-containing nanomaterials and could lead to analytical problems for carbon-based nanomaterials. The use of synthetic dispersants could have an influence on the organisms and render the ecotoxicity data less ecological relevant. The dispersant could also interact with the coating and thus change the nanoparticle characteristics. Furthermore, sonication and mixing/stirring also seems less ecologically relevant and can fragment multiwall carbon nanotubes, increase the production of ROS, remove coatings and hydroxylate surfaces (Handy et al., 2012, OECD, 2012a). No protocol has been developed to standardise dispersion protocols.

The OECD expert meeting report (2014a) notes that the discussion group on ecotoxicity testing examined the practicality of creating a technical guidance (TG) and considered the following points: type of dosimetry, the degree of monodispersiveness of the stock suspensions, the renewal of the test media and the use of stabilisers. However, no consensus was reached on sample preparation. The workgroup decided to update the OECD guidance on the sample preparation of nanomaterials (2012a) and to develop a decision tree(s) that would guide the user to a decision on the dispersion protocol based on nanoparticle characteristics. Gaps could also be identified based on such a decision tree(s). Critical variables include the type or category of material and the presence of a coating. Work on the decision trees would be based on data-rich substances such as nanosilver and titanium dioxide.
The discussion on the fate and behaviour of nanomaterials at this OECD expert meeting (2014a) also addressed a tiered approach for the testing of nanomaterials. Participants concluded that the first step should be to describe the dissolution and dispersion of nanomaterials and the second step should be to determine the agglomeration state and dispersion stability. The third step would include testing biodegradation and the fourth step would include abiotic degradation. For the first three steps, a new technical guidance must be developed. Regarding the TG 305, the expert group also concluded that the use of BCF (bioconcentration factors) is not applicable for tests with nanomaterials. The octanol-water partition coefficient (K_{OW}) value is not suitable for predicting bioaccumulation and is not an appropriate endpoint for physico-chemical characterisation of nanomaterials.
Water solubility/dispersibility and dissolution seem to be the main parameters affecting fate and behaviour in the environment and therefore have to be tested before testing ecotoxicity. To represent the main environmental conditions, these tests must be conducted under different conditions, considering four variables:

- pH (i.e. pH 4-7-9);
- With and without organisms;
- With or without NOM (natural organic matter/proteins); and
- Using filtration to isolate certain particle size ranges, e.g. to eliminate agglomerates.

In a second step, dispersion stability and aggregation state should be analysed if the material is dispersible and not soluble (OECD, 2014a). These suggestions from the expert meeting report must be further developed to create practical testing protocols. For example, no OECD test guideline exists to measure the dispersion of primary or agglomerated nanoparticles. Some methods are available or under development (OECD, 2014a).

5.2.1.2 Aquatic media characteristics

The behaviour of the nanomaterials in the aquatic environment (and in the aquatic media tested) will strongly depend on environmental

conditions, such as pH, organic matter and ionic strength (Ma and Lin, 2013). OECD (2012a) has observed that the media quality should be sufficiently addressed during intervals during a test. At the OECD expert meeting (2014a), however, the parameters that influence the nanomaterial characteristics could not be fully addressed. Yet, as described in several papers, the above-mentioned parameters could influence nanoparticle ecotoxicity and should therefore be taken into account when applying a read-across strategy.

5.2.2 Nanoparticle characteristics that determine ecotoxicity effects

The nanoparticle characteristics that determine ecotoxicity effects are summarised in Table 8 and briefly described below according to trophic level (i.e. algae, daphnia and fish).

Table 8 Parameters Critical to Ecological Endpoints Based on Current Research

Property	**Summary of Relevance**
Chemical identity	
Chemical composition	Chemical composition can fundamentally determine the effects of exposure.
Crystalline structure	Crystalline structure may influence reactivity for some materials in a way that affects toxicity.
Surface characteristics (and surface charge): • Coating • Functionalisation • Capping agents	Surface characteristics will influence sorption to environmental or biological media and the reactivity of a nanomaterial.
Impurities	Impurities can substantially contribute to ecotoxicity.
Particle characteristics	
Particle size/range	The size of the nanoparticle impacts other physico-chemical properties, and can determine whether it can be internalised into an organism. Not a static parameter; may change during the course of ecotoxicity testing.
Shape	Particle shape can enhance the internalisation of a nanoparticle and potentially its ecotoxicity.
Porosity	Not identified as a primary determinant in ecotoxicity.
Surface area	The increase in relative surface area with decreasing particle size can increase the reactivity per unit mass of the nanoparticle.

Property	Summary of Relevance
Fundamental behaviour	
Water solubility • Rate of dissolution • Equilibrium solubility	Fundamentally affects the bioavailability of substances in the aquatic environment.
Hamaker constant	Parameter can influence the degree of agglomeration and sorption, but is not typically characterised in ecotoxicity studies.
Zeta potential	Parameter can influence the degree of agglomeration and sorption, but is not typically characterised in ecotoxicity studies.
Dispersiveness	Parameter can influence the degree of exposure but is often not characterised in ecotoxicity studies.
Dustiness	Parameter is not relevant to aquatic exposures.
Activity and reactivity	
Physical hazards	Parameter may be relevant to the risk of injury in occupational exposures, but is not a primary variable in ecotoxicity studies.
Reactivity	The reactivity of a nanomaterial – particularly relative to the non-nanoform of the substance – can impact the generation of ROS, induce inflammation and elicit cellular toxicity.
Photoreactivity	Increased photoreactivity with decreasing particle size may affect ecotoxicity.

5.2.2.1 Chemical Composition

The expression *surface chemistry* (generally speaking, the chemical nature and composition of the outermost layers of the nanomaterial) may need to be considered in greater detail or perhaps in a hierarchical manner, including coatings, functional groups and capping agents; these may be involved in surface reactions in different media (e.g. redox reactions, coordination chemistry, catalysis) (OECD, 2012c).
In an experiment with copper oxide (CuO) nanoparticles and polymer coated CuO nanoparticles, Perrault et al. (2012) observed that polymer-coated CuO nanoparticles were more toxic than the uncoated CuO nanoparticles for *Chlamydomonas reinhardtii*. This was associated with the capacity of the polymer coating to penetrate the cell wall. Coatings can also influence the surface charge of the nanoparticles and different coatings can have different toxicity effects on algae. Coatings can also determine toxicity for daphnids. Allen et al. (2010) showed that the toxicity of uncoated silver particles was slightly higher than that of coated particles. The review paper of Jackson et al. (2013) also indicated that the presence of functional groups on MWCNT (multiwall carbon nanotubes) is important for daphnid mortality. Exposure to

hydroxylated and carboxylated functional groups on MWCNT resulted in lower toxicity compared with raw MWCNT, whereas alkylated and aminated functional groups on MWCNTs increased toxicity compared with raw MWCNTs (Jackson et al., 2013).
Coatings can also influence agglomeration processes (next to other environmental characteristics such as pH, ionic strength) (OECD, 2012a). The physico-chemical properties of the coating will also affect nanoparticle dispersion in an aqueous medium. When organic-coated, engineered nanomaterials are tested, the standard biodegradation TG is not applicable due to the low concentration of carbon used for the coating. It was concluded in the OECD expert meeting (2014a), therefore, that the TG dealing with biodegradation is not directly applicable for engineered nanomaterials and it was decided that a specific TG for the biodegradation of engineered nanomaterials or different groups of engineered nanomaterials is needed.
Surface charge is also important; this property can depend on the nature of the nanoparticle and/or its coating. A study from Rodea Palomares et al. (2011) indicated that the tendency to form nanoparticle aggregates strongly depends on the surface charge. Ceria particles in this test were positively charged in the water bioassay medium and the cyanobacteria were negatively charged, favouring adsorption of nanoparticles on the organism and triggering toxicity. The effects of photocatalytic TiO_2 on algae were compared to those of non-photocatalytic nanoparticles (e.g. Al_2O_3 and SiO_2) in a study from Metzler et al. (2012). TiO_2 was toxic at particle sizes within a range of 30 to 60 nm; but the most important factor determining toxicity was the surface charge, as this had an effect on the agglomeration between algae and nanoparticles. In a test with *Pseudokirchneriella subcapitata* and uncharged or negatively charged, coated gold nanoparticles, Van Hoecke et al. (2013) observed that the particles were not attracted to the algal cells. The surface charge of a given particle may depend both on pH and solution composition, and may be measured as zeta potential. Clearly, ecotoxicologists conducting research in this area will need to ensure that the surface charge is measured and that the exact measurement conditions are given within the bounds of the fluid properties likely to occur in the medium of interest (OECD, 2012a).

5.2.2.2 Particle characteristics

The size of a nanoparticle will determine whether it can be internalised in an organism. The cell wall of algae is an efficient barrier that prevents most nanomaterials internalisation via endocytosis. However, cell wall pores with diameters ranging from 5–20 nm can be a potential uptake port for small nanomaterials (von Moos and Slaveykova, 2014). Smaller particles can cross the gut lumen of the daphnids. Studies from Ebert et al. 2005 suggested that particles of less than 50 µm are more easily ingested by daphnids. As particles aggregate into masses, there is a decrease in ingestion and toxicity (Zhu et al., 2009).
Particle size can change during the course of a test. Introducing the nanoparticle into an aqueous media can lead to the formation of

agglomerates or aggregates, which could also then cause shading or influence the photosynthetic capacity of algae and may affect uptake at higher trophic levels. A number of media parameters or environmental parameters will influence the agglomeration behaviour (and thus size) of the nanoparticle, e.g. ionic strength, media composition (OECD, 2014a), pH, solvent, the presence of proteins, sonication (OECD, 2012a). The participants at the OECD workshop (2014a) could not identify all of the relevant parameters for this process. OECD (2012a) had previously recommended that particle and/or agglomerate size distribution and material concentration should be assessed at intervals sufficient to quantify exposures throughout the course of a test. It is also desirable to measure particle/aggregate/agglomeration distribution using two or more methods: SEM (Scanning Electron Microscopy), TEM (Transmission Electron Microscopy), dynamic light scattering (DLS) and other microscopy techniques. DLS measures size based on hydrodynamic diameter and electrophoretic mobility. Microscopy provides information for the visual measurement of physical size (OECD, 2012a).
The National Institute of Standards and Technology (NIST) has commented on the appropriate procedures for characterising dispersed nanomaterials. NIST advises that experimenters should ensure that particle size distribution is stable from the point that treatment to sustain the dispersion (e.g. sonication) is suspended to the point of measurement, and throughout the duration of relevant tests to be conducted with the material (Taurozzi et al., 2012).
The shape of a nanoparticle may enhance the internalisation of a nanoparticle. Needle-like nanoparticles may perforate cell membranes or cell walls, or damage the gut or gill epithelium (e.g. Handy et al., 2011; Ivask et al., 2013; Jackson et al., 2013; Ma and Lin, 2013). Furthermore, rod-shaped or fibre particles can have a greater contact area with the cell membrane, can more easily get through capillaries, adhere to blood vessels, stimulate platelet aggregation and block potassium ion channels, compared with spherical carbon nanoparticles such as fullerenes. Carbon nanotubes in fibrous structures may be difficult to engulf by macrophages. Longer carbon nanotubes may show a higher inflammatory response. The shape of nanometal can have an effect on fish embryos. A study with spherical nickel nanoparticles of different sizes and dendritic structures consisting of aggregated 60 nm particles indicated that dendritic clusters were more toxic than the soluble nickel and the nanoparticles of the different sizes. In addition, it seemed that the toxicity of the spherical nanoparticles manifested as organ defects (Shaw and Handy, 2011).
The surface area of a nanoparticle relates to its size and porosity. Smaller particles have a larger surface area and there a larger surface energy relative to the volume of the particle, compared with the corresponding ratio for larger particles. The surface area of a particle determines the particle reactivity and affects the generation of ROS and radical activity (von Moos and Slaveykova, 2014). This indicates that the smaller the size (the larger the surface area), the higher the relative potential for oxidative stress. And, as been noted by a study with silver

nanomaterials and algae, the larger the surface area, the larger the number of reaction sites for UV (ultraviolet) adsorption.
The extent of the influence of aggregation/agglomeration on available surface area is still unclear (OECD, 2014a).

5.2.2.3 Fundamental behaviour

"Nanosizing" a substance can increase the rate of dissolution of a soluble material; it can also increase the equilibrium solubility concentration of certain substances, although that effect may not be seen within the duration of most standard ecotoxicity tests. The dissolution/solubility rate of the metal ions from metal-containing nanoparticles is crucial. Studies with fish indicate poorly soluble metal oxide nanoparticles may have low toxicity (Shaw and Handy, 2011). Studies have shown, for example, that nanosilver was less toxic than silver to adult zebrafish. The opposite was found for a test with nanocopper and dissolved copper with respect to juvenile zebrafish (the reverse was seen with adult fish) (Shaw and Handy, 2011).
The rate of dissolution is a key factor affecting environmental behaviour and test performance (OECD, 2014a). If a nanoparticle can be dissolved in the test media within a given timeframe, nano-specific testing should not be considered and testing methodologies for traditional chemicals can be applied. However, TG 105 (Water Solubility) is considered not to be appropriate for nanomaterials and the development of a new TG has been suggested (OECD, 2014a).

5.2.2.4 Activity and Reactivity

Functional groups and the charge reactivity and/or photoactivity of the nanoparticle can play a role in toxicity. The surface area of a particle determines the particle reactivity and the generation of oxidants and radical activity (von Moos and Slaveykova, 2014). This indicates that the smaller the size (the larger the surface area), the higher the relative potential for oxidative stress. Photoactivity may also be influenced by other particle properties (defect sites, structural disorder). A study involving titanium dioxide and *Daphnia magna* indicated that the effects observed in a test under UVA light are likely to be due to ROS, compared with a test performed in the dark (Amiano et al., 2012).

5.2.2.5 Summary

The most important environmental factors affecting the environmental fate of nanoparticles are the following:

- pH.
- Ionic strength and conductivity, particularly due to the presence of divalent ions.
- The presence of natural organic matter, dispersants, or biomolecules.
- Temperature.
- The presence of other contaminants.
- Dissolved oxygen concentration.

- Illumination.

Due to the complex interactions between nanomaterials and environmental characteristics, it is extremely difficult to generate comparative results (e.g. for different nanoforms of a single substance in a single test organism). This requires a standardised methodological approach in which the best attempt possible is made to understand the most important environmental fate processes in the test medium and to analytically verify actual nanomaterial exposure under the conditions of the test.
From the recent review papers summarised above, it became clear that the relationship between nano-specific characteristics and toxicity is not always straightforward. Table 8 and Figure 7 through Figure 10 summarise how the physico-chemical characteristics of nanomaterials influence their fate and transport in the environment and may affect ecotoxicity at three trophic levels, i.e. algae, daphnia and fish.
As discussed in this report and illustrated in those figures, interactions with the environment – internal and external – affect the extent and consequences of exposure to nanomaterials. The precise mechanisms for toxicity are not yet well known. Research to date suggests that toxic effects may essentially relate to three mechanisms:

- Reactivity, including the generation of ROS, catalytic/redox-activity of the particle, dissolution followed by reaction of the ionic form, and other, as yet undefined, reactions.
- Physical hindrance, which may occur at particularly high concentrations that may not be environmentally relevant.
- Trojan horse effects.

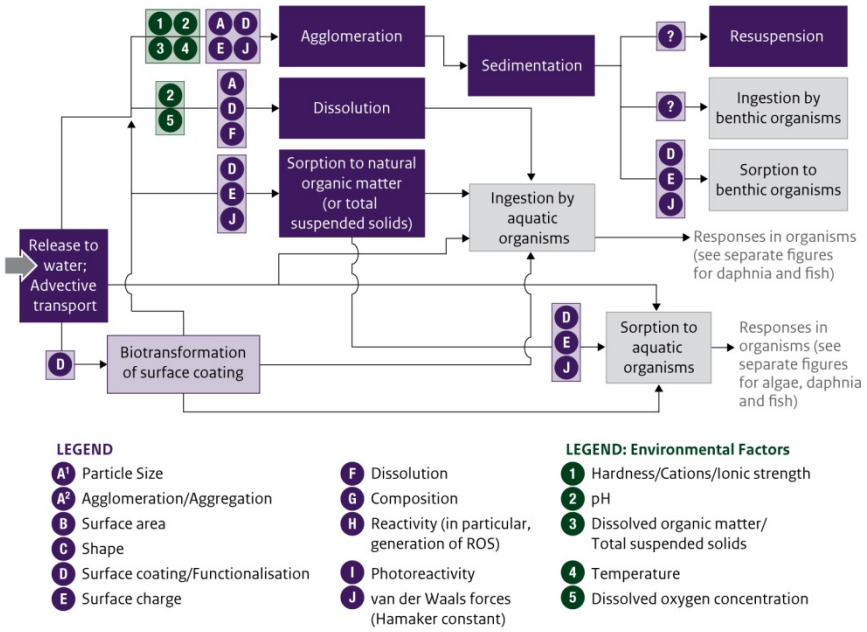


Figure 7 Environmental Transport and Exposure Pathways

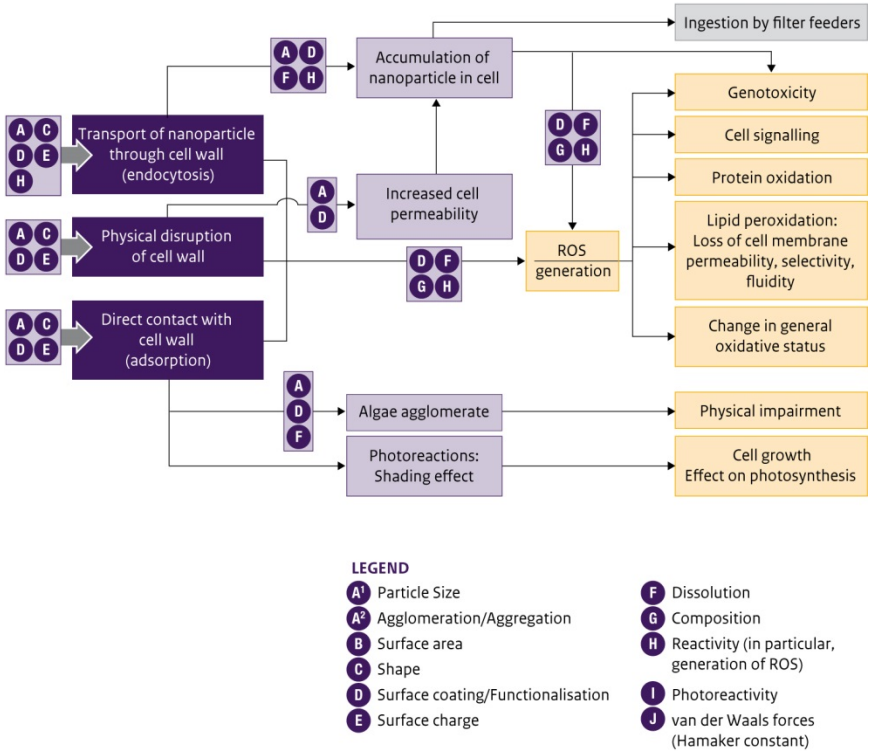


Figure 8 Algae

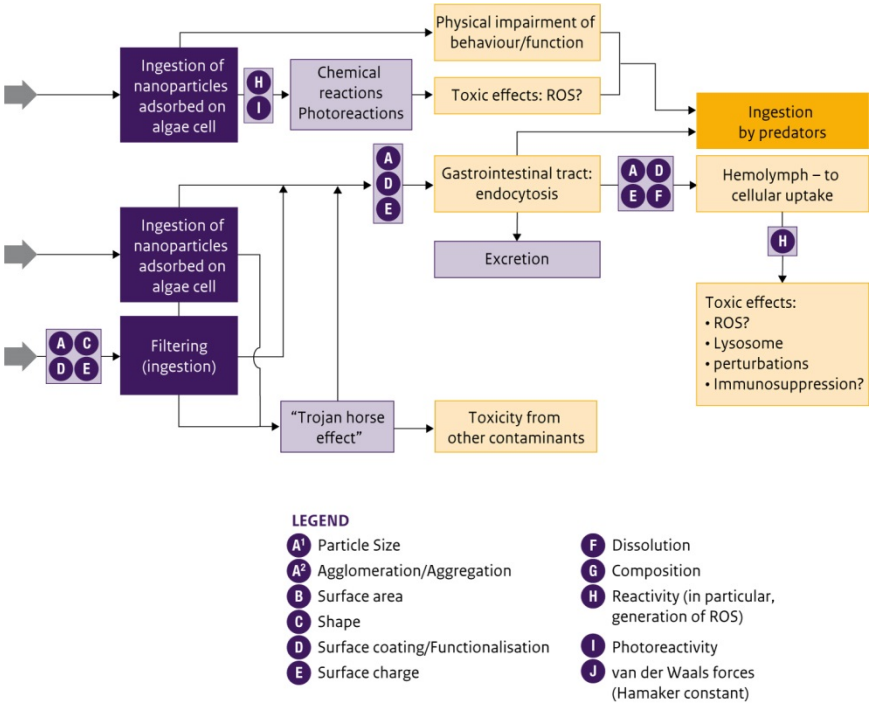


Figure 9 Daphnia

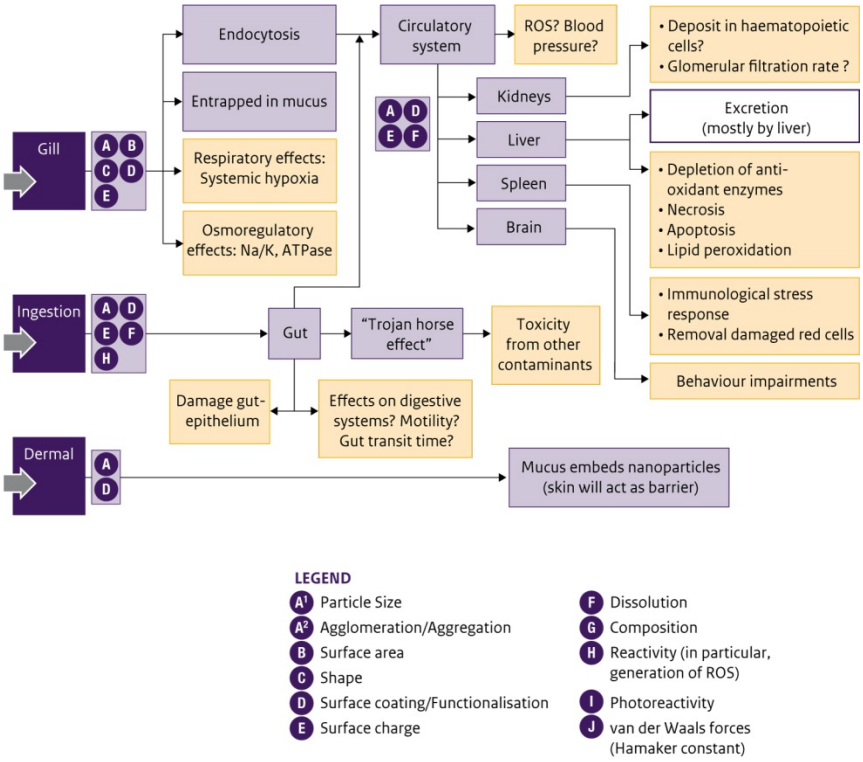


Figure 10 Fish

Adding to the uncertainty, opposite outcomes could be obtained in some studies, depending on the interaction with environmental characteristics and the organism under consideration.

5.3 Uncertainties

Despite the increasing body of research into the environmental fate and toxicity of nanoparticles, some uncertainties remain.
In the real exposure environment, the physico-chemical behaviour of nanoparticles cannot be easily predicted because the intrinsic parameters of nanoparticles are modified as a result of the complex nature of the test environment (Ivask et al., 2013). Caution should therefore be taken when interpreting test results obtained in a different test environment.
At the level of the organism itself, in most cases it is not feasible to measure the level of nanomaterials in the tissues (Handy et al., 2011). The analytical methods that are currently used to reveal the physical form of nanomaterials and distinguish between adsorption on versus absorption into tissues or cells are time-consuming, which hinders routine analysis; this limitation and the lack of standardised test protocols for the accumulation of nanoparticles may contribute to the inconsistency in data (Hou et al., 2013). It is therefore difficult to distinguish between the direct effects of nanomaterial exposure within the tissue and secondary effects (Ivask et al., 2013). For example, a

study with *Oncorhynchus mykiss* showed evidence of oxidative damage with branchial pathology in the gills. Hypoxia arising from gill injury could, however, also cause oxidative stress in the internal organs or ROS released from gill damage could damage other tissues (Handy et al., 2011).
In general, Hou et al. (2013) indicated that there is a lack of observation of engineered nanomaterial absorption into animal tissues. There is a discrepancy between what is observed *in vivo* versus *in vitro* studies, which suggests that other biological barriers (e.g. epithelium) may play a role.
As indicated by Ivask et al. (2013), although numerous studies investigate the mechanisms of the biological action of nanoparticles, a clear toxicity pathway for nanoparticles has not yet been proposed. QSARs could be of help as a tool to clarify the mode of action of nanoparticles to cells or organisms. QSARs are also accepted by REACH as an alternative to toxicity testing under certain conditions. However, because of the lack of accurate characterisation of nanoparticles in the experiments or the narrow number of nanoparticle types tested, few published data can be used for the development of this tool (Ivask et al., 2013). Initial QSAR work focusing on metal oxides suggests that different parameters relating to either the band gap or to the enthalpy of formation of a gaseous cation may be relevant parameters.
In addition, information on dietary exposure to nanomaterials in fish is very limited (Handy et al., 2011) because the target organs for dietary exposure to trace metals are often restricted to gut and liver. In addition to this, the lack of data on different species also represents a gap in the context of food-chain effects (Handy et al., 2011).

6 Human health effects and critical characteristics of nanomaterials

With the background on nanomaterial characteristics and the concepts of read-across and grouping provided above, we will now turn to examining the effects of exposure to nanomaterials and the nanomaterial properties that influence those effects.

6.1 Evaluation

The human health toxicity of any material, whether nanosized or not, depends on the exposure level or concentration in the target organs or tissues and the material's interaction with the biological system in the target organs/tissues. The exposure of an organ or tissue to a nanomaterial depends, in turn, on the material's specific toxicokinetic profile. Once in contact with the target organ or tissue, the properties of the material will dictate the specific biological response, which may reflect toxicity. The critical characteristics of nanoparticles that influence toxicokinetics and toxicity are discussed in the following sections.

6.1.1 Toxicokinetics

The toxicokinetic profile of a nanomaterial dictates the exposure in the target organ/tissue. Toxicokinetics encompasses absorption, distribution, metabolism, and excretion (ADME). In addition, deposition needs to be considered for the inhalation route of exposure. The specific toxicokinetic profile of a nanomaterial depends on its composition, size, shape, agglomeration/aggregation state, surface properties (including surface charge), hydrophobicity and dissolution, as well as the route of exposure (inhalation, dermal, oral).
Landsiedel et al. (2012) stated that the toxicokinetics of nanomaterials depends on the size of the nanomaterial, potential for proteins to bind to the nanomaterial, the agglomeration state, hydrophobicity and surface charge. In addition, solubility and route of exposure are important to consider, although not uniquely important for nanomaterials (Landsiedel et al., 2012). Finally, particle shape can have some influence, for example with respect to deposition in the lungs upon inhalation. The specific properties of nanomaterials that impact a nanomaterial's toxicokinetic profile are discussed in the sections below.
While discussed separately, these various aspects of toxicokinetics are interrelated. For example, the interaction of nanomaterials with proteins in the formation of a corona is considered an aspect of metabolism. However, the composition and properties of the protein corona impact the absorption, distribution and elimination of the nanomaterial in the body.

6.1.1.1 Deposition

The respiratory tract consists of the airways (conducting zone), which includes the trachea, bronchi and bronchioles, and the alveoli (respiratory zone). Upon inhalation, particles may deposit at different locations in the respiratory tract, depending on the size and shape of the particle. Nanoparticles deposited in the upper airways may be cleared through mucociliary mechanisms in the upper airways or macrophage clearance in the lower parts of the respiratory tract.
The smaller the particle, the farther down in the lung it is likely to be deposited (Zhang et al., 2013). Geraets et al. (2012) stated that the deposition of airborne particles within the lungs highly depends on the aerodynamic diameter. Bakand et al. (2012) suggest that, while the size distribution is the most important parameter, other properties such as shape, density and linear dimensions are important in characterising the aerodynamic properties of particles.
With respect to particle size and deposition pattern, particles in the size range of 5–30 micrometres (μm) are usually deposited in the nasopharyngeal region; particles that are 1–5 μm that are not deposited in the nasopharyngeal region are deposited in the tracheobronchial region; and submicron particles (<1 μm) and nanoparticles (<100 nm) penetrate deeply into the alveolar region, where removal mechanisms maybe insufficient (Bakand et al., 2012). At the nanoscale and as summarised by Landsiedel et al. (2012), mathematical models indicate that 90 % of inhaled 1 nanometre (nm) particles are deposited in the nasopharyngeal compartment, 10 % in the tracheobronchial region and almost none in the alveoli, while 5 nm particles are deposited about equally at around 30 % in the three regions; and approximately 50 % of 20 nm particles are deposited in the alveolar region with an approximately 15 % deposit in the tracheobronchial and nasopharyngeal regions.

6.1.1.2 Absorption

Absorption is the process by which the nanomaterials cross membrane barriers and enter the systemic circulation. Particle size, shape, dissolution, surface charge and hydrophobicity affect absorption. The mechanisms of absorption differ, depending on the route of exposure. The primary sites of absorption are the lungs, skin and gastrointestinal (GI) tract. Absorption from each route of exposure is discussed below.

Inhalation

As noted previously, inhaled nanoparticles are deposited throughout the respiratory tract. The specific sites of deposition within the lungs influence the systemic absorption of nanoparticles (Zhang et al., 2013). Nanoparticles deposited in the conducting airways are primarily cleared through mucociliary transport, with some macrophage clearance (Geiser and Kreyling, 2010). However, nanoparticles deposited in the respiratory zone are primarily cleared by macrophages.
Upon uptake by macrophages, nanoparticles may be degraded or carried to the mucociliary escalator, where nanoparticles may enter the

gastrointestinal tract (Landsiedel et al., 2012). Nanoparticles deposited in the respiratory zone that are not taken up by macrophages may be taken up by the epithelial cells or translocated through the epithelial barrier (Landsiedel et al., 2012). Zhang et al. (2013) noted that translocation from the pulmonary alveolar capillary bed to the circulatory system works as the main outlet of nanoparticles after respiratory exposure and the translocation efficiency is governed by the nanoparticle's physico-chemical properties, such as size and surface chemistry. One example provided was that of gold nanoparticles, in which larger nanoparticles (18, 40 and 100 nm) were trapped in the lungs, rather than transported to the circulatory system after repeated intratracheal instillations, yet smaller nanoparticles (1.4 and 2 nm) penetrated the alveolar barrier and were distributed to distant organs. Furthermore, a study using quantum dots of different compositions, shapes, sizes and surface charges showed that translocation depended on size and surface charge (Zhang et al., 2013). In addition to size and material type, other characteristics, such as surface charge and surface structures, which influence interactions with proteins and other cellular components, are also likely important factors (Geiser and Kreyling, 2010).

Another important factor affecting the absorption of a nanomaterial is its dissolution. Dissolution is the process by which a particle goes into the solution phase to form a homogeneous mixture and is dependent on the solubility of the material in the local environment (Borm et al., 2006). Although not unique to nanomaterials, this property influences internal exposure. The dissolution of a nanomaterial can affect its absorption and systemic availability by all routes of exposure. The dissolution profile (i.e. dissolution over time) of a nanomaterial depends on the route of exposure as well as the size, surface area and composition of the nanomaterial itself (Borm et al., 2006). As discussed previously in this report, nanomaterials are anticipated to dissolve faster than larger-sized materials of the same mass and may reach a greater equilibrium solubility concentration.

As noted by Borm et al. (2006), the kinetics of the dissolution of inhaled particulates determine whether a low toxicity particle, such as amorphous silica, will dissolve in the epithelial lining fluid or whether such particles as carbon blacks or iron oxides are engulfed by alveolar macrophages.

Nanoparticles have a strong affinity for macromolecules. The interactions with macromolecules, such as proteins within the respiratory tract, may facilitate the absorption of nanoparticles. The factors influencing the binding of proteins, including surface chemistry and charge, are discussed further elsewhere in this report. Geiser and Kreyling (2010) postulated that nanoparticles may bind with lung-lining layer proteins to form a complex that potentially facilitates transport across membranes.

Oral

The gastrointestinal tract comprises the oral cavity, oesophagus, stomach and intestine (Frohlich and Roblegg, 2012). All areas of the gastrointestinal tract are mechanically protected by epithelium and a layer of mucus of variable thickness and composition that is produced by specialised gastrointestinal epithelial cells (Bergin and Witzmann, 2013). The epithelium generally represents the highest resistance against the passage of nanomaterials and can be permeated either by passage through the cells (transcellular) or by passage between the cells (paracellular) (Frohlich and Roblegg, 2012). While most ingested nanoparticles reaching the gastrointestinal tract are excreted with the faeces, some absorption has been observed (Zhang et al., 2013). The absorption of particles through the intestinal barrier involves diffusion through the mucus layer, contact with enterocytes and/or M-cells, and uptake via cellular entry or paracellular transport (Bergin and Witzmann, 2013). Specifically, absorption of nanoparticles in the small intestine begins with the uptake of nanoparticles by enterocytes and M cells, which depends on the size of the nanoparticles (Zhang et al., 2013). In one study summarised by Bergin and Witzmann (2013), 50 nm polystyrene particles were absorbed to a greater extent than 100 nm particles. The 300 nm particles evaluated in the study were not absorbed at all. As presented in Zhang et al. (2013), following oral administration gold nanoparticles with diameters of 4 nm and 10 nm cross the small intestine more readily than do gold nanoparticles with diameters of 28 nm and 58 nm. Similarly, zinc oxide nanoparticles were absorbed much more readily than zinc oxide microparticles following oral administration (Zhang et al., 2013).

As noted by Zhang et al. (2013), positively charged nanomaterials are absorbed more efficiently than negatively charged and neutral materials. Conversely, for dendrimers, greater diameter (in later generation nanoparticles) and negative surface charge correlated with greater absorption (Bergin and Witzmann, 2013). Bergin and Witzmann (2013) also indicated that, for carbon nanotubes, there was decreased absorption with longer axis ratio.

In addition, surface coating may also affect the absorption of nanoparticles in the gastrointestinal tract. Some molecules on the surface of nanoparticles intended for pharmaceutical use are designed to bind to the surface receptors on enterocytes or M cells, such as some lectins and *Salmonella* extract, to enhance the absorption efficiency of nanoparticles in the gastrointestinal tract (Zhang et al., 2013).

Dose may be another important variable. As noted by Bergin and Witzmann (2013), metal nanoparticles with increasing percent dissolution, smaller size and higher dose appeared to have greater absorption. For carbon nanotubes, however, higher dose decreased absorption, possibly by facilitating agglomeration.

Dermal

The skin is composed of the epidermis, dermis and hypodermis. The outermost layer of the epidermis, the stratum corneum, is the primary

physical barrier of the skin (Liang et al., 2013). Nanomaterials must penetrate each of these layers of the skin in order to enter the systemic circulation. Particle size, shape, surface charge, composition and any surface coatings likely play a role (Liang et al., 2013).
Much of the research to date suggests that very little of dermally applied nanomaterials become systemically available. Landsiedel et al. (2012) noted that permeation of nanomaterials through the skin, when applied topically, was not observed. Zhang et al. (2013) concluded that the absorption of various nanoparticles through the skin depends on the integrity of the skin, based on data from different *in vitro* and *in vivo* models in which penetration was observed in animals that had skin damage, but not in animals with intact skin. Furthermore, several studies have demonstrated that metal oxide nanoparticles do not penetrate the stratum corneum, but can only lodge into hair follicles, sweat glands or skin folds (Zhang et al., 2013).
Although much of the available data indicate that skin penetration of nanoparticles is not likely, some data do indicate the potential for dermal absorption. One study, as summarised in Zhang et al. (2013), found that ^{68}Zn was detected in the blood of humans after the application of sunscreens containing radiolabelled ZnO nanoparticles with diameters of 19 and 110 nm. It may be possible, however, that the Zn detected may not be in the form of particles, but rather Zn ion as a result of dissolution.
The most important characteristic contributing to skin penetration is particle size (Liang et al., 2013). However, other characteristics may also contribute the penetration of nanoparticles through the skin, including shape and surface properties (e.g. charge, polarity). In one study summary, it was shown that spherical carboxylic acid-coated quantum dots penetrated into epidermis of weanling pig skin much more rapidly than ellipsoid shaped carboxylic acid-coated quantum dots (Liang, et al., 2013). It was postulated by Liang et al. (2013) that, because the surface of skin and/or hair under physiological conditions are negatively charged, surface charge may play a role in penetration and that positively charged particles may be most preferred for penetration. However, in one study that was summarised, only negatively charged latex particles at 50 nm and 500 nm could permeate the stratum corneum and reach the viable epidermis using a pig skin model, while positively charged and neutral particles of all sizes and negatively charged 100 and 200 nm particles did not show any permeation (Liang et al., 2013). But, as noted in a review of the available data, "the interrelationship between various types of external physical forces and shape of nanoparticle on skin penetration has yet to be fully investigated." (Liang, et al., 2013).

6.1.1.3 Distribution

Distribution refers to the translocation of a material throughout the body following absorption by any route of exposure. The distribution of nanomaterials depends on the affinity of the nanomaterial for specific tissues. The specific properties of nanomaterials likely contributing to

their distribution include their size, agglomeration/aggregation, surface charge, hydrophobicity and dissolution.
Following inhalation exposure, the extrapulmonary transport of nanoparticles is thought to occur via three routes: (1) airway and alveolar macrophage uptake, in concert with mucociliary transport or cough clearance to the gastrointestinal tract;(2) particle translocation through the alveolar wall, with subsequent transfer into blood and extrapulmonary organs;(3) phagocytic uptake of nanoparticle by pulmonary alveolar macrophages, with intracellular particle dissolution and subsequent transfer into blood (Wang et al., 2013).
The distribution of gold nanoparticles depends on the size of the nanoparticles (Zhang et al., 2013), as illustrated in a study in which the liver absorbed more 18 nm diameter gold nanoparticles than 1.4 nm diameter particles, whereas those with a diameter of 15 nm had a wider organ distribution than did larger ones. Furthermore, as discussed by Landsiedel et al. (2012), nanoparticles with a diameter of approximately 100 nm showed a low rate of uptake by the mononuclear phagocyte system and a prolonged half-life in the blood (compared with those a diameter of less than 50 nm or more than 250 nm). In addition, nanoparticles with a diameter less than 50 nm, had a higher uptake in the liver, but in the spleen, nanoparticles less than 100 nm in size had less uptake than large particles. A study summarised by Reidy et al. (2013) found that, following intravenous administration of silver nanoparticles, particle sizes 80 nm and 110 nm accumulated primarily in the spleen, liver and lungs, while 20 nm particles accumulated primarily in the liver, kidneys and spleen. Researchers have also examined the potential for nanoparticles to cross the placenta and some data indicate that such distribution is size dependent. Pregnant mice treated with 70 nm silica nanoparticles or 35 nm titanium dioxide nanoparticles suffered damage to the placenta and foetus, with particles larger than 80 nm being partially or totally excluded. However, other work, such as a study of fluorescently labelled polystyrene beads with diameters of 50, 80, 240 and 500 nm in an *ex vivo* human placental perfusion model, found no size dependence (Wick et al., 2010).
The agglomeration/aggregation state of nanomaterials also impacts the potential distribution. Wang et al. (2013) summarised one study that evaluated the systemic distribution of primary, agglomerate (loosely bound particles) and aggregate (strongly bound particles) gold nanoparticles. The study found that aggregate nanoparticles are most likely to translocate to pleural mesothelia cells in the lungs and show significantly higher accumulation in the lungs and heart of rats than primary nanoparticles and that, because of their size and surface characteristics, primary nanoparticles tended to have a wider organ and cellular distribution, and a higher systemic blood level over time than agglomerated or aggregated nanoparticles.
Surface characteristics are also an important factor in nanoparticle distribution. In one study by Lankveld et al. (2011), the clearance and distribution of gold nanoparticles that were either capped with cetyl trimethyl ammonium bromide (CTAB) or coated with polyethylene glycol

(PEG) were evaluated after intravenous administration. The CTAB-capped gold nanorods were almost immediately (<15 min) cleared from the blood circulation, whereas the PEGylation of gold nanorods resulted in a prolonged blood circulation with a half-life of 19 hours and more widespread tissue distribution. In addition, the CTAB-capped gold nanorods had tissue distribution limited to the liver, spleen and lungs; the PEGylated gold nanorods also distributed to kidney, heart, thymus, brain and testes (Lankveld et al., 2011).
Nanoparticles may be taken up by the phagocytic cells of the mononuclear phagocyte system (Landsiedel et al., 2012). The binding of specific proteins (opsonins) to the nanoparticles may increase their uptake by these phagocytic cells. Opsonins have a lower affinity to those particles, which are more hydrophilic and have a neutral surface charge (Landsiedel et al., 2012). Furthermore, hydrophobic particles are generally opsonised more quickly than hydrophilic particles. Opsonisation of nanoparticles with neutral surfaces proceeds more slowly than that of charged particles (Landsiedel et al., 2012).
In addition, with regard to systemic distribution following inhalation, Wang et al. (2013) found that, upon intranasal instillation of iron oxide (Fe_2O_3) nanoparticles and titanium dioxide nanoparticles, these particles entered the olfactory bulb via the olfactory nerve layer along the secondary nerve of the glomerular layer and were deposited in the hippocampus region of the mouse brain, which suggests that nanoparticles may translocate to the central nervous system via the olfactory bulb. Furthermore, in one study summarised by Yokel et al. (2013), 50 nm colloidal silver-coated gold nanoparticles were taken up from the nasal cavity into the olfactory nerve, olfactory bulb and across synapses to connecting neurons of the brain.
Another important consideration in the distribution of nanoparticles is their dissolution. Dissolved ions may distribute differently in the system than particles.
In addition to dissolution, the binding of proteins and the formation of protein corona will have a large impact on the distribution of the nanoparticles. The formulation of protein coronas is discussed further in the next section.

6.1.1.4 Metabolism

Because of their typical composition (metal or carbon-based nanoparticles), many nanomaterials do not undergo typical metabolism. Rather, their interaction with biological systems affects their properties. The interactions may include protein corona formation, degradation (under acid conditions) and/or dissolution.
Within the body, nanoparticles may contact biological fluids, proteins, phospholipids and nucleic acids (Wang et al., 2013). Interactions with these biological components can alter the size, aggregation state and interfacial properties of the nanomaterial. Various proteins may bind to the nanomaterial, forming a protein corona (Wang et al., 2013). Protein coronas can affect the biodistribution and translocation of nanomaterials, as well as their excretion (Wang et al., 2013). The

protein binding can alter the agglomeration status, dissolution kinetics, surface charge and surface chemistry. The composition of the protein corona may change over time due to continuous protein association and dissociation (Kettinger et al., 2013).
The specific surface properties of the nanomaterial impact the composition of the protein corona. Hydrophobic nanomaterials more easily adsorb proteins, whereas hydrophilic ones are less prone to protein binding (Kettinger et al., 2013). In addition, positively charged nanomaterials adsorb a different set of proteins on their surface than negatively charged ones, which may influence the mode of cell entry, biodistribution and biocompatibility (Kettinger et al., 2013). Saptarshi et al. (2013) found that size may impact protein binding in some instances, while in other studies size was not a factor. Saptarshi et al. (2013) also suggested that shape may influence protein binding after summarising data in which titanium dioxide nanorods and nanotubes adsorbed different plasma proteins. While shape and size may have some influence on protein binding, hydrophobicity and surface charge generally have the greatest influence on protein binding (Landsiedel et al., 2012).
The degradation of nanoparticles within the body can alter their surface chemistry. In a study summarised by Zhang et al. (2013), iron oxide nanoparticles were found to be taken up by phagocytic cells *in vivo* and were degraded within the liver and transferred to the spleen as ferritin or hemosiderin. Furthermore, Zhang et al. (2013) noted that some carbon nanomaterials can be degraded *in vitro* and summarised one study in which pristine single-walled carbon nanotubes (SWCNTs) were not degraded upon incubation with horseradish peroxidase, but carboxylated SWCNTs were degraded under these conditions. Any degradation of nanoparticles will also likely impact the proteins binding to the surface of the nanoparticles.

6.1.1.5 Excretion

Excretion is the elimination of xenobiotics from the body. This may occur through the urine, faeces, perspiration, seminal fluids, mammary glands, saliva or exhaled breath. Based on available data, excretion of systemically absorbed nanoparticles occurs primarily through the kidneys, liver or mammary glands; however, additional research is needed to identify other excretion pathways (Zhang et al., 2013).
The excretion of nanoparticles depends on their size and surface properties. As summarised by Zhang et al. (2013), highly dispersed carbon nanotubes (with average diameters of 20-30 nm) are excreted through the kidneys. For spherical nanoparticles (quantum dots and gold), the elimination threshold through the kidneys is determined by their size and surface properties (Zhang et al., 2013). Furthermore, the size of nanoparticles may impact their excretion through bile and faeces (Zhang et al., 2013). This was illustrated from data in which approximately 5 % of gold nanoparticles with a diameter of 1.4 nm were excreted through the faeces, but only 0.5 % of gold nanoparticles with a diameter 18 nm were excreted in 24 hours.

Surface charge may also impact the excretion of nanoparticles through the bile and faeces (Zhang et al., 2013). In one study, positively charged mesoporous silica nanoparticles were rapidly excreted from the liver into the gastrointestinal tract in a surface-charge-dependent manner: the more positive the surface charge on the mesoporous silica nanoparticles, the more they clear through bile and faeces (Zhang et al., 2013).

6.1.2 Toxicity

The toxicity of a given nanomaterial depends not only on the specific properties of the material, but also on which organs/tissues it is in contact with. The nanomaterial may elicit effects at the port of entry (lungs, skin, gastrointestinal tract) or at organs/tissues distal from the port of entry (liver, brain, kidneys, etc.). As previously discussed, the specific toxicokinetic profile of a nanomaterial will dictate the specific organs/tissues that may be exposed to the nanomaterial. The following sections summarise some of the potential effects in the target organs/tissues, the mechanisms of toxicity and the properties of the nanomaterial that may impact the toxicity.

6.1.2.1 Port of Entry Effects

Port of entry effects are described below according to the exposure route.

Lungs

Once a nanomaterial enters the lungs, it may elicit direct effects on the respiratory tract. Bakand et al. (2012) suggested that these effects may depend on the particle size, surface characteristics, composition and dissolution of the nanomaterial. Shape or aspect ratio may also be an important factor for some particles (Nagai and Toyokuni, 2012).
The deeper the particles are deposited, the longer it takes to remove them from the lungs and the higher the probability of adverse health effects due to particle–tissue and particle–cell interactions (Bakand et al., 2012). Furthermore, it appears that the phagocytosis function of alveolar macrophages removes large particles more effectively than inhaled nanoparticles (Bakand et al., 2012).
Iavicoli et al. (2012) noted that size is a critical factor in toxic effects on the respiratory tract, describing several studies that found greater inflammatory effects of nanoparticles compared with their fine counterparts in both acute and chronic studies.
An inhaled nanomaterial may dissolve in the lung to yield constituents with biologic activity, which may produce a toxic effect if present in excessive amounts (Borm et al., 2006). For nanomaterials that are poorly soluble, the lack of dissolution may result in persistence within the lung and could cause longer-term effects such as pulmonary inflammation (Borm et al., 2006).
The potential biopersistence and similarity in shape to asbestos have been a concern with some nanoparticles such as carbon nanotubes. Nagai and Toyokuni (2012) evaluated the mechanisms of action of

carbon nanotubes and asbestos. Carbon nanotubes and asbestos have a similar shape (needle like) and both have a high biopersistence in the lung. However, the authors found that despite these similarities, carbon nanotubes and asbestos differ in their mechanism of entry into mesothelial cells. Non-functionalised, multiwall carbon nanotubes enter mesothelial cells by directly piercing through the cell membrane in a diameter-dependent and rigidity-dependent manner, whereas asbestos mainly enters these cells through the process of endocytosis, which is independent of fibre diameter. They concluded that small particles are phagocytosed well by macrophages and removed from the respiratory system via the lymphatic system; however, long or large fibres may not be taken up by macrophages and may persist inside the lung for a long period of time, which can lead to chronic inflammation (Nagai and Toyokuni, 2012). Furthermore, fibres remaining in the lung can penetrate through alveolar epithelial cells and visceral mesothelial cells and reach the parietal mesothelial cells due to negative pressure in the pleural cavity (Nagai and Toyokuni, 2012). For carbon nanotubes, a positive charge on the surface and a thin diameter appear to be two important factors in facilitating the membrane piercing. In addition, the presence of specific ligands on the carbon nanotube surface may induce ligand-mediated endocytosis, which allows large-sized nanotubes to enter non-phagocytic cells (Nagai and Toyokuni, 2012).

Particle shape may also be an important factor for nanoparticles other than carbon nanotubes. Iavicoli et al. (2012) noted the critical role of shape in titanium dioxide nanoparticle bioactivity and indicated the increased markers of inflammation detected in the bronchoalveolar lavage of anatase nanobelt aspiration-treated mice, compared with those determined in animals treated with titanium dioxide nanospheres. The nanomaterial form (crystallinity) of titanium dioxide may also play a role in any potential respiratory tract toxicity. As discussed by Iavicoli et al. (2012), some data are available suggesting that anatase titanium dioxide nanoparticles may elicit greater pulmonary toxicity in comparison with rutile.

Surface chemistry and surface area may also impact the potential respiratory toxicity of nanoparticles. Bakand et al. (2012) suggested that the novel surface characteristics and large surface area of nanomaterials may contribute to ROS generation, leading to potential oxidative stress, inflammation, and damage to cells, proteins and DNA. One study summarised by Bakand et al. (2012) found that silicon dioxide nanoparticles induced ROS generation and glutathione depletion in A549-human pulmonary epithelial cells. Manke et al. (2013) indicated that, in addition to being self-oxidative in nature, nanoparticles can react with cells and induce oxidative stress through intracellular ROS generation involving mitochondrial respiration and activation of NADPH-like enzyme systems. ROS in the lungs can be activated by nanoparticles through the induction of alveolar macrophages and neutrophils (Manke et al., 2013).

In addition to oxidative stress, nanoparticles may also induce generation of reactive nitrogen species through the induction of inflammatory

phagocytes (Manke et al., 2013). Through nitric oxide synthase activity, phagocytes can produce nitric oxide (NO) and peroxynitrite (ONOO), which can cause DNA fragmentation, lipid oxidation and protein dysfunction, which can contribute to particle-induced lung injury (Manke et al., 2013).

Gastrointestinal Tract

Based on the review of articles evaluated as part of Phase 1 of this project, few *in vivo* data were identified that suggested toxic effects in the gastrointestinal tract following oral exposure or that provided sufficient information on the potential characteristics of nanoparticles that would contribute to the potential toxicity. Bergin and Witzmann (2013) determined, upon reviewing the primary literature, that additional research is needed to understand the potential effects on the gut microbiome. Although there is a paucity of data that indicate toxicity on the port of entry following oral administration, the properties of nanoparticles that contribute to general toxicity mechanisms, as discussed elsewhere in this report, may contribute to potential toxicity in the gastrointestinal tract.

Skin

Little *in vivo* information was identified that indicated that nanoparticles are toxic to the skin upon direct application. One study summarised by Johnston et al. (2010) indicated that carbon nanotubes, when applied dermally to mice, did result in an increase in bi-fold thickness, which is a measure of oedema and inflammation. In addition, it was suggested by Lu et al. (2008) that titanium dioxide (TiO_2) may be phototoxic through free radical generation. TiO_2 can generate hydroxyl free radicals from water in the presence of UV light, the degree of which is dependent on the crystalline structure (anatase TiO_2 has higher photocatalytic activity than rutile). Nano-TiO_2 promoted the formation of protein tyrosine nitration (photocatalytic effect in presence of NO_2 (Lu et al., 2008). However, acute dermal irritation studies in rabbits and local lymph node assay results in mice indicated that ultrafine-TiO_2 (i.e. particles of average primary size of roughly 100 nm) was not a skin irritant or dermal sensitizer (Warheit, et al., 2007).
Although few data are available that indicate toxicity to the skin, the properties of nanoparticles that contribute to general toxicity mechanisms, as discussed elsewhere in this report, may be relevant.

6.1.2.2 General Mechanisms of Toxicity of Nanoparticles

Following absorption systemically, nanoparticles may be distributed to a number of different tissues/organs. Once a nanomaterial reaches target organs/tissues, different mechanisms may be responsible for the toxicological effects of nanoparticles, including ROS generation, oxidative stress, mitochondrial perturbation, inflammation, uptake through mononuclear phagocyte system, protein denaturation, phagocytosis impairment, endothelial dysfunction, the generation of neoantigens, altered cell cycle regulation and DNA damage (Bakand et

al., 2012). It was further emphasised by Wu et al. (2012) that nanomaterials in contact with target organs/tissues can elicit biological changes in DNA and proteins, as well as changes to the cellular membrane and cytoskeleton.

The specific properties of nanomaterials that affect the apparent mechanisms of toxicity include: chemical composition, size (primary and agglomerated), shape, crystalline structure, surface area, surface chemistry, surface charge and solubility (Magdolenova et al., 2014; Bakand et al., 2012; Wu et al. 2012). Multiple mechanisms can cause nanoparticles to affect various cellular structures and cause toxicity. The key influences on cellular toxicity are discussed further below.

Cellular uptake by nanoparticles is a fundamental determinant of toxicity. Kettinger et al. (2013) noted that nanoparticle size is an important factor dictating nanoparticle uptake. Nanoparticles with a diameter of 50 nm are more efficiently internalised by cells than smaller particle sizes (approximately 15–30 nm) or larger particles (approximately 70–240 nm); nanoparticles with a diameter of 30–50 nm interact with membrane receptors and are subsequently taken up by receptor-mediated endocytosis. Iversen et al. (2011) noted that nanoparticles of 20-50 nm are taken up more rapidly by cells than smaller or larger particles. This was further supported by Zhu et al. (2012), who stated that the most important physical property of a nanomaterial in determining cellular uptake is its size.

In addition, nanoparticle shape is a factor in cellular uptake, with spherical nanoparticles taken up much faster and efficiently than rod-shaped nanoparticles (Kettinger et al., 2013). Surface charge and functional groups also play a role in cellular uptake (Kettinger et al., 2013). This was supported by Reidy et al. (2013), who indicated that negatively charged gold nanoparticles appear to enter cells through the endocytic pathway, resulting in higher cytotoxicity compared with positively charged silver nanoparticles of similar size. It is also important to note that the specific cell type is an important factor in determining whether positively or negatively charged particles are more likely to be taken up (Iversen et al., 2011).

One of the targets of nanoparticles may be the cell membrane. Cellular membrane integrity can be affected by the size, surface charge and surface chemistry of nanoparticles (Wu et al., 2012). Fröhlich et al. (2012) noted that positively charged nanoparticles appear to cause membrane damage either directly or by the detachment of adsorbed polymers (e.g. polyethyleminine), whereas anionic particles cause intracellular damage. This was further emphasised by Kettinger et al. (2013), who noted that positively charged particles interact strongly with the anionic membrane and may disrupt membrane integrity. In addition, positively charged particles may induce a more fluid state for easier penetration, but negatively charged nanoparticles induce gelation of the membrane (Wu et al., 2012). A cationic surface charge correlates with higher cellular uptake and greater cytotoxicity in non-phagocytic cells, whereas cationic nanoparticles appear to cause plasma-membrane disruption to a greater extent than anionic nanoparticles (Fröhlich et al.,

2012). However, anionic nanoparticles are taken up and are more cytotoxic in phagocytic cells (Fröhlich et al., 2012). The size of nanoparticles may also contribute to the membrane toxicity. Nanoparticles in the size range of 1.2 to 22 nm were found to induce holes in lipid membranes, whereas those nanoparticles that are less than 1.2 nm or greater than 22 nm did not have a similar effect (Wu et al., 2012). In one study summarised by Wu et al. (2012), gold nanomaterials (approximately 6 nm in diameter) that had the same chemical composition, but different surface ligand organisation (sub-nanometre striations of alternating anionic and hydrophobic groups or same moieties, but in random distribution) showed dramatic differences in cell membrane response.
In addition to effects on the cellular membrane, nanoparticles may elicit effects on the cytoskeleton[3] (Soenen et al., 2011). Disruption of the cytoskeleton is associated with nanoparticle composition, size, shape, surface modification, as well as exposure and time (Wu et al., 2012). It was found that silica nanoparticles with a diameter of 100 nm did not disturb the filaments of the cytoskeleton, whereas silica nanorods could disrupt the filaments of the cytoskeleton (Wu et al., 2012). Furthermore, experimental data have shown that gold nanomaterials have induced cytoskeletal defects and have profound effects on the morphology of several cell types, such as A549 human lung carcinoma cells; furthermore, gold nanoparticles have a concentration-dependent effect on the actin fibrils of human dermal fibroblasts (Wu et al., 2012).
Nanoparticles may also have an effect on the nucleus. Because of their size, charge, surface area, composition and surface chemistry, nanoparticles may be able to enter cell nuclei and induce genotoxicity (Wu et al., 2012). The effects on DNA result from the nanoparticle binding directly to DNA (because of the surface charge of the nanoparticle) or indirectly through the generation of ROS (Wu et al., 2012). These biological effects on DNA may result in genotoxicity. One study of four sizes of amorphous silica particles, one microsized (498 nm) and three nanosized (68, 43 and 19 nm) showed DNA damage to be size-dependent: the level of DNA damage in cells increased with decreasing particle size (Magdolenova et al., 2014). Furthermore, it was noted by Magdolenova et al. (2014) that surface properties, including chemistry and charge, shape, chemical composition and crystalline structure (i.e. rutile and anatase TiO_2), are also important factors in determining potential genotoxicity. Fröhlich et al. (2012) stated that the generation of ROS or surface activity through Ti-O or Ti-N bonds could cause DNA alterations induced by silver and TiO_2 nanoparticles.
The mitochondria may also be a potential target for nanoparticles. As discussed by Fröhlich et al. (2012), mitochondrial swelling was observed following exposure to quantum dots and decreased mitochondrial membrane potential was found following exposure to silver, titanium dioxide and alumina nanoparticles. The increase in mitochondrial

[3] The cytoskeleton is the network of protein filaments and tubules in the cytoplasm of many living cells that lends shape and coherence to the cell.

membrane permeability was induced either by disruption of the respiratory chain or by changes in Bax and Bcl-2 expression, leading to disruption of mitochondrial metabolism, increased ROS production, adenosine diphosphate-induced depolarisation, release of cytochrome C and induction of apoptosis (Fröhlich et al., 2012).
Because of the affinity of nanoparticles for macromolecules, they may bind to cellular proteins and disrupt cellular function. As summarised by Fröhlich et al. (2012), intracellular titanium dioxide induced conformational changes in tubulin and inhibited tubulin polymerisation, which could potentially lead to impairment of cell division, cellular transport and cell migration. Furthermore, it was noted that dendrimers, carbon nanotubes, alumina nanoparticles and chitosan nanoparticles could disrupt intracellular tight junctions and potentially decrease the transepithelial electrical resistance of cell monolayers (Fröhlich et al., 2012).
The generation of ROS is one of the primary mechanisms of nanoparticle toxicity. Nanoparticles may generate ROS by different processes. The first is the reactivity of the nanoparticle itself. The relatively large surface area and increased reactivity of many nanoparticles can enhance their formation of ROS (Soenen et al., 2011). Manke et al. (2013) noted that surface-bound radicals, such as $SiO^{\bullet}$ and $SiO_2^{\bullet}$, present on quartz particles are responsible for the formation of ROS, such as $OH^{\bullet}$ and $O_2^{\bullet}$.
The second factor that may be at work is the body's natural defence system. Under stress, certain cells can produce chemically active oxygen-containing molecules that can "defend" the cell by oxidising the foreign substance. Prolonged generation of ROS, however, can damage the cell itself. The ROS formed as a result of nanoparticle exposure may be radical ROS (nitric oxide or hydroxide radicals) or non-radical ROS (hydrogen peroxide) (Soenen et al., 2011). Soenen et al. (2011) describe four general mechanisms for the generation of ROS:

(a) Nanomaterials present in the acidic environment of lysosomes can induce ROS by direct reactivity of their surface coating, degradation of the coating and direct interaction of the acidic media on the metal surface or degradation of the whole nanoparticle and production of ions (Fe^{2+}, Cd^{2+}) which can induce ROS by various chemical reactions.

(b) Nanomaterials can also directly interact with oxidative organelles such as the mitochondria by destabilising the outer membrane, deregulating the mitochondrial membrane potential and hereby disrupting the electron transport chain of the oxidative phosphorylation.

(c) Nanoparticles can directly interact with redox-active proteins such as NADPH oxidase and hereby stimulate large ROS production in cells of the immune system.

(d) Interaction of nanoparticles with surface located receptors can lead to receptor activation and triggering of intracellular signalling cascades (activation of second messenger or calcium

waves), finally resulting in expression of stress response genes which can upregulate ROS.

This summary identifies several critical variables, including the chemical identity (reactivity) of the nanoparticle, its surface coating, and solubility to generate metal ions. It is important to note that the mechanism for ROS generation may differ, depending on the specific nanomaterial (Manke et al., 2013).

The contribution of size and surface area to ROS generation was suggested by Ivask et al. (2013), who found that high levels of ROS in various mammalian cells were induced by silver nanoparticles less than 20 nm. Furthermore, as summarised by Magdolenova et al. (2014), a higher potency of particles of 10 and 20 nm titanium dioxide was observed, compared with particles of 200 and >200 nm titanium dioxide, in inducing oxidative stress in the absence of photoactivation.

In addition, surface chemistry can influence ROS generation. Zhang et al. (2012) found that, for fumed silica nanoparticles, there was a positive correlation of toxicity with hydroxyl concentration and its potential to generate ROS. Manke et al. (2013) noted that nanoparticles with smaller particle size may induce higher ROS due to high surface-area-to-volume ratio and high surface charge.

While not all potential mechanisms of toxicity were summarised above, the mechanisms as described consistently suggest some common nanomaterial characteristics that may contribute to cellular toxicity. It is also important to note that the characteristics contributing to toxicity for one type of nanomaterial may not be the same as for another nanomaterial and that each should be evaluated on a case-by-case basis when developing a potential read-across or grouping strategy.

Table 9 Parameters Critical to Human Health Toxicity Based on Current Research

Property	**Summary of Relevance**
Chemical identity	
Chemical composition	Chemical composition can fundamentally determine effects.
Crystalline structure	Crystalline structure may influence reactivity, for some materials, in a way that affects toxicity.
Surface characteristics (and surface charge): • Coating • Functionalisation • Capping agents	The surface chemistry of a nanomaterial affects its systemic absorption upon inhalation route. The surface coating may determine the biomolecules that adhere to the nanomaterial, its distribution and cellular uptake, and the effects it may have on cellular toxicity. Surface charge may influence the systemic distribution and cellular uptake of a nanomaterial and ultimately its toxicity.
Impurities	Impurities can substantially contribute to toxicity.

Property	Summary of Relevance
Particle characteristics	
Particle size/range	The size of the nanoparticle impacts other physico-chemical properties, influences the degree of exposure and may also affect the systemic bioavailability, the distribution within the body and the toxicity at both the point of entry and distally.
Shape	Particle shape can influence deposition within the lungs and can also influence the persistence of a nanomaterial in the lungs. Shape may also affect the ability of a nanomaterial to penetrate into a cell.
Porosity	Not identified as a primary determinant in toxicity; however, this is important to the extent that an increase in surface area may increase the reactivity of a nanomaterial relative to its mass.
Surface area	The increase in relative surface area with decreasing particle size can increase the reactivity per unit mass of the nanoparticle.
Fundamental behaviour	
Water solubility • Rate of dissolution • Equilibrium solubility	The rate of dissolution depends on particle size, coating, stability, manufacturing process and biological environment; for nanomaterials that have a high rate of dissolution, where the ion may be dictating the toxicity, this will be an important aspect of the evaluation.
Hamaker constant	Parameter can influence the degree of agglomeration and sorption, but is not typically characterised in toxicity studies.
Zeta potential	Parameter can influence the degree of agglomeration and sorption, but is not typically characterised in toxicity studies.
Dispersiveness	Parameter can influence the degree of exposure (particularly by the oral route), but is not a primary variable characterised in toxicity studies.
Dustiness	Parameter can influence the degree of exposure (particularly by inhalation), but is not a primary variable characterised in toxicity studies.
Activity and reactivity	
Physical hazards	Parameter may be relevant to the risk of injury in occupational exposures, but is not a primary variable characterised in toxicity studies.
Reactivity	The reactivity of a nanomaterial – particularly relative to the non-nanoform of the substance – can impact the generation of ROS, induce inflammation and elicit cellular toxicity.

Property	Summary of Relevance
Photoreactivity	Parameter may be relevant for some effects by dermal exposure, but is not relevant to oral or inhalation exposures.

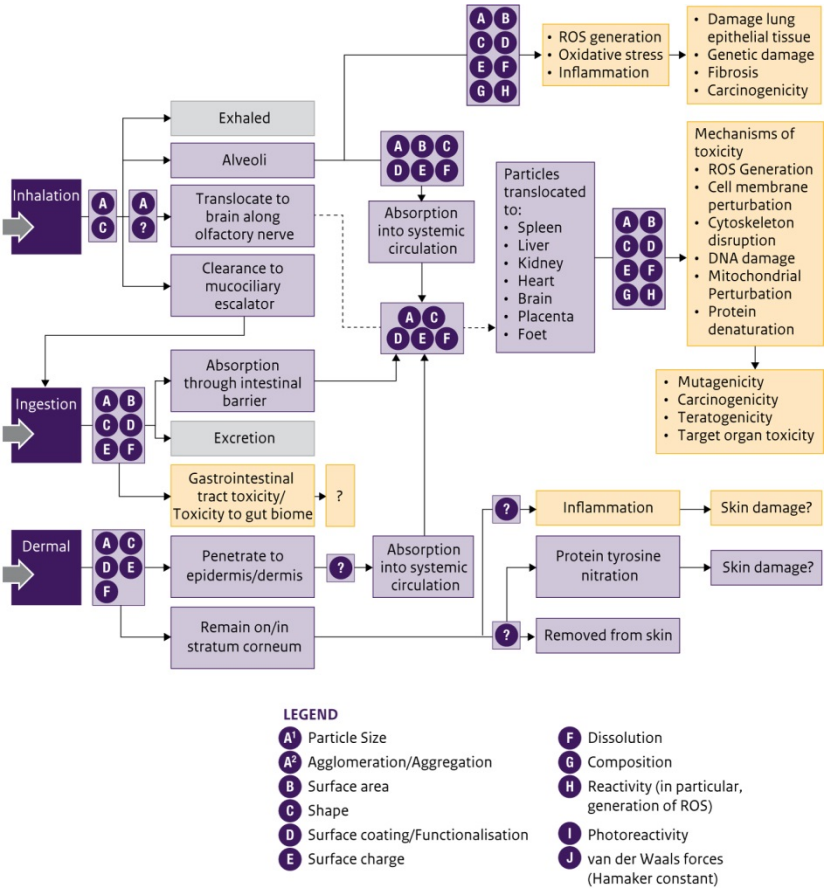


Figure 11 Human Health

6.2 Summary of Characteristics Critical to Human Health Endpoints

The key nanomaterial characteristics potentially affecting human health are summarised below. This information was synthesised from the analyses of each endpoint and is illustrated in Figure 11.
Table 9 summarises how the physico-chemical characteristics of nanomaterials affect toxicokinetics and human health toxicity for different routes of exposure. The human health toxicity of nanomaterials is dependent on its toxicokinetic profile, which dictates the concentration and characteristics in target organs or tissues and the biological interactions with the target organs or tissues. The toxicokinetics of a nanoparticle depend on the route of exposure, the material composition, size (which can change with agglomeration), shape, surface charge,

surface chemistry and dissolution. Likewise, these physico-chemical properties also affect the potential toxicity at the target organ/tissue. It should be noted that, while these are the key characteristics that should be the initial focus for any read-across or grouping strategy, there may be properties that are substance-specific that will need to be considered, but which are not tabulated above or depicted in Figure 11.

6.3 Uncertainties

Uncertainties remain with regard to the human health toxicity of nanoparticles. Some of the main uncertainties are listed below:

- Many studies have lacked adequate substance characterisation. When evaluating whether a toxicity study for one nanomaterial (or non-nanomaterial) may be used for another based on read-across, the nanomaterial evaluated as part of the study should have adequate characterisation to compare to the nanomaterial of interest.
- More data are still needed to understand the toxicokinetics of nanomaterials and the factors that dictate a nanomaterial's toxicokinetic profile. Few data are available on elimination, for example.
- While the formation of protein coronas has been noted as an important factor in the toxicokinetics and toxicity of nanomaterials, this needs to be studied further. While many nanomaterials investigated for pharmaceutical purposes may have data on potential nanomaterial protein interactions, those nanomaterials used for other applications may not have as robust a dataset.
- Additional study is needed on the impact of a single nanoparticle characteristic (e.g. size, shape, surface properties, etc.) on a particular endpoint in order to know how modifying such a characteristic can impact the potential biological effect.
- Particularly for metallic nanomaterials, there is a need to understand metal forms in target tissues/organs and whether it is ionic or particulate (e.g. silver). The current analytical methods used in many of the studies do not differentiate.
- It is important to use and understand whether doses of nanoparticles used in many of the studies are relevant from a human exposure standpoint.
- There remains a need to further standardise test methodology for the toxicity of nanomaterials so that results are repeatable and comparable.
- Much of the data available for nanomaterials have been generated from *in vitro* studies. While *in vitro* data can provide valuable insights into potential mechanisms of toxicity and, recognising the importance of limiting animal testing, more *in vivo* data are needed to substantiate findings from *in vitro* data for nanomaterials. Furthermore, much of the *in vitro* data were developed using methods for which international standards have

not yet been developed. It will be important to have standard methods so that results are comparable between substances.

7 Framework for Development of Testing Strategies

In the simplest terms, a testing strategy must answer the question '*What information do I need to reduce the amount of testing?*', while meeting the objectives of the test programme. For example, if one were considering a material comprised of nanosilver particles between 10 and 200 nm in size, what would one need to know regarding the influence of size and coating on the behaviour of nanomaterials to determine whether solubility testing was necessary to support a risk assessment? The development of a test strategy must begin with defining the objectives of the programme. This report refers to objectives associated with registering a substance under REACH. Much of the logic of the testing strategies described in this report, however, could be adapted to achieve other objectives.

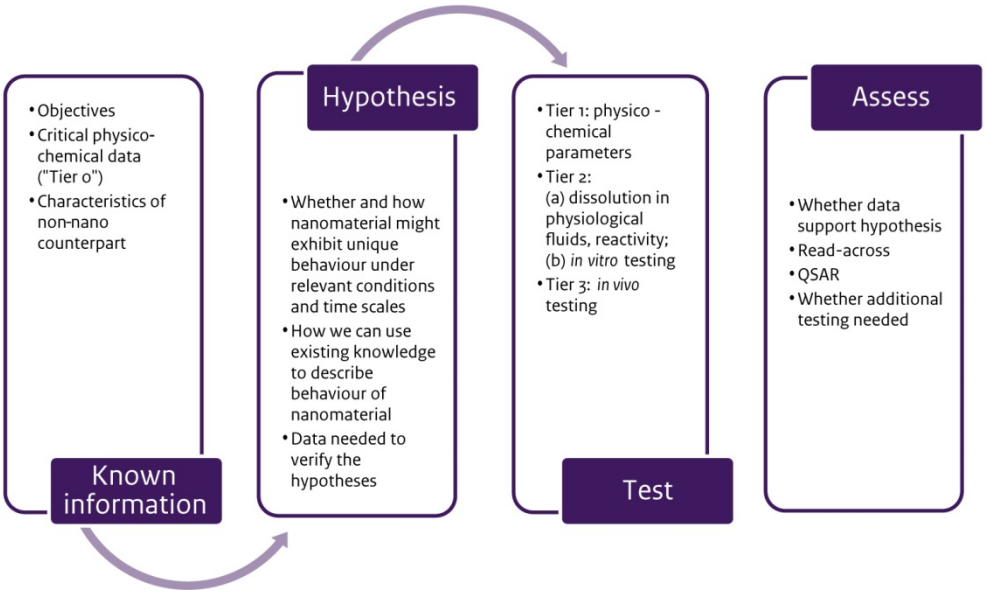


Figure 12 Framework for Testing Strategy

Figure 12 illustrates a conceptual framework for (eco)toxicology testing strategies. It shows a logical progression from compiling available information to increasingly complex testing designed to minimise the use of animal testing. This framework is somewhat idealised, as it does not reflect the reality that test methods are not commercially available for some important parameters. Furthermore, the simple linear structure of the figure does not represent the reality that this framework would require some iterative thinking. As data were collected in each tier, one would likely return to the testing hypothesis and assess whether additional data were needed.
Each of the steps in this conceptual framework is discussed briefly below, followed by a brief description of critical physico-chemical data

and discussions of how this framework might be applied for human health and ecological endpoints.

7.1 Compile known information

The process begins by compiling available information, both qualitative and quantitative.

7.1.1 Qualitative information

- What is the purpose of the nanomaterial?
- How was the nanomaterial designed to give it unique properties?
- Is the material tightly specified or relatively heterogeneous (to the extent that could lead to variability in its properties)?
- Is a single nanomaterial or are multiple modifications of the same nanomaterial under consideration?
- Is the nanomaterial organic or inorganic?
- Does the nanomaterial have a coating?
- Based on knowledge of the manufacturing process or based on analysis, does the nanomaterial potentially have impurities that are of (eco)toxicological concern?
- Is there a non-nanoform of the material?
- Does the manufacturer make any claims regarding the special properties of this material that are related to its purpose, but may also be relevant to this inquiry (e.g. transparency, reactivity, antibacterial)?
- What is the tonnage to be manufactured or imported under REACH or other pertinent regulations?
- How might the manufacture and use of this substance result in exposures?
- What physico-chemical data are available for this substance?
- Is any information available about how this nanomaterial or its properties change as it ages?
- Are (eco)toxicological data available for this substance?

7.1.2 Essential Physico-chemical Data

Some physico-chemical data are essential to characterising a nanomaterial. Other data may be less important, particularly during an early phase of evaluation; such data might be collected in the testing phase if thought to be necessary based on analysis of all available information. Table 10 indicates which data may be essential ("Tier 0") and which data may be appropriate to collect later ("Tier 1" data to be collected during the Test step in the framework). It is important to note that the data considered to be essential in this discussion exceed the information required under REACH.

Table 10 Physico-chemical Data

Property	Tier 0	Possible Tier 1
Chemical identity		
Chemical composition	X	
Crystalline structure		X
Surface characteristics (and surface charge): o Coating o Functionalisation o Capping agents	X	
Impurities	X	
Particle characteristics		
Particle size/range (reflecting agglomeration)	X	
Shape	o[1]	X
Porosity		X
Surface area	X	
Fundamental transport behaviour		
Water solubility o Rate of dissolution o Equilibrium solubility	X[2]	
Hamaker constant	o[3]	X
Zeta potential	o[4]	X[4]
Dispersiveness		X
Dustiness		X
Activity and reactivity		
Physical hazards		X
Reactivity		o[5]
Photoreactivity		o[5]

[1] Shape may be essential information, particularly for needle-like particles and/or for ecological endpoints.
[2] Information on the rate of dissolution is essential. The difference in equilibrium solubility concentration between a nano- and non-nanoform of a substance may manifest over a timescale too long to be relevant over the course of a test.
[3] While knowledge of these specific parameters may not be critical to the substance evaluation, understanding the agglomeration that may occur over the course of ecotoxicity testing is essential information.
[4] Medium dependent.
[5] May be tested in Tier 2.

As for any effort to create an overarching testing framework, some exceptions to these general rules may exist and expert knowledge of a particular nanomaterial should be used to modify the plan shown in Table 10 when appropriate.
While these parameters and relevant test methods have been discussed previously in this report, a brief summary follows to orient the reader in the testing strategy.

Chemical identity reflects several factors, including composition, impurities, surface coatings and functionalisation. These factors can be determined by knowledge of the particle's synthesis and/or by chemical analysis.
Surface charge (which reflects the substance identity) fundamentally affects the fate, transport and (eco)toxicity of nanoparticles. Zeta potential represents the surface charge of a nanoparticle in a given test solution. While experimental methods exist, including an ISO standard, no OECD guideline is available. The isoelectric point may also be relevant. It can be determined as the pH at which the zeta potential is zero. (OECD, 2010; 2014b)
Particle characteristics include primary particle size, degree of agglomeration and/or surface area. Particle shape and porosity may also be relevant. Many authorities have evaluated the appropriate methods to measure particle size, considering the particular aspect of size to be measured, the particle shape and the availability and capability of analytical equipment (OECD, 2010; ECHA, 2012a; OECD, 2014a; Linsinger et al., 2012; OECD, 2014b). An OECD working group has concluded that SEM and TEM (Scanning/Transmission Electron Microscopy) methods are preferred for spheroidal nanoparticles (OECD, 2014b). SEM and TEM methods are also preferred for non-spheroidal, agglomerate and aggregate nanoparticles, although analytical methods have more limitations for these applications. In any event, the working group felt strongly that size measurements should be compared to the results from complementary methods. Surface area[4] can be measured using the Brunauer Emmett Teller (BET) method for dry, powdered nanomaterials. An ISO standard exists and OECD is round-robin testing BET. Small angle x-ray scattering (SAXS) may be used to measure the surface area of nanoparticles in liquids, but there are no standard methods and the equipment is not commonly available; one could also calculate the surface area from theoretical considerations (ECHA, 2012a; OECD, 2010; 2014b). Shape includes diameter and length, and could also be considered to include porosity. Standard measurement methods are not currently available for these parameters. OECD (2010) suggests electron microscopy as a method for describing aspect ratio and offers a scheme from Heywood (1947) to define particle shape that uses three measures (length, breadth and thickness) to evaluate elongation ratio, flatness ratio, sphericity, circularity and rugosity. However, OECD (2010) notes that shape descriptors may need to be more complex than these and recommends a review of geological/mineralogical literature for guidance. No standard method is available to test the porosity of nanomaterials, although several ISO methods may be applicable or could be adapted for that purpose (ECHA 2012; OECD, 2010; 2014b).

[4] The OECD Expert Meeting on the Physico-chemical Properties of Manufactured Nanomaterials and Test Guidelines (OECD, 2014b) concluded that surface area may be an important predictor of toxicity when reading across from one nanoparticle to another, as long as they were of the same chemical composition, but cautioned that surface area was not likely to be appropriate for extrapolating between different nanomaterials.

The fundamental transport behaviour of a nanomaterial may strongly reflect its solubility. The particle size can affect the rate of dissolution or, if it occurs within a time period relevant to the endpoint under discussion, the equilibrium solubility concentration. Decreasing the size of a particle generally increases the rate of dissolution. Over a period of time that may be on the order of months, one might also find that, for certain compounds, the equilibrium solubility concentration also increases with decreasing particle size (Sellers and Hassinger, 2012). OECD TG 105, Water Solubility, describes the measurement of this parameter. However, the method needs to be adapted to nanomaterials (ECHA, 2012a; OECD, 2014a, 2014b). An OECD method is reportedly under development.

7.2 Develop hypothesis

The next step in the process is to develop a hypothesis about how the nanomaterial might behave and the effects of exposure. For a nanoform of a highly soluble and toxic substance, for example, the hypothesis might be that the nanoform would be similarly toxic and that the increased rate of dissolution resulting from nanosizing might affect the rapidity of the exposure effects. In other cases, the hypothesis might focus on the ways in which the small size of the particle might result in hazards. For example, with respect to mammalian toxicity, one might consider findings reported in the literature that state:

- Depending on size range, the inhalation route of exposure might be of greatest concern, as the small size of nanoparticles can allow them to penetrate deep into the lungs.
- The effect of dermal contact is generally not influenced by size (except in case of damaged skin or perhaps certain forms of reactive substances).
- While less information is available about the consequences of ingestion, the results of ingesting a nanomaterial may not be markedly different from ingesting the non-nanoform of the substance, except that nanosizing may change the dissolution rate or perhaps enhance reactivity.

Therefore, if no toxicity data were available for the nanomaterial, one might hypothesise that it could be appropriate to read-across to other similarly-sized nanomaterials for inhalation and the non-nanoform of the substance for ingestion and dermal contact (if the latter was relevant based on tonnage band and exposure).
To test this hypothesis, it would be appropriate to ask:

- Might any other characteristics of this substance (besides its size) affect inhalation toxicity?
- What factors other than size may affect distribution of nanoparticles within the lungs?
- Does nanosizing (or any other characteristics of this substance, such as coating) markedly affect the rate or extent of dissolution?

The answers to such questions would direct the next step in the process, tiered testing.

7.3 Testing

Regulatory requirements provide the basis for determining the need for testing. A testing programme might also reflect the need to collect data that would support read-across to another substance or to the non-nanoform of a nanomaterial.

7.3.1 Overview of approach

The proposed testing strategy reflects a tiered approach of collecting data at increasing levels of complexity as needed to characterise a substance directly or by read-across. The first tier would address additional physico-chemical data needed to understand the behaviour of a nanomaterial. After obtaining Tier 1 data, the assessor might consider whether to obtain Tier 2/3 testing data or whether the Tier 1 data would support a read-across strategy.
Tier 2 testing would include more complex testing of physico-chemical characteristics or the behaviour of a nanomaterial under relevant conditions, such as solubility in physiological fluids or *in vitro* testing using cell lines. Such data would primarily be used to assess whether the dissolution or reactivity of a nanomaterial were similar to the behaviour of the substance at a different size. (Some *in vitro* data are used directly to fulfil REACH testing endpoints, e.g. for mutagenicity.) The potential concerns regarding *in vitro* assays and nanomaterials are detailed further in Section 7.3.3. Finally, Tier 3 studies would be performed, if necessary, to characterise the (eco)toxicity of a nanomaterial.
This approach of collecting and evaluating data based on physico-chemical tests or cell lines before proceeding to animal testing is consistent with the objectives of REACH. ECHA (undated) has noted that:

> *Registrants are obliged by REACH to limit new studies using vertebrate animals for REACH registration as they are to be conducted only as a last resort. Registrants must first collect and assess all existing data. They then have to identify data gaps and consider whether these can be filled by using either in vitro/ex vivo studies or other alternative approaches including prediction methods before any new animal tests are conducted.*

Effectively applying this tiered strategy to testing requires that scientists assess the data as they are collected, as described below.

7.3.2 Tier 1: Filling gaps in physico-chemical data

As indicated in Table 10, some physico-chemical data ("Tier 0") are so essential to characterising a nanomaterial that they should almost always be collected early in a testing programme. Other data ("Tier 1") may be appropriate to collect at this stage in order to better understand

the potential for exposure or the possible effects of exposure by read-across.

7.3.3 Selected Tier 2 tests

Tier 2 testing, in concept, would provide information on the behaviour of nanomaterials that bridged the gap between physico-chemical properties and biological effects. Such tests would focus on two key aspects of nanomaterials: solubility (rate of dissolution) in relevant media, which pertains to availability and persistence/biopersistence, and reactivity.
Various tests of the rate of dissolution in relevant media are discussed in the sections of the report on human health effects and ecotoxicity. These tests may examine dissolution in simulated bodily fluids such as sweat. For metals in particular, transformation/dissolution testing may be appropriate to characterise environmental fate processes. Transformation/dissolution testing could arguably be considered either a Tier 1 or Tier 2 test.
Reactivity or, more precisely, whether 'nanosizing' has changed the intrinsic reactivity/photoreactivity of a material, is also relevant to testing strategies. The ability to generate reactive oxygen species (ROS) or induce an organism to generate ROS is a critical determinant of the effect of exposure to nanomaterials.
A review of the literature (Sellers and Hassinger, 2012) provided some insight into the effect of particle size on reactivity and photoreactivity. Such studies often focus on metals or metal compounds well below 100 nm in size and the following summary should be read with that limitation in mind.
Studies have shown that reactivity generally increases as particle size decreases, with maximum activity often occurring below 15 to 20 nm. Some studies reported the opposite effect and some noted a size corresponding to peak reactivity (with lesser reactivity of particles above and below that size).
At least two phenomena, electronic and geometric, may explain the size dependence of reactivity. Decreasing the particle size increases the relative number of atoms at the surface of the particle; not only does that mean that a higher proportion of atoms are available to participate in reactions, but they are less energetically stable than the atoms in the centre of the particle. In addition, some reactions depend on geometry. A reaction may require a certain type of surface atom, e.g. a crystal edge or face, in order to occur. Reactivity may also be affected by the presence of capping agents, solution conditions or the sorption of reactants to the particle surface.
Scientists have also studied the effect of particle size on the photoreactivity of titanium dioxide (TiO_2), cadmium sulfide (CdS), and gold and various gold composites. This work has generally showed that photoreactivity increases with decreasing particle size. In some cases, the behaviour of the material changes at a particle size of approximately 5 to 10 nm. For example, some studies of TiO_2 showed that photoreactivity reached a maximum at a particle size of approximately 7 to 11 nm and decreased at smaller sizes. A study of CdS showed that

particles above 6 nm in size were not photoreactive at all, but that smaller particles effectively catalysed the dehydrogenation of methanol. Braakhuis et al. (2014) identified several techniques that can be used to characterise chemical reactivity "cell free" and biochemical reactivity. In cell-free conditions, one can measure the oxidation potential of nanoparticles by electron spin resonance (ESR) techniques. "These techniques use a spin-trapping agent to detect the nanoparticle-elicited generation of hydroxyl radicals in the presence of hydrogen peroxide." Such testing does not perfectly predict the reactions within a cell. OECD (2014b) has noted that chemists can test photocatalytic activity using fluorescence-based analysis for reactive oxygen species (DCFH, described below) or colorimetric methods, though the presence of nanoparticles may interfere with these tests; other techniques may also be applicable. However, no standardised method currently exists to characterise photoreactivity.

In vitro assays can provide information about the reactivity of nanoparticles within a cell, although no single validated assay is appropriate for all types of nanomaterials. Testing options to determine the intracellular induction of ROS include the following (Braakhuis et al., 2014):

- ESR techniques in combination with *in vitro* cellular exposure;
- 2'-7'-dichlorodihydrofluorecein diacetate (DCFH-DA) assay, which uses a fluorescent probe to visualise the induction of ROS in cells exposed to nanoparticles;
- Free radical analytical system (FRAS) assay, which measures the formation of reactive oxygen metabolites (ROM) after exposure to nanoparticles;
- Erythrocyte haemolysis assay, which measures the amount of haemoglobin released after exposure of red blood cells to nanoparticles; and
- Vitamin C yellowing assay, which measures the chemical reactivity of nanoparticles toward an anti-oxidant.

In vitro models offer clear benefits: they can provide valuable mechanistic information and can be done in a rapid and cost-effective manner. However, some limitations of the use of *in vitro* models must be considered. While *in vitro* models can provide information on a specific endpoint, they may not represent actual effects *in vivo*. Furthermore, some nanomaterials may not be compatible with a particular assay. Arora et al. (2012) noted that some dye-based assays, such as tetrazolium dye (MTT), and neutral red assays, which determine cell viability, may produce invalid results with some nanomaterials because of the interaction between the nanoparticles and the dye or dye products. Furthermore, it was found that silver nanoparticles were incompatible with the lactate dehydrogenase (LDH) assay (Oh et al., 2014). In summary, *in vitro* models must be used cautiously due to their inherent limitations and, in some cases, limitations specific to their use with nanomaterials. Nonetheless, such assays can provide a measure of the *in situ* reactivity that can result in toxicity.

While these deficiencies do need to be taken into account when interpreting results from *in vitro* studies, these data are likely going to be an important aspect of the hazard assessment of nanomaterials and the development of read-across strategies.

7.4 Assessment

This brief discussion of assessing data to determine gaps is not meant to be a comprehensive treatise, but rather is meant to put the practices used for conventional chemical substances into the context of this work.

7.4.1 General concepts

Scientists assess whether data are fit for the purpose of characterising a substance under REACH by assessing their reliability and relevance to the substance, its uses and consequent exposures, and the endpoint under consideration. The reliability of tests with nanomaterials deserves careful assessment, given the lack of standardised or routine methods for many analyses and the challenges of adapting bioassays and toxicity tests to nanomaterials. The methods used to keep nanomaterials in suspension during testing, the distinction between nominal and actual (agglomerated) particle size tested, and the matrix effects in ecotoxicity testing, in particular, warrant careful scrutiny.
This data assessment is often an iterative process that begins with an initial set of data and evolves as each additional piece of data is collected. For nanomaterials, this assessment must balance two competing possibilities: firstly, that a nanoform may not present unique hazards, and secondly, that it may. Each of those two opposing cases may be true for certain nanomaterials under different conditions, routes of exposure or endpoints.
The data assessment may also reflect the need to fill gaps by read-across to other substances or quantitative structure-activity relationships (QSAR). Such assessments typically reflect the weight of evidence.

7.4.2 Filling gaps by read-across and QSAR

The purpose of reading across is to reduce animal testing, potential costs and time to market. Read-across from one substance to another may be supported by physico-chemical testing to establish that they have similar properties or by *in vitro* testing to identify a possible link between effects. The pharmaceutical industry utilises *in vitro* testing, for example, to identify potential drug candidates that may cause human health effects early in the development. McKim (2010) states that "it is unlikely that any one *in vitro* model would be sufficient as a final decision point for toxicity, but rather a series of models that provide important information at the right time in the discovery pipeline should be used in a tiered approach". This same logic can be applied to the use of *in vitro* testing to substantiate a read-across strategy for nanomaterials. However, such tests must be considered carefully, as

test methods are not always standardised and the link between *in vitro* results and biological (*in vivo*) effects are not always clear.
QSAR evaluations are currently an important consideration for many non-nano substances for which read-across is being considered. However, the development and regulatory acceptance of QSAR models for nanomaterials is still in its infancy. Consequently, the testing strategies for nanomaterials discussed in this report do not rely on QSAR.

7.4.3 Weight of evidence

"Weight of evidence" refers to the practice of applying expert judgment to limited data in order to draw reasonable conclusions. Annex XI of REACH provides a context for using the weight of evidence in substance notifications, i.e.

> *There may be sufficient weight of evidence from several independent sources of information leading to the assumption/conclusion that a substance has or has not a particular dangerous property, while the information from each single source alone is regarded insufficient to support this notion.*
>
> *There may be sufficient weight of evidence from the use of newly developed test methods, not yet included in the test methods referred to in Article 13(3) or from an international test method recognised by the Commission or the Agency as being equivalent, leading to the conclusion that a substance has or has not a particular dangerous property.*
>
> *Where sufficient weight of evidence for the presence or absence of a particular dangerous property is available:*
>
> - *further testing on vertebrate animals for that property shall be omitted,*
> - *further testing not involving vertebrate animals may be omitted.*
>
> *In all cases adequate and reliable documentation shall be provided.*

With respect to read-across and grouping, Annex XI also states the following requirement relevant to considering the weight of evidence:

> *In all cases results should:*
>
> - *be adequate for the purpose of classification and labelling and/or risk assessment,*
> - *have adequate and reliable coverage of the key parameters addressed in the corresponding test method referred to in Article 13(3),*
> - *cover an exposure duration comparable to or longer than the corresponding test method referred to in Article 13(3) if exposure duration is a relevant parameter, and*

- *adequate and reliable documentation of the applied method shall be provided.*

Given the state of the science regarding the measurement of nanomaterial characteristics and the definition of their (eco)toxicological effects, most testing strategies must reflect the weight of evidence rather than absolute certainty. The recommendations on testing strategies in this report illustrate how the weight of evidence may be applied. For further guidance on weighing scientific evidence under REACH, the reader is referred to *Practical guide 2: How to report weight of evidence* (ECHA, 2010).

8 Recommendations on Testing Strategies: Ecotoxicity

The ecotoxicological testing strategy reflects a fundamental difference between the mammalian toxicity of nanomaterials and aquatic ecotoxicity: the nature of the test medium and the behaviour of the nanomaterial in that test medium are of fundamental importance to ecotoxicity. The testing strategy that follows takes that factor into consideration.

8.1 Known Information

The first step in the testing strategy is to compile known information about the nanoparticle and any potential read-across substance. A brief review of the data that may be relevant follows. This includes data relevant to the nanosilver case study presented below.

The **size** of the nanomaterial(s) is the first key parameter to consider. Particle size can influence the dissolution rate and equilibrium solubility of nanomaterials and affect the photoactivity and reactivity of a nanoparticle. The rate of dissolution increases with decreasing particle size, yet no clear threshold of size-related effect exists. This is important for inorganic nanoparticles, as the ionic form can trigger ecotoxicity effects. Franklin et al. (2007) observed similar dissolution rates between zinc oxide nanoparticles and a non-nano counterpart. Lopes et al. (2014) observed a different effect. Zinc oxide particles > 200 millimetres (mm) in size showed a higher dissolution rate in 48 hours compared with the two nanomaterials considered (of 30 nm and 80-100 nm); while these data are different from what might be expected (i.e. nanoparticles generally dissolve more quickly than larger particles), the authors of the study inferred that the effect related to the structure of the nanoparticles.

The influence of particle size on the rate of dissolution can be complicated by other test variables. The rate of dissolution and dispersiveness also depend on the methodology used for production and functionalisation and the media in which the nanoparticles were dispersed (Lopes et al., 2014), although studies on these phenomena and their implications for ecotoxicity testing are quite rare (Reidy et al., 2013). Reidy et al. (2013) also indicated that the OECD reference media for the different species used in ecotoxicological studies (daphnia, algae, etc.) are significantly different and that dispersion can also be significantly different in those media, leading to significant problems of comparing data and assessing their relevance to real exposures. Finally, the influence of particle size on the solubility of a metal salt can be influenced by the counter anion. With decreasing size of silver particles, the potential for releasing silver ions increases, with silver sulphide having the least release and silver nitrate having the maximum release.

The nanoparticle **coating** is a particularly critical variable. The coating affects nanoparticle solubility and therefore also nanoparticle ecotoxicity

(SCENIHR, 2014). Griffitt et al. (2008) found that 0.07 % of a silver nanoparticle with a metallic coating dissolved, leading to a LC_{50} (lethal concentration that causes a 50 % effect) of 7 milligrams per litre (mg/L) in zebrafish exposed to silver nanoparticles with a size of 44.5 and 216 nm in suspension. In contrast, Bilberg et al. (2010) tested PVP (polyvinyl pyrrolidone)-coated particles and found that 40 % of the added silver (Ag) was present as silver ions, having a 48-h LC_{50} of 84 µg/L. In addition to affecting the solubility of the coated metal, different coatings may also lead to different toxicities. In a test with zinc oxide, nanoparticles coated with polyvinyl alcohol increased the permeability of the cell membrane of bacteria and thus promoted the uptake of the nanoparticle (Ivask et al., 2013). Polymer coatings can enhance toxicity compared with the uncoated form in some tests (Perrault et al., 2012), but not in others (Allen et al., 2010). This indicates that the type of coating is of importance when considering read-across as part of the testing strategy. In fact, an OECD working group has recommended that coating (or the lack of coating) should be considered in categorising nanomaterials for the purpose of read-across and grouping coated and naked material (OECD, 2014a).
Tejamaya et al. (2012) studied the stability of several coated silver nanoparticles in ecotoxicology media. In the test using citrate, PVP (polyvinyl pyrrolidone)-coated and PEG (polyvinyl glycol)-coated silver nanoparticles, it became clear that changes in **surface functionalisation, shape, agglomeration** and dissolution occur as a function of surface coating, **media composition and ionic strength**.
Shape can have an influence on toxicity. Rigid or semi-rigid fibres may cause cell toxicity and death by perforating cell membranes. For example, dendritic-clusters of nanonickel could induce higher toxicity than spherical nanonickel in zebrafish. Concerning **surface charge**, Du et al. (2013) mention that cationic nanoparticles could induce stronger toxicity than anionic nanoparticles.
As described in the recommendation for nanomaterials applicable to endpoint-specific guidance on information requirements (ECHA, 2012), the recommendations set out in the OECD Guidance Manual for testing (OECD, 2010) and Preliminary Guidance Notes on Sample Preparation and Dosimetry for nanomaterials (OECD, 2012) needs to be taken into consideration, especially with regard to methods of suspension, the method of the nanomaterials' introduction, storage and the stability of test material, the chemical composition of the test media, the characterisation of stock dispersions, and the characterisation of samples (prepared from stock dispersions prior to administration/testing and, if possible, during and/or at the end of the test).
In summary, the following parameters of the nanomaterial are particularly crucial, both as the nanomaterial is manufactured and as the nanomaterial is transformed in a test medium or the environment: substance identity (with coatings and composition as main characteristics), particle size, surface area and shape, and solubility (rate of dissolution and equilibrium solubility) are mostly identified with ecotoxicity effects. Less information is available on the direct

relationship between the parameters of surface charge and reactivity with the outcome of the ecotoxicity effects; such data may be used more in the weight of evidence or as a possible explanation for the observed effects or discrepancy with other test results.
Based on this information, the following questions should be considered regarding the nanomaterial:

- Are there impurities that could result in an ecotoxicity effect?
- Is the nanomaterial coated or naked and what is the effect of the coating on shape, dissolution rate, particle size, reactivity and photoreactivity?
- What is the rate of dissolution and equilibrium solubility of the nanomaterial of interest?
- What other physico-chemical characteristics are known?
- Are there other ecotoxicity data available on the nanomaterial of interest? (If so, are details available on the dispersion method, preparation of stock suspensions and aquatic test medium?)

The following questions may be relevant to a potential read-across substance:

- Is the potential read-across substance a nanomaterial or the non-nanoform of the nanomaterial?
- If the potential read-across substance is also a nanomaterial, what are the physico-chemical characteristics of this substance and were those also measured during the ecotoxicity test?
- If the potential read-across substance is also a nanomaterial, what was the dispersion method used in the test?
- Is it clearly described how stock suspensions were prepared (and are those comparable to the test method used for the nanomaterial)?
- In which medium is the test performed and are the abiotic parameters (organic matter, hardness, pH, conductivity) known?
- If the potential read-across substance is the non-nanoform, then the rate of dissolution is of main importance.

8.2 Hypothesis

During this step, a scientist must assess whether and how a nanomaterial might exhibit unique behaviour under conditions and timescales relevant to ecotoxicity testing. As described above, information about particle size, coating, shape, surface area and water solubility is fundamental to this assessment and, if information about those variables is not clearly known, then testing might be appropriate. Testing will be necessary in many cases with regard to solubility, as described below. In addition to this, as physico-chemical characteristics, i.e. particle size (degree of agglomeration), shape, reactivity of the nanomaterials, can change once added into the aquatic test medium, the unique behaviour of the nanomaterial has to be assessed.

8.3 Testing

To read-across or to group nanomaterial(s), certain physico-chemical characteristics of the nanomaterial and the potential read-across substances must be fully known. As discussed above, however, many parameters could influence the outcome of ecotoxicity tests. Therefore a tiered approach is recommended in testing the hypothesis.

8.3.1 Tier 1: Physico-chemical testing

Essential data include: chemical composition, particle size, surface area, surface characteristics (coating and functionalisation, capping agents), impurities, shape, and water solubility.
Water solubility/dispersibility and dissolution seem to be the main parameters affecting fate and behaviour in the environment, and are thus also of relevance for ecotoxicity testing (see Figure 13)
A first step would be to indicate **the solubility of the test materials** (or the non-nanoform, or the nanoparticles). In the OECD meeting report (2014a) on "Ecotoxicology and environmental fate of manufactured nanomaterials: test guidelines", it was decided that the dissolution as well as the dispersibility should be tested. However, the water solubility test (described in Technical Guideline 105 (OECD, 1995)) is not applicable to nanoparticles (OECD, 2014a). As noted previously in this report, methods are under development for solubility and dispersiveness.
Relevant to such testing or to comparing the results of different ecotoxicity tests, the parameters for the dispersion of nanomaterials are: ultrasonication procedures, water quality and composition, pre-wetting steps, stabilising/dispersing agents and stock concentrations. (For example, a pre-wetting step with ethanol can be used to disperse hydrophobic nanomaterials in a water-based system, although the use of ethanol was critically discussed during the OECD expert meeting.) The choice of an appropriate dispersion method, however, depends on the type of test that should be performed and the type of nanomaterials. For example, sonication may not be necessary for hydrophilic nanomaterials.
To standardise results between laboratories (and also to compare them with, for example, the outcome of a read-across substance), the material dispersion and preparation of the suspension must be described. In addition, it is recommended that the supernatant of the dispersion be characterized after 24 hours to cover the kinetics of the aggregation process. For the testing of dissolution, the test should be performed in a time-dependent manner to cover the kinetics of dissolution and also filtration should be used (OECD, 2014a).

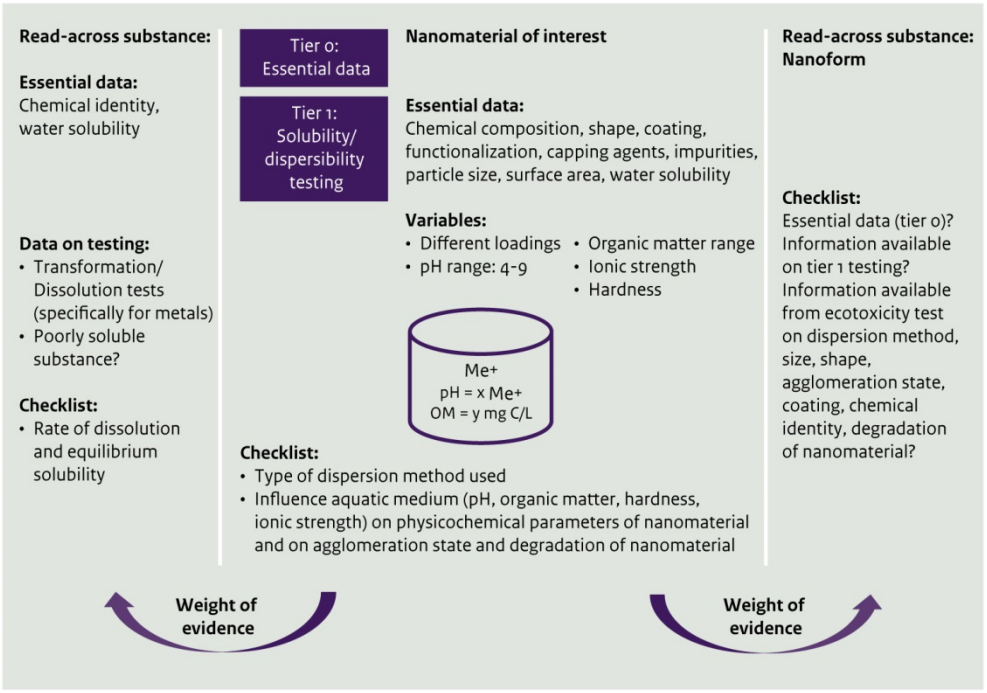


Figure 13 Schematic overview of read-across between read-across substance and nanomaterial of interest based on Tier 1 testing

The potential for the nanomaterial to change during ecotoxicity testing or, more precisely, the **agglomeration state** and **degradation rate** (abiotic and biotic) should be understood. The agglomeration state depends on different parameters: media, suspension preparation, and the concentration of the nanomaterials in the media. It may therefore be appropriate to measure the net particle size (agglomeration) throughout an ecotoxicity test to determine whether the agglomeration state is stable. The degradation rate can refer to the degradation of the nanomaterial itself or its coating. The technical guidelines available on biodegradation tests are not applicable for nanomaterials because of the low concentration of organic material. For this reason, technical guidelines specific to nanomaterials must be developed. According to the OECD meeting expert report of 2014, it could be advised that the information on the coating as a chemical can provide information on the surface modification on the nanomaterial.
Comparison of nanomaterial behaviour under laboratory and environmental conditions indicates that the agglomeration state, dissolution and dispersibility of nanomaterials are more variable under environmental conditions. These parameters must be assessed in different media, with varying pH, hardness, ionic strength and natural organic matter (NOM) content or proteins that reflect the most common natural conditions (OECD, 2014a). These conclusions can be important with respect to the testing strategy in performing a read-across between nanoparticles and may lead to additional testing in Tier 1. While information on the solubility of a nanomaterial is so crucial that it has

been designated "Tier 0", it may be appropriate to perform Tier 1 testing of the nanoparticle solubility under different environmental conditions (e.g. pH, NOM, etc.) in order to support read-across.
In the risk assessment of conventional chemicals and, more precisely, of metals, the standard OECD guideline for the transformation/dissolution testing of metal (OECD, 2001) may be of use. In this test protocol, the rate and extent to which metals and sparingly soluble metal compounds can produce soluble, available ionic and other metal forms in aqueous media are determined. The test conditions should be representative of the aqueous environment. The test media is reconstituted water with a pH range between 5.5–8.5. A screening transformation/dissolution test is available for sparingly soluble metal compounds, having the smallest representative particle size available on the market. The loading in this test is 100 mg/L. After 24 hours of agitation, the dissolved metal ion concentration can then be measured. The full transformation/dissolution test determines the level of the dissolution or transformation of metal and metal compounds after a certain period at different loadings. As noted in this protocol, the surface area of the particles in the test sample has an important influence on the rate and extent of transformation/dissolution, and powders are tested at the smallest representative particle size placed on the market. The specific surface area should be determined in order to characterise and compare similar samples (OECD, 2001). However, the ring test (OECD, 2008) did not provide for comparisons of transformation/dissolution performance for different specific surface areas (m^2/g) of a single metal. The relationship of metal concentration to surface area loading in such experiments is important for assessing the validity of the transformation/dissolution protocol for classification purposes. Such data are, however, becoming available (Skeaff et al., 2008). Fraunhofer (2012) tested this transformation/dissolution protocol for silver nanoparticles to study the dissolution kinetics of the silver nanoparticles in aqueous media. The data indicated that reliable results can be achieved when tests are carried out according to the OECD 29 guidance.
Data regarding the solubility and dispersibility of a nanoparticle indicate how the nanomaterial of interest could behave in the aquatic medium. The results of those tests also give an indication of whether results of potential read-across substances are comparable. For example, if read-across is based on a literature study where no sonication of the read-across substance is used, the outcome of those results could not be directly related to the unique behaviour of the nanomaterial of interest because sonication may have altered the behaviour of the nanoparticle in suspension.
On the potential read-across substance, information should also be available. If the read-across substance is a **non-nanomaterial**, then information on transformation/dissolution tests (if the material is a metal compound) should be gathered if available. This will provide information on the dissolution rate and equilibrium solubility of the substance. Information on ecotoxicity tests from this non-nanoform can also be used to determine the solubility of this test item. Information

should also be available if the non-nanoform is a difficult substance to test. (An OECD guidance document (2000) is available.) If this information indicates that the read-across substance is poorly water-soluble (defined as a substance with a solubility of < 100 mg/L) and the first test indicates that the nanomaterial of interest is soluble, then a read-across cannot be performed. If the potential read-across substance is a soluble substance and the equilibrium solubility of the nanomaterial has been reached, then a potential grouping or read-across could be possible. However, the other physico-chemical characteristics of the nanomaterial, (i.e. shape, type of coating, reactivity) can also have an effect on the aquatic organisms. It is therefore advised that further testing be conducted, as this uncertainty cannot be ruled out.
If the potential read-across substance is a **nanoform**, this tiered approach (Tier 0-Tier 1) can also be applied to this substance. Information on ecotoxicity tests and, for example, information that describes the dispersion of the nanomaterial into the aquatic test medium should be carefully considered. In addition, if information is available from testing physico-chemical parameters during ecotoxicity tests, then these data could be compared with "Tier 0" data. The information should be considered in a weight-of-evidence approach. If all of the characteristics of both nanomaterials are similar, then it is not necessary to perform an extra ecotoxicity test and a read-across can be performed. However, no real thresholds to determine this similarity have been developed yet. Therefore, all collected data should be considered using a weight-of-evidence approach.
At the end of this tier, the following questions on the nanomaterial (and if the potential read-across substance is a nanoform) should be considered:

- What is the influence of dispersibility methods on the physico-chemical characteristics of the nanomaterial?
- What is the dissolution rate and kinetics of the nanomaterial?
- What is the agglomeration state and degradation rate of the nanomaterial of interest?
- Do organic matter, pH, ionic strength and/or cations in solution have an influence on the dissolution kinetics and agglomeration kinetics of the nanomaterial?
- Does coating (if applicable) change particle size, shape and substance identity once dispersed in the medium?
- Do the impurities, if any, dissolve in the aquatic medium?

The following questions should be considered on the potential read-across substance (non-nanoform):

- What is the rate of dissolution and equilibrium solubility of this substance (via transformation/dissolution testing or OECD guidance (2000)).
- Is the chemical identity similar to the nanomaterial?

Figure 13 gives an overview of this first tier and approach on the read-across substance.

8.3.2 Tier 2

In the testing scheme described in this report, Tier 2 studies are those which help to describe the anticipated behaviour and effects of a nanomaterial short of *in vivo* toxicity testing. Tier 2 testing is generally more relevant for human health toxicity than ecotoxicity, though transformation/dissolution testing might arguably be considered Tier 2. To the extent that test methods are available, it may be important to test the reactivity and photoreactivity of a nanomaterial in Tier 2. Because reactivity may increase with decreasing particle size, understanding the change in this behaviour with size may help to weigh the evidence for read-across. However, OECD guidelines are not yet available to measure the reactivity/photoreactivity routinely.

8.3.3 Tier 3: Daphnid, algae and fish ecotoxicity studies

The following are discussions of:

- REACH requirements for testing; and
- OECD methods for ecotoxicity testing and special considerations for testing nanomaterials.

8.3.3.1 REACH requirements for testing

REACH requires the following ecotoxicity studies for substance registration:

- Annex VII (substances manufactured or imported at > 1 tonne per annum):
  - Short-term toxicity testing on invertebrates (preferred species Daphnia)
  - Growth inhibition study on aquatic plants (algae preferred)
- Annex VIII (substances manufactured or imported at > 10 tonnes per annum): Same as annex VII plus
  - Short-term toxicity testing on fish
  - Activated sludge inhibition growth test
  - Degradation
  - Hydrolysis in function of pH
  - Adsorption/desorption screening
- Annex IX (substances manufactured or imported at > 100 tonnes per annum): Same as Annex VIII plus.
  - Long-term toxicity testing on invertebrates (preferred species Daphnia)
  - Long-term toxicity testing on fish: fish early-life-stage toxicity test, fish juvenile growth test, fish short-term test on embryo and sac-fry stages
  - Soil simulation testing
  - Sediment simulation testing
  - Bioaccumulation in aquatic species, preferably fish
  - Effects on terrestrial organisms: short-term toxicity to invertebrates, effects on soil microorganisms, short-term toxicity to plants

- Annex X (substances manufactured or imported at > 1000 tonnes per annum): Same as Annex IX including:
  - Effect on terrestrial organisms: long-term toxicity testing on invertebrates, long-term toxicity testing on plants
  - Long-term toxicity to sediment organisms
  - Long-term or reproductive toxicity to birds

This work focuses on short and long-term toxicity to invertebrates, fish and algae as the most fundamental and important aspects of a testing strategy.

8.3.3.2 OECD methods for ecotoxicity testing and special considerations for testing nanomaterials

The OECD Technical Guidelines (TG) 201 (algae), 202 (short-term toxicity testing on daphnia), and 211 (long-term toxicity testing on daphnia) derived for conventional chemicals are applicable to nanomaterials with some adjustments to these test protocols (OECD, 2014a). For any test, it is recommended that stock suspensions should be stable with respect to particle size and as monodisperse as possible. Certain tests may require specific adaptations. For example, in tests with algae (TG 201),

- Shaking of the test vessels enhances the CO_2 supply to the medium. This CO_2 supply affects the pH of the medium, whereas the shaking influences the dispersion of the nanomaterial. These secondary effects (pH change and dispersion) influence the outcome of the test. For this reason, a shaking procedure is recommended only for range finding.
- The determination of algae growth by fluorescence measurement may result in artefacts (e.g. measurement of isolated chlorophyll may be a more reliable endpoint) as nanomaterials can interfere with measurements.
- As nanoparticles can absorb on algae or cause shading effects, it is recommended that those effects be tested before the ecotoxicity test[5].

When test methods are adapted to use with nanomaterials, the laboratory technicians must report all test conditions clearly, giving specific attention to shaking, media composition and the preparation of stock suspensions and the age of those suspensions before testing.
A clear description of the medium used to perform the ecotoxicity test is particularly important. OECD test guidelines allow the laboratory some

[5] Van Hoecke et al. (2010) have described a preliminary experiment to determine whether such shading might occur. An opaque plate can be placed above a white plate. In the controls, both plates are spiked with algae. When algae and nanoparticles are separated, the white plate contains the medium while the nanoparticles (using one loading) are spiked in the OECD medium in the upper opaque plate. When algal cells and nanoparticles are in direct contact, the white plate contains both algal cells and nanoparticles in the medium, while the opaque plate contain the medium only. In each plate, the chlorophyll content can be measured and compared in the different treatments. In a standardized ecotoxicity test with algae, shaking of the test flasks occurs, which also can have an influence on the dispersiveness of the nanomaterials. Therefore a shaking procedure is recommended for range finding.

latitude in test conditions. For example, TG 211 specifies that "it is further recommended that [total organic carbon] levels in the medium […] be below 2 mg/L […]. The pH should be within the range 6–9 and normally it should not vary by more than 1.5 units in any one test. Hardness above 140 mg/L (as $CaCO_3$) is recommended." These conditions – organic carbon, pH and ionic strength (hardness) – can affect the behaviour of nanomaterials. It is important for a testing strategy to consider the potential effects of varying these parameters, as described below, and to understand those effects when contemplating read-across.

The pH of the aqueous medium influences the agglomeration of the nanomaterial (RIVM, 2009). pH can also influence the dissolution rates of metal nanoparticles, whereby the solubility is enhanced at more acidic conditions (Bondarenko et al., 2013) and thus forms smaller nanoparticles during dissolution. pH changes may also affect surface charge, agglomeration and reactivity (Ivask et al., 2013). The behaviour of the released metal ions is also influenced by the test medium. The most important parameters of the test medium are pH, dissolved organic carbon and water hardness (Bondarenko et al., 2013). In general, calcium and magnesium cations will have a protective function for aquatic organisms, as they will compete with the metals for the binding sites (Ivask et al., 2013). However, cations can modify the surface properties and the charge of nanoparticles, favouring aggregation and thus decreasing their persistence and probability for contact with biota (Ivask et al., 2013). Natural organic material can bind nanoparticles, leading to the formation of colloid structures. Humic substances can also form coatings on nanoparticle surfaces, leading to a negative charge and resulting in reduced aggregation (Pronk et al., 2009).

The data may also need to be interpreted very carefully. For example, consider TG 211 for long-term testing on daphnia. According to TG 211, the actual test concentration in the highest and lowest concentrations should be measured. The loss of substance should not be higher than 20 %. Whether this is a meaningful level for engineered nanomaterials is not clear. Nanomaterials provoke physical effects on the surface of daphnids, which affect the movement of animals. In chronic tests, nanomaterials can also interact with feed (e.g. adsorption on algae).

The outcome of this second step (ecotoxicity testing itself) will lead to an effect concentration expressed as an LC_{50} or an EC_{50} (effect concentration that causes a 50 % effect) (Figure 14). Based on those results, the hypothesis can be tested if the nanomaterial had a unique behaviour or not, or read-across or grouping is possible. However, the interpretation of the effect concentrations can only be done if 1) there is information available on the dispersibility/dissolution of the nanomaterial and 2) the aquatic medium of the potential read-across substance is fully described (and it is understood what the effect is on the nanomaterial characteristics).

In summary, in this tier the following information should be checked on the nanomaterial of interest and on the potential read-across substance

- Dispersion method used in ecotoxicity testing.
- Aquatic media composition: influence of pH, organic matter and ionic strength on the solubility of nanomaterial and therefore on ecotoxicity and on other physico-chemical characteristics of nanomaterial (coating, shape, aggregation/agglomeration state and degradation state, reactivity).

The parameters cited above on the read-across substance and the nanomaterial of interest could be used in a weight-of-evidence approach. Only if those parameters are similar, can a read-across be performed. Currently, detailed data on (1) environmental fate of nanomaterial and (2) nanomaterial characteristics are mostly lacking for many nanomaterials. Such a lack of data may limit the ability to do read-across.
Figure 14 summarises the thinking described here.

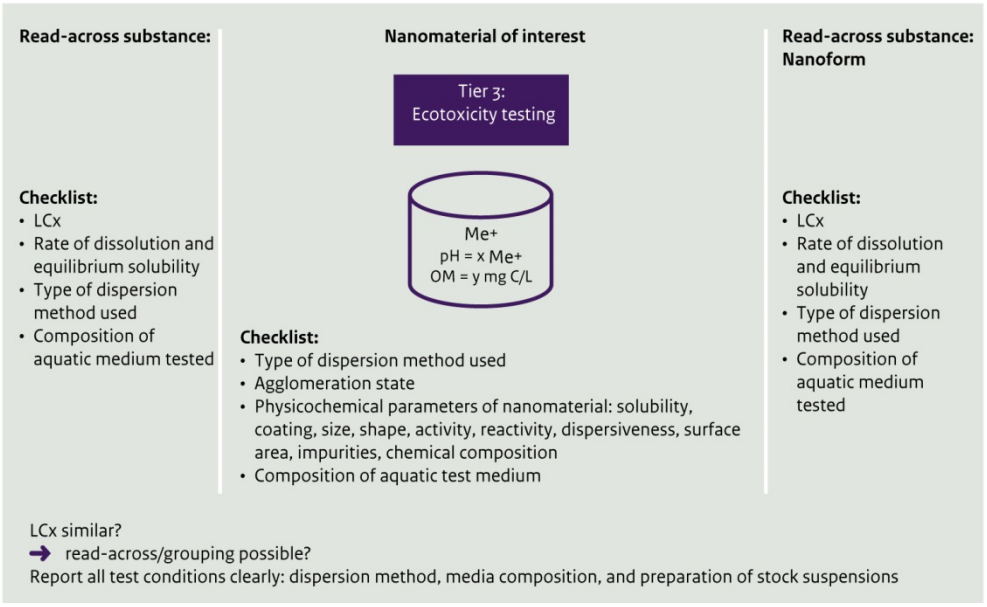


Figure 14 Read-across strategy between nanomaterial of interest and (non-) nanoform for tier 3 testing.

9 Recommendations on Testing Strategies: Human Health

The testing strategy to assess the potential human health effects of a nanomaterial is dictated by the regulatory framework being considered, the potential routes of exposure from the use of the nanomaterial and the endpoints that need to be addressed. The following sections outline the general process for collecting and assessing known information such that a testing strategy can be developed.

9.1 Overview of Process

9.1.1 Initial data collection and evaluation

The process begins with compiling available information, both qualitative and quantitative, on the nanomaterial of interest. (See Section 7.1 for the questions one might ask upon initiating this process.)
In addition to the known information on the nanomaterial of interest, it is also important to consider the endpoint(s) of interest. The key nanomaterial characteristics that should be considered will vary depending on the endpoint.

9.1.2 Considering Read-across

Read-across to another nanomaterial or to a non-nanoform of the same substance may be an integral part of a testing strategy, as it can limit the need for additional testing. The paragraphs below briefly discuss read-across considerations.

9.1.2.1 Selection of read-across substance

Once the initial collection and evaluation of data have been completed, the substance to be considered for the read-across strategy will need to be selected. It may be the same chemical in non-nanoform or a different nanoform of the same chemical, or a different substance entirely in nanoform. (If considering read-across to another nanoform, it is important to consider whether the substances may be of different size ranges, such that the difference could potentially result in any physiological effects.) During the selection process, one of the key questions is whether the substance being considered for read-across has sufficient data to fill any gaps in data for the substance of interest.
If a potential read-across substance that has sufficient data is not identified and multiple versions of the same nanomaterial are being evaluated, then a testing strategy may be developed to cover multiple versions of the same nanomaterial.

9.1.2.2 General strategies and data collection

The development of a read-across strategy requires some substance-specific data on the nanomaterial of interest. The objective of compiling or generating data is to build a case through the weight of evidence that

read-across is justified. The endpoint under consideration and the route of exposure may determine the data that need to be amassed.
A read-across strategy may be developed for a single endpoint or multiple endpoints. This distinction may affect the specific tests pursued. For example, when developing a testing strategy for the skin irritation endpoint, conducting *in vitro* dissolution testing in sweat fluid may not be warranted when an *in vitro* skin irritation assay could be conducted. However, if multiple endpoints (i.e. repeated dose toxicity, mutagenicity, carcinogenicity, etc.) are being considered for the dermal route of exposure, pursuing *in vitro* dissolution testing may help to build an argument that read-across is warranted for the group and can substantiate read-across for the skin irritation endpoint without the need to conduct *in vitro* skin irritation studies.
In addition, one of the main factors influencing the degree to which data need to be developed for substantiating read-across is the route of exposure. Effects from exposure through the dermal route, for example, are less likely to be impacted by the characteristics of nanomaterials than is the case for the inhalation route. Therefore, endpoints such as skin irritation or skin sensitisation may require fewer data to substantiate read-across than repeated dose toxicity or carcinogenicity when the route of exposure is inhalation.
The process of developing and substantiating a read-across strategy may reflect one of three scenarios:

- Some data are available on the nanomaterial of interest and a read-across substance is available from which data gaps may be filled. One would collect all available data on the nanomaterial of interest and the read-across substance and evaluate the weight of evidence to determine whether the data were sufficient to demonstrate that the nanomaterial of interest is substantially similar to the potential read-across substance. If this initial data set is not sufficient, additional data may be developed strategically to substantiate the read-across approach.
- Few or no data are available on the nanomaterial of interest, but a read-across substance is potentially available from which data may be used. This scenario would require the development of a testing strategy on the nanomaterial of interest, with the potential of using the initial data developed to substantiate read-across for other *in vivo* toxicity endpoints. In this scenario, the data would be evaluated at different points in the process to determine whether the weight of evidence is sufficient to substantiate read-across.
- Few or no data are available on the nanomaterial of interest and no obvious read-across substance is available with a complete dataset from which data can be extrapolated. This situation might occur when a new nanomaterial has been developed for which a non-nanoform is not available, other forms of the nanomaterial have not been previously studied and multiple forms of the new nanomaterial have been developed. An example of this type of scenario would be a situation in which multiple, different sizes of

a new nanomaterial have been developed. A testing strategy could be developed such that the nanomaterial forms being tested may cover the other nanomaterial forms within the range developed.

9.1.3 Development of data

If data must be developed for the nanomaterial of interest, the testing strategy may reflect:

- Endpoint-specific requirements; and
- Testing to provide data that strengthen the weight of evidence for read-across, which may include *in vitro* testing or other alternative testing strategies.

9.1.3.1 Building the weight of evidence

In instances where read-across is being considered, data may need to be developed to justify the use of read-across. This determination may need to rest on the weight of various pieces of evidence, including the nanomaterial design that gives it its unique properties, the mechanisms of action for any endpoint being assessed and the key physico-chemical properties. Furthermore, the results of *in vitro* testing or *in vivo* testing may help to make a case if the materials are substantially similar. Ideally, the data were generated from tests performed in accordance with standard methodologies. Some non-standard methods may be used to support the overall weight of evidence, but by themselves may not be sufficient to satisfy testing requirements.

9.1.3.2 Role of in vitro testing

In vitro testing is used in several contexts. The pharmaceutical industry has utilised *in vitro* assays in drug development to make decisions as to which drug candidates may be selected for further testing in animals. Because of the potential costs associated with testing, as well as the emphasis placed on the reduction of animal testing, many authors have emphasised the importance of *in vitro* testing for nanomaterials (Arora et al., 2012; Nogueira et al., 2014; Jones and Grainger, 2009; Nel et al., 2013). A number of *in vitro* assays have been used to evaluate reactivity within the context of toxicity (Wang et al., 2014; Arora et al., 2012; Park et al., 2009; Roesslein et al., 2013; Takhar and Mahant, 2011). These assays include, but are not limited to, cell viability assays (e.g. alamar blue assay, comet assay, lactate dehydrogenase assay, neutral red assay, etc.), oxidative stress assays (e.g. dichlorofluorescein assay, electroparamagnetic resonance assay, etc.), and inflammatory assays (e.g. enzyme-linked immunosorbent assay).
While certain physico-chemical characteristics of nanomaterials are critical to developing a testing strategy, collection of *in vitro* data on the reactivity of nanomaterials within the context of toxicity can also provide valuable insights. *In vitro* models offer clear benefits by providing mechanistic information in a rapid and cost-effective manner. However, some limitations in the use of *in vitro* models must be considered. While

in vitro models can provide information on a specific endpoint, they may not represent actual effects *in vivo*. Furthermore, some nanomaterials may not be compatible with a particular assay. Arora et al. (2012) noted that some dye-based assays, such as tetrazolium dye (MTT) and neutral red assays, which determine cell viability, may produce invalid results with some nanomaterials because of the interaction between the nanoparticles and the dye or dye products. Furthermore, it was found that silver nanoparticles were incompatible with the lactate dehydrogenase assay (Oh et al., 2014). While these potential compatibility issues were summarised for non-OECD guideline assays, the same caution should be applied to OECD guideline *in vitro* assays (e.g. bacterial gene mutation assay) that may be used to support the safety assessment of a nanomaterial.
While some of the available *in vitro* assays, their benefits and limitations have been presented in this section, the discussions of acute toxicity, skin irritation and other endpoints that follow do not include a specific evaluation of any *in vitro* assays. Rather, the use of *in vitro* assays to support the safety assessment of a nanomaterial should be considered on a case-by-case basis.

9.2 Human Health Endpoint Considerations

This report addresses the key considerations and potential strategies for the following endpoints: acute toxicity, skin irritation, eye irritation, skin sensitisation, mutagenicity, repeated dose toxicity and carcinogenicity. While other endpoints may need to be addressed as part of a regulatory framework, such as reproductive toxicity or neurotoxicity, they are not specifically discussed in the following sections. The general framework presented herein, however, provides a general strategy for addressing most other endpoints.

9.2.1 Acute toxicity

9.2.1.1 Known information

As described previously, one would begin by asking a series of questions that would elicit the available relevant information about the nanomaterial to be tested and about other forms of the substance which might be suitable for read-across.
In addition to the known information on the nanomaterial of interest, one should also consider the relative importance of the nanomaterial characteristics on the endpoint being considered. The relative impact of nanomaterial characteristics on human health effects depends on the route of exposure. For acute toxicity, the dermal route is a less likely concern than either the oral or inhalation routes. Therefore, when all routes of exposure are relevant, testing would likely focus on the oral and/or inhalation routes rather than the dermal route of exposure. Furthermore, because acute toxicity addresses effects, primarily death, over a short duration, the most important characteristics of a nanomaterial are the composition, solubility and reactivity of the nanomaterial. In summary, for assessing acute toxicity, it will be

important to understand the route of exposure, as well as whether the specific nanomaterial characteristics that are likely to impact effects may occur over a short duration.

9.2.1.2 Hypothesis

Depending on the likely exposures during manufacture and use of the nanomaterial and based on what is known about the hazards of exposure, the testing strategy for acute toxicity will likely focus on the inhalation or oral routes of exposure rather than the dermal route. The acute toxicity of a nanomaterial depends on the substance identity (i.e. the nanomaterial itself, surface coating or functionalisation, impurities within the nanomaterial, or metabolites). Acute toxicity also reflects the reactivity of the substance, which may increase as particle size decreases.
Data available for other endpoints may provide information on the potential acute toxicity of the nanomaterial of interest. If repeated dose toxicity data are available, for example, any effects observed within the first few days of the test may give clues as to the potential acute toxicity of the nanomaterial of interest and be used to shape the hypothesis.
In most cases, read-across will first be considered to the non-nanoform of the substance. The following questions should be considered:

- Is the potential read-across substance classified for acute toxicity? If it is, then the mechanism of action should be considered to determine if it is relevant for the nanomaterial of interest.
- Does the nanoform contain impurities, coatings, or surface functionalisation that could result in acute toxicity?
- Does nanosizing potentially change the rate of dissolution and/or affect the reactivity/photoreactivity of the substance?

9.2.1.3 Testing

The need for testing depends on the regulatory requirements and may proceed through three tiers of testing, as described below.

Regulatory Context for Addressing Endpoint

REACH specifies the following requirements for testing acute toxicity:

- Annex VII (substances manufactured or imported at > 1 tonne per annum).
  - Acute toxicity by the oral route should be tested, unless a study on the inhalation route is available.
- Annex VIII (substances manufactured or imported at > 10 tonnes per annum).
  - In addition to the oral route, for substances other than gases, data should be provided for one other route of exposure. The route to be tested depends on the likelihood of exposure and the nature of the substance.
- Annex IX (substances manufactured or imported at > 100 tonnes per annum): Same as Annex VIII.

- Annex X (substances manufactured or imported at > 1000 tonnes per annum): Same as Annex VIII.

Tier 1: Development and assessment of initial physico-chemical data

Based on the tonnage under REACH and the relevant routes of exposure, *in vivo* acute toxicity testing may need to be conducted on the nanomaterial of interest. However, should data be available for read-across, then testing the rate of dissolution in a relevant fluid may be important.
Once the initial information has been collected on the nanomaterial of interest, the weight of evidence can be assessed to determine whether additional data are needed. For example, consider the case of a nanomaterial for which the non-nanoform is considered acutely toxic as a result of the ionic form of the substance and the nanomaterial of interest dissolves more rapidly than the non-nanoform. In this case, collection of additional data may be warranted to determine whether the increase in solubility is likely to result in an increase in acute toxicity. As another example, consider the following case: the dermal route of exposure is the only relevant route of exposure; neither the nanomaterial nor its non-nanoform are particularly soluble; the non-nanoform is not classified for acute dermal toxicity; and the design of the nanomaterial is not such that it would be considered extraordinarily reactive. In this case, additional data may not be required and read-across to the non-nanoform is supported.

Tier 2: Testing and Evaluation

In general, the purpose of Tier 2 testing is to provide information on the behaviour of nanomaterials in order to bridge the gap, to the extent possible, between physico-chemical properties and biological effects. There are no standard OECD *in vitro* methods that sufficiently predict acute toxicity. So, if one is not considering read-across nor addressing other human health endpoints, the testing strategy may require conducting *in vivo* acute toxicity testing under Tier 3. However, if data are potentially available from read-across and/or other human health endpoints need to be addressed, conducting *in vitro* dissolution/bioaccessibility testing or *in vitro* reactivity studies to support this endpoint may be considered.

Tier 3: In vivo *Acute Toxicity Testing*

In cases in which the weight of evidence based on the design of the nanomaterial of interest, its physico-chemical properties or other available toxicity data are insufficient to establish read-across, *in vivo* acute toxicity testing on the nanomaterial of interest is warranted. Consideration should be given to the applicable routes of exposure, as well as to which route is likely to represent worst-case effects.

9.2.2 Skin Irritation

9.2.2.1 Known information

One would begin by compiling the available relevant information about the nanomaterial to be tested and about other forms of the substance which might be suitable for read-across (nanoform and non-nanoform). In addition, one should also consider the relative importance of the nanomaterial characteristics on the endpoint being considered. The size of the nanoparticles does not significantly affect skin penetration unless the skin is damaged. However, the size of the nanomaterial may indirectly affect skin irritation if it increases the rate of dissolution or results in increased reactivity/photoreactivity. The rate of dissolution is important in cases in which the soluble ionic species is irritating or the nanomaterial contains impurities which are potentially irritating. In addition, data from testing other endpoints may be useful. For example, data from acute dermal toxicity testing may provide an indication of the irritation potential.

9.2.2.2 Hypothesis

The working hypothesis for many nanomaterials may be that the potential for skin irritation can be predicted from the non-nanoform of the nanomaterial itself or its coating, or from the solution pH. Changes in reactivity or the rate of dissolution resulting from nanosizing may need to be taken into account. When evaluating the potential use of read-across for the skin irritation endpoint, the following should be considered:

- If the potential read-across substance is classified as a skin irritant, then the mechanism of action for irritation should be considered and the question of whether it is also relevant for the nanomaterial of interest;
- Whether impurities, coatings or surface functionalisation that could result in irritation should be considered; and
- Whether nanosizing changes the rate of dissolution or reactivity/photoreactivity of the substance should be considered.

9.2.2.3 Testing

The testing strategy for skin irritation may reflect three kinds of evidence: pH (or acid/alkaline reserve), *in vitro* testing and/or *in vivo* skin irritation testing. Should data be available on a potential read-across substance, the testing strategy may also include development of *in vitro* dissolution or *in vitro* reactivity data.

Regulatory Context for Addressing Endpoint

For the skin irritation endpoint, REACH specifies the following requirements, depending on tonnage:

- Annex VII (substances manufactured or imported at > 1 tonne per annum).

    - An *in vitro* skin corrosion or skin irritation study is required unless
        - the available information indicates that the criteria are met for classification as corrosive to the skin or irritating to eyes, or
        - the substance is flammable in air at room temperature, or
        - the substance is classified as being very toxic when in contact with skin, or
        - an acute toxicity study by the dermal route does not indicate skin irritation up to the limit dose level (2,000 milligrams per kilogram [mg/kg] body weight).
- Annex VIII (substances manufactured or imported at > 10 tonnes per annum).
    - An *in vivo* skin irritation study is required unless:
        - the substance is classified as being corrosive to the skin or as a skin irritant, or
        - the substance is a strong acid (pH ≤ 2.0) or base (pH ≥ 11.5), or
        - the substance is flammable in air at room temperature, or
        - the substance is classified as very toxic in contact with skin, or
        - an acute toxicity study by the dermal route does not indicate skin irritation up to the limit dose level (2,000 mg/kg body weight).
- Annex IX (substances manufactured or imported at > 100 tonnes per annum): Same as Annex VIII.
- Annex X (substances manufactured or imported at > 1000 tonnes per annum): Same as Annex VIII.

Tier 1: Development and assessment of initial physico-chemical data

Based on the tonnage under REACH and whether the dermal route of exposure is relevant based on the manufacture and use of a nanomaterial, skin irritation may need to be addressed. Skin irritation data may be developed using OECD guideline studies or the assessment of the pH of the material.

As pH is a potential indicator of skin irritation/corrosion, the pH of the nanomaterial of interest in aqueous solution should be assessed first. For most nanomaterials, pH may not dictate a determination of skin irritation/corrosion. If pH is not a concern for the nanomaterial of interest and there are no other data available to support a determination of skin irritation, then read-across should be considered before conducting skin irritation testing. Data such as the rate of dissolution or photocatalytic activity may support read-across.

The following examples illustrate how read-across thinking might be applied. If a poorly-soluble nanomaterial of relatively low reactivity had the same chemical composition as a non-nano substance demonstrated not to cause skin irritation, then the weight of evidence would suggest that reading across for this endpoint is warranted. Alternatively, if the nanomaterial of interest was more reactive and dissolved more quickly

than its non-nano counterpart, or contained impurities that are considered irritating, then read-across to the non-nanoform would not be warranted based on the available information and further data would be needed.

Tier 2 Testing and Evaluation

The testing strategy for the skin irritation endpoint will depend on the tonnage and the number of human health endpoints that need to be addressed for the nanomaterial of interest. *In vitro* testing of skin irritation (in lieu of *in vivo* testing) is acceptable at lower tonnage bands. At higher tonnages, conducting *in vitro* dissolution/bioaccessibility testing or *in vitro* reactivity studies to support this endpoint may not be warranted if one is only considering the single endpoint of dermal irritation. However, if there are other endpoints that are being considered for read-across for which this type of data may help substantiate read-across, then such studies might be warranted.

Tier 3: In vivo *irritation data on the nanomaterial of interest*

In cases in which the weight of evidence based on the design of the nanomaterial of interest, its physico-chemical properties or other available toxicity data are insufficient to meet the endpoint requirements or establish read-across, *in vivo* skin irritation testing on the nanomaterial of interest may be warranted, depending on the specific tonnage requirements under REACH.

9.2.3 Eye Irritation

9.2.3.1 Known information

One would begin by compiling the available information about the nanomaterial to be tested and about other forms of the substance which might be suitable for read-across.
In addition, one should also consider the relative importance of the nanomaterial characteristics for the endpoint being considered. There are few data available on nanomaterial effects on eye irritation. However, the Scientific Committee on Consumer Safety (SCCS) summarised data on nanoforms of both titanium dioxide and carbon black and, in both cases, little eye irritation was observed consistent with their non-nanoforms (SCCS, 2014a,b). Both of these substances are poorly soluble. The data may suggest that, in cases in which the nanomaterial is poorly soluble and does not significantly differ in reactivity from the non-nanoform, the eye irritation potential would likely be similar to that of the non-nanoform. It is logical to conclude, in the absence of other information, that for the eye irritation endpoint the design of the nanomaterial of interest and the key physico-chemical properties need to be assessed similarly to skin irritation. The size of the nanomaterial may indirectly affect the potential for irritation if it increases the rate of dissolution or results in increased reactivity/photoreactivity. Hence, the nanoform of a material may have the potential to cause eye irritation in instances where nanosizing results

in increased reactivity. The rate of dissolution is important in cases in which the ionic soluble form is irritating or the nanomaterial contains impurities that are potentially irritating.
Finally, if acute dermal toxicity data or repeated dose dermal toxicity data are available, any irritation effects observed may give clues as to potential eye irritation resulting from exposure to the nanomaterial of interest.

9.2.3.2 Hypothesis

Based on the limited information available on eye irritation and nanomaterials, the hypothesis for this endpoint would likely be similar to the hypothesis for skin irritation: the potential for irritation can be predicted from the non-nanoform or from the solution pH; changes in reactivity or the rate of dissolution resulting from nanosizing may need to be taken into account. When evaluating the potential use of read-across for the eye irritation endpoint, particularly to the non-nanoform of the nanomaterial, the following should be considered:

- If the potential read-across substance is classified as an eye irritant, then the mechanism of action for irritation should be considered and the question of whether it is also relevant for the nanomaterial of interest;
- Whether impurities, coatings or surface functionalisation that could result in irritation should be considered; and
- Whether nanosizing changes the rate of dissolution or reactivity/photoreactivity of the substance should be considered.

9.2.3.3 Testing

The testing strategy for eye irritation should entail an evaluation of pH (and/or acid/alkaline reserve) and potentially conducting *in vitro* and/or *in vivo* eye irritation testing. Should data be available on a potential read-across substance, the testing strategy may also include development of *in vitro* dissolution or *in vitro* reactivity data. In addition, data available for other endpoints may provide information on the potential irritation of the nanomaterial of interest.

Regulatory Context for Addressing Endpoint

For the eye irritation endpoint, REACH specifies the following requirements depending on tonnage:

- Annex VII (substances manufactured or imported at > 1 tonne per annum).
  - An *in vitro* eye irritation study is required unless
    - the available information indicates that the criteria are met for classification as being corrosive to the skin or irritating to eyes, or
    - the substance is flammable in air at room temperature
- Annex VIII (substances manufactured or imported at > 10 tonnes per annum).
  - An *in vivo* eye irritation study is required unless:

    - the substance is classified as being irritating to eyes with a risk of serious damage to eyes, or
    - the substance is classified as being corrosive to the skin and providing the registrant classified the substance as an eye irritant, or
    - the substance is a strong acid (pH ≤ 2.0) or base (pH ≥ 11.5), or
    - the substance is flammable in air at room temperature.
- Annex IX (substances manufactured or imported at > 100 tonnes per annum): Same as Annex VIII.
- Annex X (substances manufactured or imported at > 1000 tonnes per annum): Same as Annex VIII.

Tier 1: Development and assessment of initial physico-chemical data

Based on the tonnage under REACH, eye irritation may need to be addressed. Eye irritation data may be developed using OECD guideline studies or an assessment of the pH of the material. Physico-chemical data on solution pH and on the rate of dissolution may be relevant. As pH is a potential indicator of irritation/corrosion, the pH of the nanomaterial of interest in aqueous solution should be assessed first. For most nanomaterials, pH is unlikely to dictate a determination of irritation/corrosion. If pH is not a concern for the nanomaterial of interest and there are no other data available to support a determination of eye irritation, then read-across should be considered before conducting *in vivo* eye irritation testing on the nanomaterial of interest. Two examples of read-across situations follow. If the nanomaterial is designed so that it is not reactive and not soluble and test data indicate that a non-nanoform of the material is not irritating to the eyes, the weight of evidence would suggest that reading across for this endpoint is warranted. However, if the small size enhanced the reactivity and rate of dissolution of a nanomaterial and it contained impurities known to be irritating, then read-across might not be appropriate and additional data needed.

Tier 2 Testing and Evaluation

The testing strategy for the eye irritation endpoint will likely depend on the tonnage and the other human health endpoints that need to be addressed. If only considering eye irritation, then an *in vitro* eye irritation study might be considered. However, it should be noted that currently there are no validated or OECD guideline *in vitro* eye irritation assays. If the skin irritation endpoint is also being considered, then conducting an *in vitro* skin corrosion/irritation test may be considered first before conducting the *in vitro* eye irritation. If only considering the irritation endpoints, conducting *in vitro* dissolution/bioaccessibility testing or *in vitro* reactivity studies to support these endpoints may not be warranted. However, if other endpoints are being considered for which this type of data may help substantiate read-across, then other *in vitro* testing may be considered as part of the Tier 2 testing.

Tier 3: In vivo *Eye Irritation Testing*

In cases in which the weight of evidence based on the design of the nanomaterial of interest, its physico-chemical properties or other available toxicity data are insufficient to meet the endpoint requirements or establish read-across, *in vivo* eye irritation testing on the nanomaterial of interest may be warranted, depending on the specific tonnage requirements under REACH.

9.2.4 Sensitisation

9.2.4.1 Known information

One would begin compiling the available information on the nanomaterial to be tested and about other forms of the substance which might be suitable for read-across. In addition, one should also consider the relative importance of the nanomaterial characteristics for the endpoint being considered. While the size of a nanoparticle may not significantly influence skin penetration, unless the skin is damaged, changes in reactivity/photoreactivity or the rate of dissolution with size may be relevant to this endpoint. The composition of the nanomaterial, including impurities, coatings and surface functionalisation, is an important aspect to consider for the skin sensitisation endpoint. Other available data should also be considered that provide information on sensitisation, such as repeated dose dermal-toxicity studies.

9.2.4.2 Hypothesis

The hypothesis for this endpoint would likely be similar to the hypothesis for skin irritation: the potential for sensitisation can perhaps be predicted from the non-nanoform of a substance (either for the nanoparticle or its coating). Changes in reactivity or the rate of dissolution resulting from nanosizing may need to be taken into account. Data available for other endpoints may provide relevant information. For example, if repeated dose dermal-toxicity data are available, any sensitising effects observed may be useful for assessing this endpoint. In most cases, read-across would likely be considered to the non-nanoform of the substance. As for the skin sensitisation endpoint, the following points would be relevant to the hypothesis.

- If the potential read-across substance is classified as an skin sensitizer, then the mechanism of action for irritation should be considered and the question of whether it is also relevant for the nanomaterial of interest;
- Whether or not impurities, coatings or surface functionalisation that could result in sensitisation should be considered; and
- Whether nanosizing changes the rate of dissolution or reactivity/photoreactivity of the substance should be considered.

9.2.4.3 Testing

The testing strategy for skin sensitisation may include an *in vivo* skin sensitisation assay. Should data be available on a potential read-across

substance, the testing strategy may also include development of other *in vitro* assays (i.e. *in vitro* dissolution or *in vitro* reactivity data) as needed to support read-across.

Regulatory Context for Addressing Endpoint

For the skin sensitisation endpoint, REACH specifies the following requirements, depending on tonnage:

- Annex VII (substances manufactured or imported at > 1 tonne per annum).
  - An in vivo skin sensitisation assay is required unless
    - the available information indicates that the substance should be classified for skin sensitisation or corrosivity, or
    - the substance is a strong acid (pH ≤ 2.0) or base (pH ≥ 11.5), or
    - the substance is flammable in air at room temperature.
- Annex VIII (substances manufactured or imported at > 10 tonnes per annum): Same as Annex VII
- Annex IX (substances manufactured or imported at > 100 tonnes per annum): Same as Annex VII.
- Annex X (substances manufactured or imported at > 1000 tonnes per annum): Same as Annex VII.

Tier 1: Development and assessment of initial physico-chemical data

Based on the tonnage under REACH and if the dermal route of exposure is relevant based on the product's manufacture and use, the skin sensitisation endpoint may need to be addressed. Skin sensitisation data may be developed using OECD guideline studies.
As a first step, if a substance is considered to be corrosive, then conducting a skin sensitisation assay would not be warranted. Therefore, as pH is a potential indicator of skin corrosion, the pH of the nanomaterial of interest in aqueous solution should be assessed first. If pH is not a concern for the nanomaterial of interest and there are no other data available to support a determination of skin sensitisation, then read-across should be considered before conducting an *in vivo* skin sensitisation assay on the nanomaterial of interest. As noted above, the rate of dissolution of the nanomaterial should be considered.
For example, if the nanomaterial is designed so that it is not extraordinarily reactive, does not dissolve at a markedly faster rate and does not have any impurities, coatings or surface functionalisation that suggest sensitisation potential, and the non-nanoform of the material has sensitisation data indicating that it is not sensitising, then the weight of evidence would suggest that reading across for this endpoint is warranted. Alternatively, if the nanomaterial of interest is designed to be readily soluble and contains impurities that are considered sensitising, read-across may not be warranted and further data needed.

Tier 2 Testing and Evaluation

The testing strategy for the skin sensitisation endpoint will likely depend on the tonnage and the number of human health endpoints that need to be addressed for the nanomaterial of interest. Currently, there are no OECD guidelines for *in vitro* sensitisation assays. However, if the potential for the nanomaterial of interest to be corrosive is not known, then conducting an *in vitro* skin corrosion assay prior to conducting an *in vivo* skin sensitisation study is warranted. Furthermore, if one is only considering the skin sensitisation endpoint, then *in vitro* dissolution/bioaccessibility testing or *in vitro* reactivity studies may not be warranted.

Tier 3: In vivo *Skin Sensitisation Testing*

In cases in which the weight of evidence based on the design of the nanomaterial of interest, its physico-chemical properties or other available toxicity data are insufficient to meet the endpoint requirements or establish read-across, then *in vivo* skin sensitisation testing on the nanomaterial of interest is warranted.

9.2.5 Mutagenicity

9.2.5.1 Known information

As described previously, one would begin by compiling the available information on the nanomaterial to be tested and about other forms of the substance which might be suitable for read-across. Also considered would be the relative importance of the nanomaterial characteristics with respect to the mutagenicity, given the state of the science. Whether or not a nanomaterial elicits mutagenic effects depends on the composition of the material, including any impurities, coatings or surface functionalisation, and its solubility. The degree of reactivity, specifically the ability to generate reactive oxygen species (ROS), may play a role. (Other important parameters, such as surface charge and surface area, are challenging to measure.) The toxicokinetics of the nanomaterial are also important to consider with respect to the bioavailability of any mutagenic form, its ability to penetrate cells and bind to deoxyribonucleic acid (DNA), or to initiate other effects that may cause damage to DNA (e.g. generation of ROS). It is important to note that while size, shape, charge, coating, surface area and solubility likely impact the toxicokinetics of nanomaterials, the extent to which each of these parameters affects nanomaterial-specific toxicokinetics has not yet been fully understood (Discussed further in Section 6.1.1).

9.2.5.2 Hypothesis

Depending upon the tonnage band and the assessment of the limitations of some *in vitro* tests for mutagenicity, *in vitro* testing of mutagenicity is probably appropriate. The need to read-across to another substance may not be as significant for this endpoint as it is for other endpoints because the availability of standardised *in vitro* tests already limits the

need for mammalian testing. (As noted below, however, the appropriateness of some tests for nanoparticles has been questioned.) If read-across were to be considered in lieu of *in vivo* testing, the following questions would be relevant.

- Is the potential read-across substance classified as a mutagen? If the potential read-across substance is classified as a mutagen, then the mechanism of action should be considered and the question of whether it is also relevant for the nanomaterial of interest.
- Are there impurities, coatings or surface functionalisation that could result in mutagenicity?
- Does the nanosizing potentially change the rate of dissolution or reactivity/photoreactivity of the substance?
- Would the other characteristics of the nanomaterial, such as charge or shape, potentially impact the toxicokinetics of the nanomaterial or increase the potential for resulting in mutagenicity?

9.2.5.3 Testing

The testing strategy for the mutagenicity endpoint may entail *in vitro* and *in vivo* mutagenicity assays. Should data be available on a potential read-across substance and/or if other human health endpoints may need to be assessed, then the testing strategy may also include development of other *in vitro* assays (i.e. *in vitro* dissolution or *in vitro* reactivity data).

Regulatory Context for Addressing Endpoint

For the mutagenicity endpoint, REACH specifies the following requirements depending on tonnage:

- Annex VII (substances manufactured or imported at > 1 tonne per annum).

An *in vitro* gene mutation study in bacteria is required. Should there be a positive result, further studies shall be considered.

- Annex VIII (substances manufactured or imported at > 10 tonnes per annum).
  - An *in vitro* gene mutation study in bacteria;
  - An *in vitro* cytogenicity study in mammalian cells or *in vitro* micronucleus study;
  - An *in vitro* gene mutation study in mammalian cells if
    - Negative *in vitro* gene mutation data in bacteria,
    - Negative *in vitro* cytogenicity study in mammalian cells or *in vitro* micronucleus.
  - Appropriate *in vivo* mutagenicity studies shall be considered in the case of a positive result in any of the genotoxicity studies in Annex VII or VIII.
- Annex IX (substances manufactured or imported at > 100 tonnes per annum): Same as Annex VIII.

- Annex X (substances manufactured or imported at > 1000 tonnes per annum): Same as Annex VIII.

Tier 1: Development and assessment of initial physico-chemical data

No specific testing of physico-chemical parameters is recommended for this endpoint, unless warranted by read-across considerations should *in vivo* data be necessary.

Tier 2: Testing and Evaluation

The testing strategy for the mutagenicity endpoint will likely depend on the tonnage and the outcome of any initial *in vitro* mutagenicity assays. For the mutagenicity endpoint, the REACH framework outline specifies that *in vitro* mutagenicity assays should be conducted to support the endpoint based on the tonnage. However, as previously discussed, some nanomaterials may not be compatible with particular *in vitro* assays (e.g. bacterial gene mutation assay). Therefore, before conducting any *in vitro* testing for a nanomaterial, any potential issues with the assay should be assessed.

Tier 3: In vivo *Mutagenicity Testing*

In vivo mutagenicity studies would be considered in cases of a positive result in any of the *in vitro* genotoxicity studies for a material in the higher tonnage bands if read-across could not be used to fulfil the testing requirements. Consideration should be given to the applicable routes of exposure, as well as to which route is likely to represent worst-case effects.

9.2.6 Repeated Dose Toxicity

9.2.6.1 Known information

One would begin by compiling the available relevant information on the nanomaterial to be tested and about other forms of the substance which might be suitable for read-across. In addition, one should also consider the relative importance of the nanomaterial characteristics with respect to the route of exposure and the toxicological endpoint. The relative effect of nanomaterial characteristics depends on the route of exposure; with the dermal route being less impacted than the inhalation route and, to some extent, the oral route. The size and shape of a nanoparticle may profoundly affect its penetration into the lungs and the effects of exposure. These parameters are less important with respect to exposure via undamaged skin. The effect of nanomaterial properties on toxicokinetics may also be vitally important to consider. The size, shape, charge, coating, surface area and solubility of a nanoparticle may influence its toxicokinetics. However, the extent to which each of these parameters affects nanomaterial-specific toxicokinetics has not yet been fully understood.

9.2.6.2 Hypothesis

Depending on the likely exposures during the manufacture and use of the nanomaterial, the testing strategy for repeated dose toxicity will likely focus on the inhalation or oral routes of exposure, rather than the dermal route. Furthermore, the potential effects following repeated exposure from a nanomaterial may be influenced by the substance identity (i.e. the nanomaterial itself, surface coating or functionalisation, impurities within the nanomaterial or metabolites), size, shape, charge, surface area and solubility. The potential reactivity of the substance is also a factor that needs to be taken into account in the testing strategy. In order to minimise animal testing, the testing strategy for repeated dose toxicity should focus on conducting studies to support the read-across for this endpoint.
Based on the tonnage under REACH, *in vivo* repeated dose toxicity testing may be required. These data may be developed using OECD guideline studies; however, should data be available for read-across, then the testing strategy may first focus on the development of the most relevant physico-chemical data on the nanomaterial.
In most cases, read-across would likely be considered to the non-nanoform of the substance. Relevant questions include the following:

- Is the potential read-across substance classified as a repeated dose toxicant? If the potential read-across substance is classified, then the mechanism of action should be considered and the question of whether it is also relevant for the nanomaterial of interest.
- Are there impurities, coatings or surface functionalisation that could result in effects following repeated exposure?
- Does the nanosizing potentially change the rate of dissolution or reactivity/photoreactivity of the substance?
- Would the other characteristics of the nanomaterial, such as charge or shape, potentially impact the toxicokinetics of the nanomaterial or increase the potential for long-term human health effects?

9.2.6.3 Testing

Regulatory Context for Addressing Endpoint

For the repeated dose toxicity endpoint, REACH specifies the following requirements, depending on tonnage:

- Annex VIII (substances manufactured or imported at > 10 tonnes per annum).
  - Short-term repeated dose toxicity study (28 days), one species, male and female, most appropriate route of administration, bearing in mind the likely route of human exposure.
- Annex IX (substances manufactured or imported at > 100 tonnes per annum).

    - Sub-chronic toxicity study (90-day), one species, rodent, male and female, most appropriate route of administration, bearing in mind the likely route of human exposure.
- Annex X (substances manufactured or imported at > 1000 tonnes per annum): Same as Annex VIII.

Tier 1: Development and assessment of initial physico-chemical data

If the primary route of concern is inhalation, then the particle size or range of sizes (which may reflect agglomeration during testing) and shape are of primary importance, as they influence penetration of the nanoparticle into the lungs and the effect of exposure. The rate of dissolution, which affects biopersistence, is also important, but the effect on repeated dose toxicity is difficult to quantify.
In most cases, development of the key physico-chemical properties on the nanomaterial of interest, without additional *in vitro* or *in vivo* toxicity data, will not provide sufficient weight of evidence to judge the potential for repeated dose toxicity under a regulatory framework. Rather, the physico-chemical property data will help to build the case for read-across along with other data generated in Tier 2 and Tier 3.

Tier 2: Testing and Evaluation

There are not currently standard OECD *in vitro* methods that sufficiently predict repeated dose toxicity. Therefore, if one is not considering read-across nor addressing other human health endpoints, the testing strategy may require conducting *in vivo* repeated dose toxicity testing under Tier 3. But if data are potentially available from read-across and/or if other human health endpoints need to be addressed, then conducting *in vitro* dissolution/bioaccessibility testing or *in vitro* reactivity studies to support this endpoint may be considered. For example, if the form attributed to the repeated dose toxicity of the potential read-across substance is the soluble form, the dissolution of the nanomaterial of interest can be assessed in the pertinent biological fluids (i.e. lung fluid, sweat, gastric fluids) to determine whether it is similar to the potential read-across substance.

Tier 3: In vivo *Toxicity Testing*

For Tier 3 testing, the test strategy may incorporate acute toxicity testing and/or *in vivo* toxicokinetic testing to help to support read-across for the repeated dose toxicity endpoint.
In cases in which the weight of evidence based on the design of the nanomaterial of interest, its physico-chemical properties or other available toxicity data are insufficient to establish read-across, *in vivo* repeated dose toxicity testing on the nanomaterial of interest may be warranted, depending on the specific tonnage of the nanomaterial. Consideration should be given to the applicable routes of exposure, as well as to which route is likely to represent worst-case effects.

9.2.7 Carcinogenicity

9.2.7.1 Known information

Whether or not a nanomaterial may be carcinogenic depends on multiple factors, which include:

- The route of exposure,
- Whether or not it has effects at the port of entry, such as inflammation,
- Its systemic bioavailability,
- Its ability to penetrate into cells and bind to DNA or cause carcinogenicity through other mechanisms (e.g. generation of ROS).

Many of the same questions evaluated for repeated dose toxicity will also be evaluated for the carcinogenicity endpoint. As with the other endpoints, the nano-specific characteristics of the nanomaterial are going to have less impact on the dermal route of exposure as opposed to both the oral and inhalation routes of exposure. Furthermore, the toxicokinetics of the nanomaterial are going to dictate whether or not the nanomaterial may have effects at the port of entry or systemically. One would begin by compiling relevant information on the nanomaterial to be tested and about other forms of the substance which might be suitable for read-across. Furthermore, the relative effect of nanomaterial characteristics on human health depends on the route of exposure, with the dermal route less likely to be impacted than either the oral or inhalation routes. The size and shape of a nanoparticle may profoundly affect its penetration into the lungs and the effects of exposure. These parameters are less important with respect to exposure via undamaged skin. The effect of nanomaterial properties on toxicokinetics may also be vitally important to consider. The size, shape, charge, coating, surface area and solubility of a nanoparticle may influence its toxicokinetics. However, the extent to which each of these parameters affects nanomaterial-specific toxicokinetics has not yet been fully understood.

9.2.7.2 Hypothesis

The testing strategy for carcinogenicity will likely focus on the inhalation or oral routes of exposure, rather than on the dermal route. Furthermore, the potential carcinogenicity of a nanomaterial may be influenced by the substance identity (i.e. the nanomaterial itself, surface coating or functionalisation, impurities within the nanomaterial) and its metabolites, size, shape, charge, surface area and solubility. The potential reactivity of the substance is also a factor that needs to be taken into account in the testing strategy. The relationship between each of these parameters and the carcinogenic potential cannot necessarily be quantified. In order to minimise animal testing, the testing strategy for carcinogenicity should focus on conducting studies to support read-across for this endpoint.

In most cases, read-across would likely be considered to the non-nanoform of the substance. The following questions should be considered:

- Is the potential read-across substance classified as a carcinogen or mutagen? If the potential read-across substance is classified, then the mechanism of action should be considered and the question of whether it is also relevant for the nanomaterial of interest.
- Are there impurities, coatings or surface functionalisation that could result in carcinogenicity?
- Does nanosizing potentially change the rate of dissolution or reactivity/photoreactivity of the substance?
- Would the other characteristics of the nanomaterial, such as charge or shape, potentially impact the toxicokinetics of the nanomaterial or increase the potential for carcinogenicity?

9.2.7.3 Testing

Data may be developed using OECD guideline studies. The testing strategy for this endpoint, however, should either focus on determining whether carcinogenic effects are likely based on mutagenicity and repeated dose toxicity data or, alternatively, if data is available for read-across, then the testing strategy should focus on the development of the most data on the nanomaterial to support read-across.

Regulatory Context for Addressing Endpoint

For the carcinogenicity endpoint, REACH specifies the following requirements, depending on tonnage:

- Annex X (substances manufactured or imported at > 1000 tonnes per annum).
  - A carcinogenicity study may be proposed by the registrant or may be required by the Agency in accordance with Articles 40 or 41 if:
    - the substance has a widespread dispersive use or there is evidence of frequent or long-term human exposure, and
    - the substance is classified as mutagen category 3 or there is evidence from the repeated dose study/studies that the substance is able to induce hyperplasia and/or pre-neoplastic lesions.

  If the substance is classified as mutagen category 1 or 2, the default presumption would be that a genotoxic mechanism for carcinogenicity is likely. In these cases, a carcinogenicity test will normally not be required.

Tier 1: Development and assessment of initial physico-chemical data

Please see the corresponding discussion for repeated-dose toxicity in Section 9.2.6.3.

Tier 2: Testing and Evaluation

Tier 2 testing would involve mutagenicity studies to determine whether the nanomaterial of interest is classified as a mutagen. Assessment of the dispersiveness or dustiness of the material (depending on whether exposure might be to a liquid or solid suspension, respectively) might be relative to the question of whether exposure could be possible due to "widespread dispersive use or there is evidence of frequent or long-term human exposure" (according to the requirement of REACH cited above). If read-across is being considered, *in vitro* dissolution testing may also be considered. This would be important if it were a soluble form responsible for the carcinogenicity of the potential read-across substance. Dissolution of the nanomaterial of interest in the pertinent biological fluids (i.e. lung fluid, sweat, gastric fluids) could then be assessed in comparison to the potential read-across substance. Dissolution testing may also be applicable in instances in which there may be a mutagenic or carcinogenic impurity in the nanomaterial of interest and it is important to understand whether the impurity may be bioavailable. Although not part of the regulatory framework, other *in vitro* reactivity assays may be considered, which may help provide additional information on potential mechanisms of actions, such as the generation of ROS. However, the specific *in vitro* assays to be considered will depend on the nanomaterial substance being evaluated.

Tier 3: In vivo *Testing*

The weight of evidence from Tier 2 testing, exposure considerations and repeated dose toxicity testing (if available) should be assessed to determine whether additional *in vivo* data need to be generated to support the carcinogenicity endpoint. The additional *in vivo* testing may include repeated dose toxicity testing by the applicable route of exposure (if not previously performed) and/or toxicokinetic studies. The repeated dose toxicity data can provide information on whether the nanomaterial may cause any pre-neoplastic lesions or hyperplasia and, together with the mutagenicity data, can meet the regulatory requirements of this endpoint.
In cases in which the weight of evidence based on the design of the nanomaterial of interest, its physico-chemical properties or other available toxicity data are insufficient to meet the carcinogenicity endpoint or establish read-across, then *in vivo* carcinogenicity toxicity testing on the nanomaterial of interest may be warranted. Consideration should be given to the applicable routes of exposure, as well as to which route is likely to represent worst-case effects.

10 Case Studies

The two case studies that follow illustrate the thinking behind applying a generalised test strategy. The case studies are hypothetical and are not intended to approximate real products. Developing case studies for a relatively little-studied class of materials presents a paradox: without available data, case studies are not a particularly practical means to work through a conceptual framework; however, relatively well-studied and much-discussed nanomaterials are challenging to modify in believable ways in order to create a truly novel hypothetical product. The data cited in these case studies were culled from literature reviews. With the explosive pace of research into the (eco)toxicity of nanomaterials, more recent data may have superseded some of the conclusions cited in this report. With the focus on applying and testing a framework for a testing strategy, this potential inaccuracy was deemed to be acceptable.
Finally, the case studies reflect the tiered approach to data collection introduced in this report. The "Tier 0" data considered essential within this framework exceed the information requirements under REACH.

10.1 Nanosilver

The hypothetical nanomaterial is described below, followed by the application of the testing strategy to human health and ecotoxicological endpoints.

10.1.1 Hypothetical substance

Table 11 describes the nature of the substance and available data on that substance in this hypothetical case study. It includes information on the hypothetical product containing the substance and the use of the product, because that information provides clues to potential exposures. Figure 15 illustrates the conceptual life cycle of the product. It shows the major transformation processes that would occur throughout the life cycle and the potential human and environmental exposures. For simplicity, it shows only the most significant exposures likely to occur. (As shown in the figure, human exposure by inhalation might occur at some points in the life cycle. The text below focuses on the route of exposure of primary concern – by dermal contact.) The figure also, in the interest of simplicity, does not include the transformation processes that would occur within a living organism, such as the formation of a protein corona.
Based on this description, the testing strategy would need to reflect the following aspects of the product in particular: the size and chemical nature of the silver particle, and the behaviour and effects of the citrate coating.

The behaviour of nanosilver has been described in numerous publications, including Pronk et al. (2009) and SCENIHR (2014). In addition, the OECD Working Party on Manufactured Nanomaterials is sponsoring safety testing of a representative set of fourteen manufactured nanomaterials that includes nanosilver. The standard nanosilver samples for this testing programme contain 1 milligram per millilitre (mg/mL) of nanosilver particles with diameters of 10 nm and 75 nm and citrate or polyvinylpyrrolidone (PVP) surfaces (NanoComposix, 2011). As of this writing, the testing data are not available.
The presence of citrate, as well as other variables, can affect the behaviour of silver nanomaterials. For example, consider the following data.

Research teams have studied the effects of particle size and environmental variables on the rate of silver dissolution and proposed model equations that relate those variables. Time, particle size, temperature, pH, dissolved oxygen and the concentration of humic or fulvic acids all affect the dissolution rate.

Table 11 Hypothetical nanosilver case study

Purpose	Antibacterial, non-food contact
Anticipated annual tonnage manufactured or imported into EU	900 tonnes/annum
Form of the product	Liquid suspension of nanosilver; Prepared from silver nitrate with the addition of sodium citrate analogue
Anticipated human exposures based on purpose/use	Human – dermal
Substance identity, including:	
Composition	• Silver
Impurities	• No significant impurities
Surface coatings	• Citrate analogue
Functionalisation	• None
Particle size, including	
Primary particle size	• 20 – 40 nm
Degree of agglomeration	• Agglomeration untested. Citrate analogue anticipated to limit agglomeration.
Surface area	• Surface area untested
Shape, including	
Diameter	• Primary particle size 20 – 40 nm
Length	• Triangular nanoparticles
Porosity	
Surface charge	Not known
Solubility	Not tested
Reactivity	Not tested
Data on physico-chemical properties	Not tested

- Zhang et al. (2011a, 2011b) examined the effect of particle size and other variables on the rate of dissolution and extent of agglomeration. They prepared aqueous solutions of citrate-coated nanosilver particles in three sizes – 20, 40 and 80 nm – at 300 and 600 micrograms per litre (µg/L) in dilute Hoagland medium and periodically measured the concentration of silver ions (Ag^+) in solution and the particle size of agglomerates. Their data showed that the released silver ion concentration is a function of time, particle size, oxygen and hydrogen ion concentrations, and temperature. Agglomeration did not markedly affect the release of Ag^+. Agglomeration was affected by the dissolved oxygen in solution.
- Liu and Hurt (2010) measured the time-dependent release of dissolved silver ion from citrate-stabilised nanosilver colloids. The release rates increased with temperature in the range 0−37 °C, and decreased with increasing pH or the addition of humic or fulvic acids. They found that Ag^+ sorbed to nanoparticle surfaces.

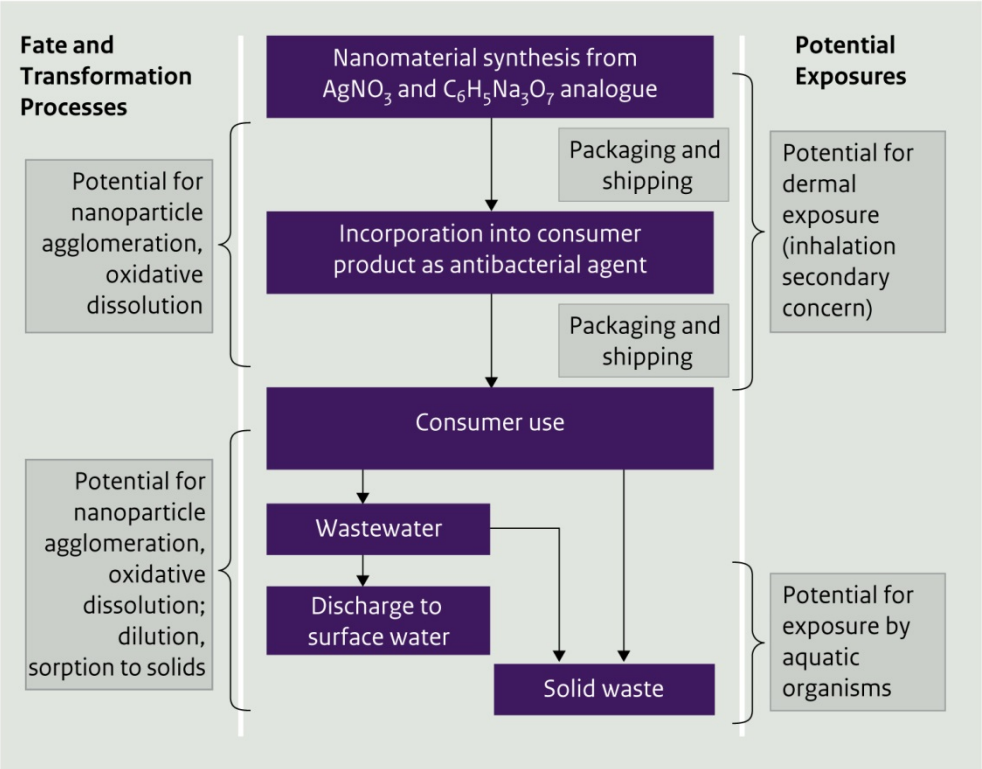


Figure 15 Conceptual life cycle of hypothetical nanosilver product

Several research teams have examined the effect of surface coatings on the dissolution or reaction of nanosilver. In short, the research showed that the presence of a coating does not preclude reactions and the type of coating can influence the rate of dissolution or reaction.

- Levard et al. (2011) studied the corrosion of PVP-coated nanosilver particles in aqueous solution with organic matter with the objective of determining whether the PVP coating stabilised the nanoparticle surface and limited the reaction. Their initial research showed that a stable silver chloride corrosion product formed, despite the presence of potential surface stabilisers like PVP or polyacrylic acid (PAA), which they used in their experiments as a proxy for natural organic matter (NOM).
- Fauss et al. (2011) investigated the effect of various capping or functionalising agents on the dissolution rate of nanosilver and the generation of reactive oxygen species (ROS). They tested three types of particles: 20 nm diameter citrate-capped nanosilver, 30 nm diameter starch (maltose)-capped nanosilver, and silver proteinate functionalised particles approximately 15 nm in diameter. Nanoparticles released dissolved silver at a rate of 0.02 to 13 micromoles per square meter per hour (μmol/m^2 hr), depending on the functionalisation and total silver concentration. ROS generation ranged from 0.01 to 400 μmol/m^2 hr; the rate was proportional to surface area and depended on the capping agent.
- Gondikas et al. (2011) examined the effect of two coatings on the oxidative dissolution and agglomeration of nanosilver. They added cysteine, an analogue for the thiol ligands which would bind nanosilver in the aquatic environment, to their suspensions of nanosilver. These suspensions contained either nanosilver coated with citrate, which stabilises the particles by electrostatic repulsion, or nanosilver coated with PVP, which causes electrosteric stabilisation. Their preliminary results indicated that the addition of cysteine enhanced agglomeration and increased the rate of dissolution of Ag. PVP-coated nanosilver was more susceptible to oxidative dissolution than citrate-coated nanosilver, perhaps because PVP coatings desorbed more readily from the particle surface.
- Ma et al. (2011) measured the aqueous solubility of synthesised and commercially produced nanosilver (transmission electron microscopy size 5-80 nm) with no surface coating, and coated with PVP, citrate or gum Arabic. Solubility did not depend on the coating.

The citrate analogue moiety is anticipated to be of low toxicity. The citrate ion is found in plants and animals and occurs in the human diet (U.S. FDA, 1977). Trisodium citrate is not classified, according to the REACH dossier.
Some evidence suggests that citrate may enhance the toxicity of nanosilver in some contexts, depending in part on the concentration of the citrate anion in solution (Djokic, 2008).

10.1.2 Ecotoxicological endpoints

10.1.2.1 Known information

The hypothetical silver nanomaterial is unique in size and has a coating. A first key question is to see whether the coating has an impact on the solubility of the nanomaterial and whether the coating can also have an effect on the aquatic organisms. It can be assumed that nanosilver forms free silver ions in aqueous solution by dissolution. This indicates that information available on silver ions can be used to represent the toxicity of nanosilver. However, the dissolution kinetics of silver nanomaterials and ageing effect should be kept in mind. Reidy et al. (2013) give an extensive overview of literature regarding the dissolution rate of nanosilver materials. One of the studies cited is by Kittler et al. (2010), who observed that citrate stabilised silver nanomaterials released 15 % (at 25 °C), whereas PVP-stabilised silver nanomaterials released 50 % (at 25 °C) and thus dissolution was never complete for both nanomaterials. Kittler et al. (2010) also indicated that the age of the silver nanomaterials in dispersion is also of importance. Time-dependent dissolution leading to higher toxicity seems to be evident, although the synthesis route and capping/stabilising layer is also an important factor. Therefore, to perform a read-across between a non-nanoform and a nanomaterial, the dissolution kinetics and the ageing effect seem to be important factors. Information on other nanosilver materials can also give an indication of the ecotoxicity of the nanosilver particle of interest. Information on the potential read-across substances is described below.
For ecotoxicity, the dispersion method used and the properties of the aquatic medium tested, as well as the properties of the nanomaterial itself (water solubility, chemical identity, particle characteristics, fundamental transport behaviour, activity and reactivity, chemical identity, see Table 10), have to be taken into account to develop a read-across and testing strategy.
The main (tier 0) physico-chemical characteristics that are important with respect to ecotoxicity endpoints are: chemical composition, surface characteristics (and surface charge), particle size, shape, surface area and water solubility. Information on the rate of dissolution is essential because, for a nanomaterial, the equilibrium solubility concentration can manifest over a longer time scale compared with, for example, the non-nanoform.

Known information on dispersibility/solubility

The literature available on the ecotoxicity of nanosilver particles describes different dispersion methods. For example, in the study of Asghari et al. (2012), the stock mixtures were added to the ecotoxicological media and were stirred using a magnetic stirrer. The powdered silver nanoparticles were prepared by vortexing and sonicating the powders, whereas the colloidal silver nanoparticles were simply purchased from the manufacturer and were not sonicated/vortexed in the media. Therefore, the transmission electron

micrographs indicated the different sizes and shapes of the nanoparticles. Fabrega et al. (2011) give an overview of the sample preparation of ecotoxicity studies involving silver nanoparticles. For example, in ecotoxicity studies with fish, the following sample preparations were used: dilution with tap water, sonication in milliQ water, washed by centrifugation and incubated in egg water for 120 hours, sonication, centrifugation and filtering, and sonication in ultrapure water (see Fabrega et al. (2011) for more details).
The citrate analogue coating used in the hypothetical example can have an effect on dissolution. As indicated by Kittler et al. (2010), polyvinylpyrrolidone (PVP)-coated silver nanoparticles dissolved more rapidly than citrate-coated nanoparticles. In general, solubility, solution chemistry, surface coating, concentration, particle size and shape may all impact agglomeration state, size and structure, leading to the fact that the effects of agglomeration on nanosilver dissolution could be more pronounced in some situations than they are in others.
The stability of silver nanoparticles strongly influences their toxicity, because silver ions are considered to be one of the main silver toxicity factors. The stability (and dissolution) of the silver nanoparticles can be determined by the following physico-chemical characteristics of the media tested: ionic strength, composition, dissolved organic matter, humidity of the environment, dissolved oxygen concentration and temperature (see Reidy et al., 2013). The coating, shape and size, and the concentration of the nanomaterial will also influence the stability of the nanoparticle (Reidy et al. 2013). The solubility of silver nanoparticles depends on the interaction with organic material in the test medium, because silver ions have a strong complexation potential with organic material like proteins, amino acids, natural organic matter, and humic substances, because they may coat and disperse nanoparticles and also complex the released silver ions. In addition to this, silver ions have a high affinity with sulphur, which decreases the toxicity of the silver nanoparticle (Ivask et al., 2013). Natural organic matter (NOM) stabilises nanoparticle suspensions and is therefore expected to increase the possibility of contact with biota (Ivask et al., 2013).
Liu and Hurt (2010) looked at the ion release kinetics and particle persistence of a citrate-coated nanosilver particle in well-defined media. The effects of dissolved oxygen, pH, temperature, oceanic salts and NOM were investigated. The silver ion release rate can be decreased by dissolved oxygen, the addition of NOM, the addition of citrate, reducing the temperature or by an increase of pH. Sea salts only had a minor effect on ion release. Based on these data, the kinetics for citrate-stabilised silver can lead to an estimation of ionic release rates in the low concentrations (for example relevant to the environment). Stabilised citrated nanosilver will not persist as a particle in environmental compartments that contain any dissolved oxygen, but its disappearance is a slow process.

Known information from ecotoxicity experiments

The overview below describes the observed toxicity of nanosilver particles in standard ecotoxicity experiments. Because the focus was only on the literature available from the reports by SCENIHR (2014), Allen et al. (2010), and Pronk et al. (2009), only short-term experiments were discussed.

Short-term toxicity to fish

Fabrega et al. (2011) reviewed the available ecotoxicity data on silver nanoparticles. The results indicated that 10-80 nm silver nanoparticles affect early life-stage development. Juvenile zebrafish and Japanese medaka have been shown to be more susceptible to silver nanoparticles than they are to equal mass concentrations of silver nitrate ($AgNO_3$) at least under conditions that maximise free ion Ag^+ concentrations. Other authors suspect that a differential uptake of Ag^+ ions has been shown, whereby silver nanoparticles aggregates were incorporated into blood vessels, skin, brain, heart and yolk, whereas Ag^+ ions were concentrated in organelles, nucleus and the yolk.
As indicated before, coatings can have an influence on the dissolution of Ag^+ ions and thus affect the observed toxicity.
Griffitt et al. (2008) tested the ecotoxicity of a metallic coated nanosilver particle to zebrafish (*Danio rerio*). The size of the nanomaterials during the test was between 44.5 and 216 nm, in which a dissolution of 0.07 % Ag was found. The LC_{50} (lethal concentration that causes a 50 % effect) was 7.2 mg/L. Bilberg et al. (2012) used the same fish species to test PVP-coated nanosilver particles with a size of 173 nm. A 40 % dissolution was found, and LC_{50} was 84 µg/L. It seems that the higher ecotoxicity found by Griffitt et al. (2008) could also be explained by the characteristics of the aquatic media used in the test. Griffitt et al. (2008) also indicated that silver nanoparticles were more toxic than $AgNO_3$ (non-nanoform) and that Ag^+ did not explain all toxicity observed for nanosilver exposure.
Kennedy et al. (2010) tested eight nanosilver materials and $AgNO_3$. When the LC_{50}s were expressed as dissolved silver, the effect concentrations were comparable to $AgNO_3$. The ASAP[6] coating led to the highest solubility and therefore led to a higher toxicity. PVP coating had the lowest effect concentration. Citrate-coated silver nanoparticles had a LC_{50} of 1.5 µg/L (expressed as dissolved Ag), which was lower than $AgNO_3$. However, similar LC_{50} values were found for *D. magna* (see further). A possible explanation of the discrepancy found between the two species could be that the particle binding and the dissolution at the gill site could be a unique feature relevant to fish. Another explanation could be that additional nanosilver dissolved during the exposure.
The studies described above could be used in a weight-of-evidence approach for testing/grouping information on the hypothetical nanosilver case. An overview of these studies is given in Table 12.

[6] ASAP is a commercially sold colloidal silver drink (ASAP, American Biotech Laboratories, Alpine, UT, USA).

Table 12 Overview of literature available on ecotoxicity of nanosilver material to fish

Organism	Size	Coating	Shape	Medium	LC_{50} values	Dosing regime	Sample preparation	Reference
Danio rerio	44.5–216 nm	Metal oxide	Spherical	ASTM	7.07 mg/L	48 h	Sonication in milliQ water	Griffitt et al. (2008)
Danio rerio	173 nm	PVP	Elliptical/ multifaceted	OECD	84 µg/L	48 h	Ag nanoparticles were sonicated, centrifuged and filtered through a 0.22 µm pore filter	Bilberg et al. (2010)
Pimephales promelas	in milliQ water: 27–185 nm in moderately hard reconstituted water (MHRW): 77-228 nm	Different coatings: ASAP, Citrate, EDTA, PVP	Circum-spherical (expect rods in citrate-nanosilver and NC2 and NC50 samples)	MHRW was used for control and dilution water	5.7- 125.6 µg/L (expressed as total Ag) 1.5–5.6 µg/L (expressed as fractionated Ag) $AgNO_3$: 5.7–6.6 µg/L	48 h	Different protocols	Kennedy et al. (2010)

Short-term toxicity to aquatic invertebrates

Griffitt et al. (2008) tested a metallic-coated silver nanoparticle with respect to *Daphnia pulex* adults and *Ceriodaphnia dubia* neonates. Because only the neonates are of relevance in terms of REACH testing, only the results of these tests will be reported. The solubility of silver ions was relatively low, leading to an LC_{50} of 67 µg/L. In this study, only one kind of nanoparticle was tested. Asghari et al. (2012) tested two colloidal nanoparticles, nanosilver powder and $AgNO_3$ to *Daphnia magna*. The results indicated that silver nanopowders suspended in test media are less toxic compared with nanosilver colloids.
Allen et al. (2010) tested several coatings on acute toxicity of *Daphnia magna*. The toxicity of the nanosilver was comparable to ionic silver. The toxicity of the uncoated silver particles was slightly higher compared with the coated particles. The variation in the observed toxicity could probably be explained by particle size. In larger particles, the surface area available to release the Ag^+ ion via oxidation processes is decreased.
Kennedy et al. (2010) showed that ionic Ag^+ from $AgNO_3$ was more toxic than total measurable Ag (which included nanosilver particulates). However, the large variation in LC_{50} values indicated that total measurable Ag is not an appropriate predictor for nanosilver toxicity. Using the fractionated nanosilver concentrations (i.e. dissolved), the nanosilver particles were comparably toxic to $AgNO_3$. The most toxic nanomaterial was ASAP-nanosilver, which had the highest dissolved fraction, while PVP-nanosilver had the lowest dissolved fraction. The citrate-nanosilver particle had an LC_{50} of 1.5 µg/L, expressed as fractionated Ag.
The citrate-coated silver nanoparticles used in the studies by Asghari et al. (2012), Allen et al. (2010) and Kennedy et al. (2010) reported LC_{50} values between 1.1–11 µg/L.
An overview of these invertebrate studies is given in Table 13. Overall the variability in toxicity could be attributed to: sample preparation, test organism, coating, the size of nanomaterial and test medium tested.

Toxicity to algae

Kennedy et al. (2010) tested the toxicity of a variety of manufactured nanosilver particles in suspension. In addition, the nanosilver toxicity was compared with toxicity of ionic Ag^+ and to relate toxicity to the particulate and ionic fractions in the nanosilver suspensions. Algae were less sensitive to Ag^+, but were more sensitive to PVP-nanosilver particles. This could be explained by the surfactant properties of ethylene glycol, as they could disrupt algae membranes. In the test of Griffitt et al. (2008), lower toxicity was found.

Table 13 Overview of literature available on the ecotoxicity of nanosilver material to aquatic invertebrates

Organism	Size	Coating	Shape	Medium	LC_{50} values	Dosing regime	Sample preparation	Reference
Ceriodaphnia dubia	44.5 nm	Metal oxide	Spherical	ASTM	67 µg/L	48 h	Sonication in milliQ water	Griffitt et al. (2008)
Daphnia magna	7.32 nm (count median diameter – CDM)		nanosilver1 colloid-spherical	OECD	4 µg/L	48 h	Suspension	Asghari et al. (2012)
	6.47 nm (CMD)		nanosilver2 colloid-spherical		2 µg/L		Suspension	
	17.97 nm (CMD) (70 % of the aggregates had a diameter from 25 nm to 100 nm)		nanosilver3 suspension-spherical		187 µg/L		Dispersion, vortexing and sonication	
			$AgNO_3$ solution		2.3 µg/L			

Organism	Size	Coating	Shape	Medium	LC_{50} values	Dosing regime	Sample preparation	Reference
Daphnia magna	Different sizes	Different coatings		Deionised water or moderate hard reconstituted water (MHRW)	0.7–16.7 µg/L	48 h	different protocols	Allen et al. (2010)
Daphnia magna	in MilliQ water: 27–185 nm in moderately hard reconstituted water (MHRW): 77–228 nm	Different coatings: ASAP, Citrate, EDTA, PVP,		MHRW	1.8 µg/L–97 µg/L (expressed as total Ag) 0.3–1.9 µg/L (expressed as fractionated Ag) $AgNO_3$: 0.7–1.6 µg/L	MHRW	Different protocols	Kennedy et al. (2010)

Table 14 Overview of literature available on ecotoxicity of nanosilver material to algae

Organism	Size (nm)	Coating	Shape	Medium	LC_{50}s	Dosing regime	Sample preparation	Reference
Pseudokirchneriella subcapitata	44.5 nm	Metal oxide	Spherical	ASTM	190 µg/L	72h	Sonication in milliQ water	Griffitt et al. (2008)
Pseudokirchneriella subcapitata	96 µm	PVP			18.4 µg/L 21 µg/L	72h		Kennedy et al. (2010)
Chlamydomonas reinhardtii	25 ± 13 nm	carbonate coating			92 µg/L-355 µg/L	1–5h	Not clear	Navarro et al. (2008)

Navarro et al. (2008) found EC_{50} values for carbonate-coated silver nanoparticles ranging from 355 µg/L after 1 hour and 92 µg/L after 5 hours, corresponding to 3.6 and 0.9 µg free Ag/L. However, adding a cysteine ligand to the silver nanoparticles tests, the EC_{50} values increased up to 6.1 and 6.6 µg/L, respectively.
An overview of these algal studies is given in Table 14.

10.1.2.2 Hypothesis:

The nanomaterial exhibits a unique behaviour under ecotoxicity testing.

10.1.2.3 Testing strategy:

Tier 1: Physico-chemical testing

Essential data (Tier 0) for this nanomaterial are: chemical composition, coating, particle size, shape, surface area and water solubility.
In the first tier (Tier 1) of testing, solubility/dispersibility should be determined under conditions relevant to the environment. The dispersibility and solubility of the nanomaterial should be investigated under different pH, cation concentrations and organic matter. These tests may provide results that differ from those obtained in standard laboratory test media. For dispersibility, there is no standard OECD guideline available (OECD, 2014). In the OECD (2014) expert meeting report on physico-chemical parameters, it was concluded that there is a need to develop new test guidelines which can refer to the existing ISO (International Organization for Standardization) standards.
The rate of dissolution should also be evaluated under those different environmental parameters, to determine the bioavailability of the silver ions under environmentally relevant conditions. The information available in the study of Liu and Hurt (2010) can be used as a starting point to estimate the dissolution of the silver ions in the aquatic medium of interest.
It is expected that the citrate coating will prevent agglomeration of the nanomaterial. However, Tejamaya et al. (2012) observed that the size of citrate nanosilver particles increased by approximately 2-fold compared with stock solutions. It is also expected that dissolution of silver nanoparticles is less likely to occur from a citrate-coated nanoparticle compared with PVP-coated silver nanoparticle, because PVP coating stabilises silver nanoparticles against aggregation more effectively than a citrate coating. However, the coating also affects dissolution: Kennedy et al. (2010) observed a higher percentage of dissolved silver in the treatment with the citrate coating compared with the PVP coating.
Data on the solubility (rate of dissolution and equilibrium solubility) and the dispersibility of the nanomaterial can then be used to support the determination of the hypothetical silver nanomaterial and potential read-across substance(s).
If the results from this first tier (solubility/dispersibility testing under environmentally relevant conditions) with the hypothetical silver nanomaterial indicates that size (and agglomeration state) and solubility

are similar to those found in the literature above, then read-across between those substances is possible and effect concentrations can be used in the dossier. Data from the other nanoforms found in literature can be used if only this first tier indicates that similar rate of dissolution (and equilibrium solubility) can be reached, which will also depend on the dispersion method used. It can be expected that, because of the citrate coating, the solubility will be limited relative to the solubility of uncoated nanosilver or non-nanoparticles. Therefore, the soluble silver compounds (the non-nanoforms like $AgNO_3$) cannot be used as a possible read-across substance (or only in a worst case approach). From the literature above, data obtained from Kennedy et al. (2010), Allen et al. (2010) can be used as a starting point to check what the solubility (rate of dissolution and equilibrium solubility if reached) is of those substances under the different dispersion methods.
The particle shape should also be considered. Based on the information available in the literature, the effect of the triangular shape on ecotoxicity cannot be predicted. To rule out the effects of shape, therefore, an additional tier (tier 3 ecotoxicity testing) can be considered. As not a lot of information is available on the reactivity and photoreactivity of the nanomaterials, although literature indicates that this is of major importance for explaining silver toxicity, ecotoxicity testing on the nanomaterial should be considered.
In general, read-across between a nanomaterial and a non-nanomaterial is possible if data on testing conditions and the characteristics of the nanomaterial are available. However, in practice such detailed information is currently often lacking, which prevents performing read-across.

Tier 2 Testing

No Tier 2 testing would be proposed in this case.

Tier 3: Ecotoxicity testing

The registration of this hypothetical nanomaterial would be an Annex IX dossier. For an Annex IX dossier the following endpoints are obligatory unless they meet waiver criteria:

- Degradation.
- Hydrolysis as a function of pH.
- Adsorption/desorption screening.
- Short-term toxicity testing on invertebrates (preferred species Daphnia).
- Growth inhibition study on aquatic plants (algae preferred).
- Short-term toxicity testing on fish.
- Long-term toxicity testing on invertebrates (preferred species Daphnia).
- Long-term toxicity testing on fish: fish early life-stage toxicity test, fish juvenile growth test, fish short-term test on embryo and sac-fry stages
- Activated sludge inhibition growth test.
- Soil simulation testing.

- Sediment simulation testing.
- Bioaccumulation in aquatic species, preferably fish.
- Effects on terrestrial organisms: short-term toxicity to invertebrates, effects on soil micro-organisms, short-term toxicity to plants.

Based on the solubility/dispersibility, using a weight-of-evidence approach it can be decided if additional ecotoxicity testing is needed. If results from this first tier indicate that size (and agglomeration state) and solubility are not similar to those found in the literature cited above, then read-across between those substances is not possible and ecotoxicity testing on the nanomaterial of interest should be performed. The specific testing should take into account the endpoints that need to be considered for the regulatory framework. For this hypothetical nanomaterial, we will only focus on the ecotoxicological testing (acute) of daphnid, algae and fish.
Data available on nanosilver particles with citrate coating indicate that differences in reported LC_{50} values could be attributed to: the dispersion method used, the aquatic test medium used, the preparation of stock suspensions, test species, and the size of the nanomaterial. The other physico-chemical characteristics that are of importance for nanomaterials, such as shape, functionalisation, surface charge and reactivity, are not discussed in the publications reviewed. It seems that the coating, size and rate of dissolution of the nanosilver material are the most important parameters to determine ecotoxicity (or at least are mostly described). Higher toxicity can be observed for nanosilver in comparison with the non-nanoform $AgNO_3$, although the observed discrepancy is not yet fully understood. It seems that additional dissolution of the nanosilver particles could be contributing to the Ag^+ toxicity.
The publications from Kennedy et al. (2010) and Allen et al. (2010) could be used to perform a read-across, as those studies tested nanomaterials in a similar size range and with a citrate coating. The data obtained from Kennedy et al. (2010) are relevant to two endpoints: acute toxicity to fish and invertebrates. Allen et al. (2010) studied short-term toxicity to aquatic invertebrates. However, to read-across to these data (which were obtained for test substances similar to the hypothetical case study substance with respect to particle size and coating) one must also consider the particle shape.
As indicated above, shape is a main parameter that could contribute to ecotoxicity effects. The shape of the nanomaterial of interest is not the same as described in the publications of Kennedy et al. (2010) and Allen et al. (2010). It would be prudent, therefore, to perform an additional ecotoxicity test. Testing results obtained by Kennedy et al. (2010) indicate that *Daphnia magna* is more sensitive to citrate-coated silver nanoparticles than fish (*Pimephales promelas*). It would be advisable, therefore, to perform an additional study with the nanomaterial of interest, with *Daphnia magna* as the test species. The observed LC_{50} value obtained from this Tier 3 testing can then be compared with the data available in the literature cited above. If the result of this test is

similar to the results from Kennedy et al. (2010) and Allen et al. (2010), then a short-term toxicity test to fish should not be performed. Using a weight-of-evidence approach on all data and information gathered, read-across between the nanomaterials can then be considered.
An Annex IX dossier under REACH for this hypothetical substance (tonnage band is 900 tonnes/annum) would also require data on long-term toxicity to fish and invertebrates and an algal growth inhibition growth study. As the focus was only on the literature available from the reports by SCENIHR (2014), Allen et al. (2010), and Pronk et al. (2009), only short-term experiments were discussed in this report.

10.1.3 Human Health Toxicity Endpoints

10.1.3.1 Known information

The first step in the testing strategy is to collect all the known information about the nanomaterial of interest, including the design and purpose of the nanomaterial, the regulatory framework and the endpoints that need to be addressed as part of this framework. One would also consider how nanomaterial properties affect the applicable endpoints generally, whether there are data for potential read-across, and mechanisms of action for the read-across substance.
In addition to the information presented above, we know the following for this hypothetical nanosilver material. The regulatory framework on which this proposed nanomaterial is being assessed will be REACH. Based on the tonnage, the following human health endpoints will be considered for this case study, with the testing requirements under REACH paraphrased.

Table 15 Requirements for human health endpoints[1]

Endpoint	**Testing Requirement under REACH**
Acute toxicity	Oral route and one other route relevant to exposure (dermal)
Skin irritation	*In vivo* skin irritation test, with some exceptions (e.g. acute toxicity test shows no irritation)
Eye irritation	*In vivo* test required, with some exceptions
Skin sensitisation	*In vivo* test required, with some exceptions
Mutagenicity	Battery of *in vitro* tests; appropriate *in vivo* test if *in vitro* test is positive
Repeated dose toxicity	Subchronic study (90 days) via relevant exposure route (dermal)

[1] Although reproductive/developmental toxicity does need to be addressed in this tonnage band, for the purposes of this case study, these endpoints are not considered. See Section 9.2.
For the purposes of this case study, the toxicity data for silver and nanosilver as summarised in Pronk et al. (2009) are presented in this report and are considered for read-across for the hypothetical silver nanomaterial. The key parameters to consider for each endpoint are discussed in the following sections, as well as the available data from which read-across may be performed.

10.1.3.2 Evaluation of initial information

The hypothetical silver nanomaterial is unique in size and has a coating. The key questions that will need to be considered are whether the coating itself is reactive or is likely to cause a toxicological effect or whether the coating may potentially enhance the toxicity of the nanoform. Because there are not any significant impurities present in this nanomaterial, the impact of impurities will not need to be considered. The design of the nanomaterial is such that it has antibacterial effects. Furthermore, the coating is used to help limit agglomeration that would increase the net particle size.
For each endpoint discussed below, this report presents the general nanomaterial characteristics relevant to the endpoint, the data available for read-across and the specific aspects of this hypothetical product that should be considered. Because multiple human health endpoints are being evaluated rather than a single endpoint, the development of a testing strategy and subsequent data evaluation would require a weight of evidence for all the endpoints being considered.
It should be noted that the main human health concern with regard to silver toxicity is with the ionic form. As such, the human health toxicity information presented in Pronk et al. (2009) is primarily on the silver thiosulphate salt.

Acute toxicity

Acute toxicity is dictated by the route of exposure, as well as by those properties that may affect toxicity over the short term, such as composition (of a particle itself and its coating), solubility and reactivity. The potential route of exposure for this silver nanomaterial is the dermal route[7].
Systemic toxicity is less of a concern with the dermal route of exposure than it is with either the inhalation or oral routes of exposure because significant systemic availability through dermal absorption has not been observed in studies on other nanomaterials. However, the characteristics of the nanomaterial should be evaluated to determine whether the characteristics are such that 'nanosizing' would enhance the systemic bioavailability.
As presented by Pronk et al. (2009), the acute dermal and oral lethal doses of 50 % (LD_{50}) for silver (as silver thiosulphate) are > 2000 mg/kg. Therefore, inherently, silver does not appear to be acutely toxic following exposure through either the oral or dermal routes.
For the acute toxicity endpoint, it will be important to understand whether the coating itself is acutely toxic or has any impact on the potential acute toxicity of silver. Based on the available information presented above on citrate, it is not considered acutely toxic. The

[7] Note that REACH Annex IX would require acute oral data for a substance at the tonnage band of this hypothetical substance. Given the lack of oral exposure in this hypothetical case and the REACH requirement to minimize animal testing, one would carefully consider the need for testing acute oral toxicity.

coating itself, therefore, does not present a concern for acute toxicity. As described previously, the citrate analogue enhances the dispersion of silver nanoparticles, which may in turn affect the rate of dissolution of silver. Considering the possibility that the citrate analogue may increase the potential bioavailability of silver ions and the silver ion is the form primarily responsible for the toxicological effects of silver, the potential effect on the acute toxicity should be considered for the testing strategy. It should also be noted that, because additional human health endpoints are being considered for this nanomaterial, these other endpoints should be considered for development of the testing strategy as described in the next section of this report.

Skin irritation, eye irritation, skin sensitisation

Because the key nanomaterial characteristics for these endpoints are similar, they will be assessed together for purposes of this case study. For these endpoints, the key characteristics to consider include composition, reactivity, solubility, and pH.
As presented in the RIVM report on nanomaterials under REACH (Pronk et al., 2009), silver (as silver thiosulphate) is not irritating to the skin or eyes or sensitising to the skin.
Based on the available information presented above on citrate, it is not considered irritating to the eyes or skin or sensitising to the skin. The coating itself, therefore, does not present a concern for these endpoints. As described previously, the citrate analogue enhances the dispersion of silver nanoparticles, which may in turn affect the rate of dissolution of silver. Although the citrate analogue may increase the potential bioavailability of silver ion, based on the data from Pronk et al. (2009), silver is not irritating or sensitising. The use of the citrate analogue coating, therefore, is unlikely to impact the irritation or sensitising effects of the nanomaterial. But because other endpoints are being considered for the testing strategy, development of additional data may further support the use of read-across for these endpoints.

Mutagenicity

For this case study, one needs to consider whether the composition, size and coating may affect mutagenicity.
As presented in the RIVM report on nanomaterials under REACH (Pronk et al., 2009)[8], silver (as silver thiosulphate) was negative *in vitro* for mutagenicity in the Ames assay and chromosome aberration test. It was, however, positive in the mouse lymphoma gene mutation assay. Yet it was noted that the likely mechanism for this positive response was due to silver binding to the enzymes involved in DNA synthesis, rather than the direct interaction of silver with DNA. Silver (as silver thiosulphate) was also negative *in vivo* in an unscheduled DNA synthesis assay. Furthermore, nanosilver was found to be negative in an *in vivo*

[8] A more recent review of the relevant literature is provided by SCENIHR (2014). For the purposes of illustrating the testing and read across strategy, the discussion in this report relied on the information presented by Pronk et al. (2009).

micronucleus assay (Pronk et al., 2009). Based on the weight of evidence from the data presented in Pronk et al. (2009), silver is not inherently expected to be mutagenic.
It will also be important to understand whether the coating itself is mutagenic or has any impact on the potential mutagenicity of silver. Based on the available information presented above on citrate, it is not considered mutagenic. The coating itself, therefore, does not present a concern for mutagenicity. As described previously, the citrate analogue enhances the dispersion of silver nanoparticles, which may in turn affect the rate of dissolution of silver. Although the citrate analogue may increase the potential bioavailability of silver ion, based on the data from Pronk et al. (2009), silver is not mutagenic. The use of the citrate analogue coating, therefore, is unlikely to impact the mutagenic effects of the nanomaterial. But because other endpoints are being considered for the testing strategy, development of additional data may further support the use of read-across for this endpoint.

Repeated dose toxicity

For the repeated dose toxicity endpoint, the route of exposure, as well as the composition, size, and coating need to be evaluated this case study.
For this hypothetical nanomaterial, the dermal route of exposure is the only relevant route. As noted previously, the systemic toxicity is less of a concern for the dermal route than it is for either the oral or inhalation routes of exposure because significant dermal absorption into the systemic circulation has not been observed following dermal exposure to nanomaterials. However, as summarised by Pronk et al. (2009), although low, there is a potential for bioavailable silver following dermal exposure to the ionic form (approximately 0–2.4 %).
A 28-day dermal repeated dose toxicity study on silver (as silver thiosulphate), as summarised by Pronk et al. (2009), indicated a systemic, no observed adverse effect level (NOAEL) of 300 mg/kg based on changes in clinical chemistry parameters observed at the 1,000 mg/kg dose group, the lowest observed adverse effect level (LOAEL) of 1,000 mg/kg, and a LOAEL of 100 mg/kg for local effects (irritation).
As with the other endpoints, the potential impact of the coating needs to be assessed and the question of whether the coating itself is toxic through repeated exposure or has any impact on the potential repeated dose toxicity of silver. Based on the available information presented above on citrate, it is not considered toxic through repeated exposure. The coating itself, therefore, does not present a concern for repeated dose toxicity. As described previously, the citrate analogue enhances the dispersion of silver nanoparticles, which may in turn affect the rate of dissolution of silver. Considering the possibility that the citrate analogue may increase the potential bioavailability of silver ion and the silver ion may be the form primarily responsible for the toxicological effects of silver observed in the 28-day repeated dose toxicity study, the potential effects of the coating on repeated dose toxicity should be considered for the testing strategy.

10.1.3.3 Testing Strategy

Tier 0 Testing

For this case study, the most important property for Tier 0 testing should be size. While the primary particle size is 20–40 nm as manufactured, it will be important to know the particle size at the point of potential exposure because the size may impact the rate of dissolution. The coating with a citrate analogue is intended to stabilise the particles and may prevent significant agglomeration.
In addition, the solubility and rate of dissolution should be evaluated for the nanomaterial to determine the likelihood of an increase in the bioavailability of silver ions.

Tier 1 testing

While the pH of a suspension of the silver nanomaterial is not expected to be in the range that would dictate classification as a corrosive, the pH of the nanomaterial in solution should be evaluated. Development of this piece of information can help support a determination that the hypothetical silver nanomaterial and the potential read-across material are substantially similar.

Tier 2 testing

Based on the current understanding of the mode of action of nanosilver, the Tier 2 focus would be on dissolution and required *in vitro* testing rather than on measuring reactivity. The Tier 2 testing should involve any *in vitro* assays that are required under the regulatory framework and/or may support read-across for *in vivo* data. For this case study, *in vitro* irritation and *in vitro* mutagenicity assays should be conducted at the proposed tonnage. However, any potential compatibility issues with nanosilver and these types of assays should be considered before pursuing testing. The use of these types of *in vitro* assays can help meet the endpoint-specific requirements and potentially support read-across for required *in vivo* data.

Tier 3 Testing

Based on the results of the Tier 2 testing, additional *in vivo* testing may be warranted. The specific *in vivo* testing should take into account the endpoints that need to be considered for the regulatory framework, as well as whether the data can be filled through read-across.
Based on the tonnage of the nanomaterial, acute toxicity testing would be required. As dictated by the regulation, acute oral and dermal toxicity testing would be required. But data are available from read-across for both routes of exposure. The results from the Tier 1 testing should be considered in order to determine whether read-across is justified. Considering that repeated dose toxicity testing by the dermal route is required for this material, *in vivo* acute dermal toxicity data may help substantiate read-across for the repeated dose toxicity testing. If *in vivo* acute dermal toxicity testing were conducted, the data could provide

further justification that *in vivo* irritation studies may not be warranted and that data from read-across could be used.
If the results of the *in vitro* mutagenicity assays conducted in Tier 2 are negative, *in vivo* mutagenicity testing would not be warranted. However, if any of the *in vitro* assays has a positive result, *in vivo* mutagenicity testing would be required.
An *in vivo* toxicokinetics study would provide a context for the effects observed in the 28-day repeated dose dermal toxicity study on silver thiosulphate, by characterising the relative systemic bioavailability between the silver nanomaterial of interest and that of the read-across substance. Toxicokinetic data might help support the case that systemic toxicity between the two is likely to be similar and read-across is justified.

10.2 Nanotitanium Dioxide

The hypothetical nanomaterial is described below, followed by the application of the testing strategy to human health endpoints. Ecotoxicity endpoints are not addressed in this case study.
While actual data for nanotitanium dioxide are used as part of this case study to illustrate the process by which a testing strategy is developed for a nanomaterial considering read-across, this does not represent a comprehensive evaluation of all available data on nanotitanium dioxide. The data summarised are from peer reviewed sources, which implies that the data reliability has been assessed.

10.2.1 Hypothetical substance

Table 16 describes the nature of the substance and available data on that substance in this hypothetical case study. It includes information on the hypothetical product, which would incorporate the substance because the nature of the product and its use would affect exposures. Figure 16 illustrates the conceptual life cycle of the product, including the major transformation processes and potential human exposures. Based on the description of the hypothetical nanomaterial, the testing strategy for human health toxicity would need to reflect the following aspects of the product in particular: the size, crystallinity of the titanium dioxide nanoparticle (i.e. anatase), potential routes of exposure (dermal and inhalation), and the behaviour and effects of the manganese doping. In accordance with REACH, the toxicity of only the nanotitanium dioxide material is considered. Because the polyurethane matrix is associated with the final product and not the substance itself, the polyurethane matrix is not considered a part of this evaluation.
While manganese as the doping agent should be considered, it is not classified according to the REACH dossier. The levels as present or that may be released in biological fluids are unlikely to result in significant toxicity; however, the specific levels of manganese that may be released should be confirmed. It is likely that the impact that the doping may have on the reactivity of the nanotitanium dioxide is of greater importance in regard to potential toxicity than is the presence of manganese itself.

In addition, shape is not a significant property of concern for this material. As discussed in previous sections of this report, shape is unlikely to affect dermal toxicity. For the inhalation route, shape, in general, may be a concern. But, given that the shape is spherical-like and not needle-like, shape is not expected to be a significant factor in the toxicity of the hypothetical nanotitanium dioxide material.

Table 16 Hypothetical titanium dioxide case study

Purpose	Spray coating on building
Anticipated annual tonnage manufactured or imported into EU	7000 tonnes/annum
Form of the product	Liquid suspension of nanotitanium dioxide in polyurethane, dries upon curing after application
Anticipated human exposures based on purpose/use	Human – dermal; inhalation (potential during application) Preliminary studies suggest release of titanium dioxide upon weathering of the coating is not detectable
Substance identity, including:	
Composition	• Titanium dioxide (TiO_2) (anatase)
Impurities	• No significant impurities; doped with manganese at 5 %
Surface coatings	• None
Functionalisation	• None
Particle size, including	
Primary particle size	• Up to 100 nm
Degree of agglomeration	• Agglomeration untested
Surface area	• Surface area untested
Shape, including	
Diameter	• Shape irregular (but not needle-like)
Length	
Porosity	
Surface charge	Not known
Solubility	Not tested
Reactivity	Not tested
Data on physico-chemical properties	Not tested

Data obtained from testing nanotitanium dioxide have been evaluated and summarised in a number of publications. The data presented by the Scientific Committee on Consumer Safety (SCCS, 2014a) will be used as the primary basis for the assessment and development of a testing strategy presented herein; however, information from other publications, such as U.S. EPA (2010), the National Institute for Occupational Safety and Health (NIOSH, 2011) and the European

Commission (EC, 2014), were also used in the assessment where applicable. Only those data that SCCS (2014a) deemed relevant for assessing the risks associated with nanotitanium dioxide (i.e. had sufficient material characterisation) were summarised for this case study.

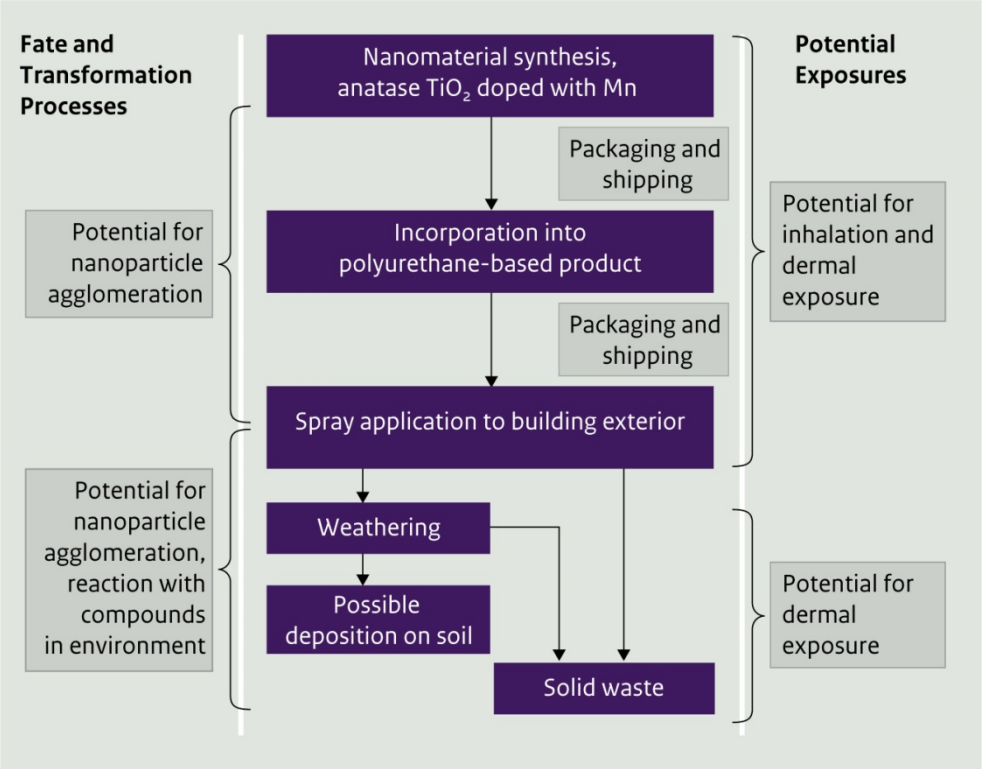


Figure 16 Conceptual life cycle of hypothetical nanotitanium dioxide product

The data presented by SCCS (2014a) include toxicity data on different nanotitanium dioxide materials. Most of the data pertain to coated and undoped nanotitanium dioxide materials, with some of the materials being mainly rutile, some being mainly anatase and others a combination of the two crystalline forms.
Titanium dioxide particles are poorly soluble. Dissolution experiments summarised in EC (2014) found that nanotitanium dioxide particles were nearly insoluble.
Pure titanium dioxide is photocatalytic when exposed to ultraviolet radiation, with anatase being more photoreactive than rutile (EPA, 2010). Zaleska (2008) summarised the doping methods for titanium dioxide. Doping with transition metals, such as manganese, can enhance the photocatalytic properties of titanium dioxide. However, the degree to which the photocatalytic properties are enhanced by doping with manganese is not currently available. The SCCS (2014a) considers a 10 % deviation in photocatalytic activity, in comparison with

corresponding non-coated or non-doped reference, as acceptable in regard to potential human health effects.

10.2.2 Human Health Toxicity Endpoints

10.2.2.1 Known information

As with the nanosilver case study, the first step in the testing strategy is to collect all the known information about the nanomaterial of interest, including the design and purpose of the nanomaterial, the regulatory framework and the endpoints that need to be addressed as part of this framework. One would also consider how nanomaterial properties affect the applicable endpoints generally, whether there are data for potential read-across and mechanisms of action for the read-across substance. In addition to the information presented above, we know the following for this hypothetical nanotitanium dioxide material. The regulatory framework on which this proposed nanomaterial is being assessed will be REACH. Based on the tonnage, the following human health endpoints will be considered for this case study, with the testing requirements under REACH paraphrased as indicated in Table 17.

Table 17 Requirements for human health endpoints[1]

Endpoint	**Testing Requirement under REACH**
Acute toxicity	Oral route and one other route relevant to exposure (inhalation/dermal)
Skin irritation	*In vivo* skin irritation test, with some exceptions (e.g. acute toxicity test shows no irritation)
Eye irritation	*In vivo* test required, with some exceptions
Skin sensitisation	*In vivo* test required, with some exceptions
Mutagenicity	Battery of *in vitro* tests; appropriate *in vivo* test if *in vitro* test is positive
Repeated dose toxicity	Subchronic study (90 days) via relevant exposure route (dermal)
Carcinogenicity	A carcinogenicity study may be required, depending on the results of mutagenicity and repeated dose toxicity studies

[1] Although reproductive/developmental toxicity does need to be addressed in this tonnage ban for REACH, for the purposes of this case study, these endpoints are not considered. See Section 9.2.
The key parameters to consider for each endpoint are discussed in the following sections, as well as the available data from which read-across may be performed.

10.2.2.2 Evaluation of initial information

The hypothetical titanium dioxide nanomaterial is unique in size and has been doped with manganese to enhance its photocatalytic properties for use as a self-cleaning coating. Some of the key questions that will need to be considered include:

- What is the impact of size on the toxicokinetics and toxicity?
- How does the crystalline structure (100 % anatase) affect toxicity?

- What is the effect of doping with manganese on the toxicity of the nanotitanium dioxide particles?

Besides the use of manganese, there are no other significant impurities present in this nanomaterial. The impact of the photocatalytic activity will need to be considered in the testing strategy.
For each endpoint discussed below, this report presents the general nanomaterial characteristics relevant to the endpoint, the data available for read-across and the specific aspects of this hypothetical product that should be considered. Because multiple human health endpoints are being evaluated rather than a single endpoint, the development of a testing strategy and subsequent data evaluation would require a weight-of-evidence assessment of all the endpoints being considered.
The next section of this report synthesises the conclusions of this evaluation with respect to specific recommendations for testing.

Acute toxicity

Acute toxicity data on nanotitanium dioxide available from SCCS (2014a) are summarised in Table 18.

Table 18 Summary of Acute Toxicity Data

Route	**Species**	**Particle Size (nm)**	**Crystalline Form**	**LD_{50}/LC_{50} (mg/kg)**
Oral	Rats	49	85 % Anatase, 15 % rutile	>2150
Oral	Rats	49	85 % Anatase, 15 % rutile	>2150
Intragastric intubation	Rats	49	85 % Anatase, 15 % rutile	>11000

Acute toxicity is dictated by the route of exposure, as well as by those properties that may affect toxicity over the short term, such as composition, solubility and reactivity. The potential routes of exposure for this hypothetical titanium dioxide nanomaterial are the inhalation and dermal routes.
As previously discussed in this report, systemic toxicity is less of a concern for the dermal route of exposure than it is for either the inhalation or oral routes of exposure because significant systemic availability through dermal absorption has not been observed in studies. The lack of significant systemic bioavailability following dermal exposure has been summarised specifically as it relates to nanotitanium dioxide (EPA, 2010; Shi, 2013).
Inhalation is also a potentially relevant route of exposure for this hypothetical nanomaterial. Because nanotitanium dioxide is poorly soluble, significant bioavailability of titanium is not expected following acute inhalation exposure. However, local effects could occur and the impacts of the photoreactivity, as well as the manganese doping, need to be considered. NIOSH (2011) concluded that the particle surface properties related to the crystalline structure of titanium dioxide,

including photoactivation, can influence acute lung responses, particularly with regard to local effects. The extent to which the photoreactivity of the hypothetical nanomaterial may influence acute inhalation toxicity, therefore, should be considered. (While an inhaled nanoparticle would obviously not be illuminated *in situ*, photoreactivity that is enhanced by illumination just before inhalation may have an effect after inhalation.) The size of the nanoparticles also needs to be considered, as that may impact the potential reactivity or, specifically for inhalation exposure, the potential deposition within the lung.
No acute inhalation toxicity data of sufficient quality, as determined by SCCS, were documented in the SCCS (2014a) report. However, NIOSH (2011) summarised some acute inhalation toxicity studies. In one such study, mice were exposed to fine titanium dioxide (2–5 nm primary particle size) for 4 hours. No adverse effects were observed in the mice (Grassian et al., 2007). But no other details were provided on the study nor was it stated whether or not it was conducted to standard methodology. In another acute study summarised by NIOSH (2011), rats were exposed to either fine (1 µm particle size) or ultrafine (21 nm particle size) titanium dioxide via inhalation (Nurkiewicz et al., 2008). No evidence of pulmonary inflammation or lung damage was noted; however, systemic microvessel dysfunction was observed. These studies provide some information on the potential acute toxicity of titanium dioxide following inhalation exposure; but because sufficient details were not provided on the methodology, the studies would need to be evaluated further for reliability for REACH purposes.
No acute dermal toxicity data of sufficient quality, as determined by SCCS, were described in the SCCS (2014a) report.
Acute toxicity data obtained in accordance with standard methodologies following oral exposure are available as summarised in SCCS (2014a). Based on those data, nanotitanium dioxide does not appear to be acutely toxic following exposure by the oral route.
Because insufficient acute inhalation and dermal toxicity data were available for potential read-across, acute toxicity testing may need to be conducted for these routes of exposure. However, acute oral toxicity data are potentially available for read-across. The impact of size, the crystalline structure and manganese doping would need to be considered if these acute oral data were used for read-across.

Skin irritation, eye irritation, skin sensitisation

Because the key nanomaterial characteristics for these endpoints are similar, they will be assessed together for the purposes of this case study. The key characteristics to consider include composition (crystalline structure), reactivity/photoreactivity, solubility and pH. The available data on nanotitanium dioxide from SCCS (2014a) are presented in Table 19.

Table 19 Summary of Irritation and Sensitisation Data

Endpoint	Animal	Particle Size (nm)	Crystalline Form	Result
Skin irritation	Rabbit	49	85 % Anatase, 15 % rutile	Non-irritant
Skin irritation	Rabbit	49	85 % Anatase, 15 % rutile	Non-irritant
Eye irritation	Rabbit	49	85 % Anatase, 15 % rutile	Non-irritant
Eye irritation	Rabbit	49	85 % Anatase, 15 % rutile	Non-irritant
Skin sensitisation	Guinea pig	49	85 % Anatase, 15 % rutile	Non-sensitizer
Skin sensitisation	Guinea pig	49	85 % Anatase, 15 % rutile	Non-sensitizer

As presented in SCCS (2014a), nanotitanium dioxide is not irritating to the skin or eyes or sensitising to the skin. Because irritation and sensitisation data are available, they may potentially be used for read-across. Because of the exposure to UV light, the potential effects of photoreactivity on the skin and eyes are of concern. The photoreactivity of the hypothetical nanomaterial would need to be evaluated in the testing strategy to determine whether it would likely be more reactive than that of the nanomaterials for which data are currently available.

Mutagenicity

The mutagenicity/genotoxicity data on nanotitanium dioxide from SCCS (2014a) are summarised in Table 20. Based on the available *in vitro* and *in vivo* mutagenicity/genotoxicity data, SCCS (2014a) concluded that "the potential to cause DNA damage has been clearly demonstrated for some TiO_2 nanomaterials" and, as such, "TiO_2 nanoparticles have to be considered genotoxic".
Because data are available for the genotoxicity endpoint, read-across may be considered. However, to support read-across for other endpoints and establish equivalency to tested nanotitanium dioxide materials, *in vitro* genotoxicity testing may be considered. It would be important to consider any potential compatibility issues with the specific *in vitro* assays used.

Table 20 Summary of Mutagenicity/Genotoxicity Data

Test	*In vitro / in vivo*	Particle Size (nm)	Crystalline Form	Result
Bacterial Gene Mutation Assay	*In vitro*	49	85 % anatase, 15 % rutile	Negative
Bacterial Gene Mutation Assay	*In vitro*	49	85 % Anatase, 15 % rutile	Negative

Test	*In vitro / in vivo*	Particle Size (nm)	Crystalline Form	Result
Bacterial Gene Mutation Assay	*In vitro*	10 x 50 nm; mean agglomerates 200 nm	100 % Rutile	Negative
Chromosome aberration test in mammalian cells	*In vitro*	49	85 % anatase, 15 % rutile	Negative
Chromosome aberration test in mammalian cells	*In vitro*	49	85 % Anatase, 15 % rutile	Negative
Micronucleus test in human epidermal cells	*In vitro*	Not available	Anatase, 99.7 %	Positive
Fpg modified Comet assay in human epidermal cells	*In vitro*	Not available	Anatase, 99.7 %	Positive
Comet assay in human lymphocytes	*In vitro*	Not available	Not Reported	Positive
Mammalian cell gene mutation test	*In vitro*	5 and 40	Anatase	Positive
Micronucleus test in mammalian cells	*In vitro*	10 x 50 nm; mean agglomerates 200 nm	Rutile	Negative
Alkaline Comet assay in mammalian lung cells	*In vitro*	12, 21, 24, 68, and 142	Multiple forms	Positive
Alkaline Comet assay in mammalian liver cells	*In vitro*	9, 10, 100	Multiple forms	Positive
Micronuclei in peripheral blood erythrocytes after oral uptake	*In vivo*	21	85 % anatase, 15 % rutile	Positive
DNA double strand breakage in bone marrow cells after oral uptake	*In vivo*	21	85 % anatase, 15 % rutile	Positive
Comet assay *in vivo* in rat lungs	*In vivo*	10 x 50 nm; mean agglomerates 200 nm	Rutile	Positive

Repeated dose toxicity

The repeated dose toxicity data on nanotitanium dioxide from SCCS (2014a) are summarised in Table 21.

Table 21 Summary of Repeated Dose Toxicity Data

Route	Duration	Species	Particle Size (nm)	Crystalline Form	Results
Intragastric	30 days	CD-1 Mice	5	Anatase	NOAEL: 62.5 mg/kg bw/d.
Intragastric	60 days	CD-1 Mice	5	Anatase	LOAEL: 5 mg/kg bw/d

The only relevant repeated dose toxicity data as presented by SCCS (2014) were following oral exposure. However, the oral route is not relevant for the hypothetical nanotitanium dioxide material. Rather, for this hypothetical nanomaterial, both the inhalation and dermal routes of exposure are relevant.

As noted previously, systemic toxicity is less of a concern for the dermal route than it is for either the oral or inhalation routes of exposure because significant dermal absorption into the systemic circulation has not been observed following dermal exposure to nanomaterials. However, because the hypothetical nanomaterial is photocatalytic, potential local effects following repeated exposure should be considered. The SCCS (2014a) report noted that most dermal absorption studies on titanium dioxide have indicated that nanoparticles are not able to penetrate live cells of the epidermis/dermis; however, they may penetrate into the stratum corneum, in hair follicles and/or sweat glands. If the titanium dioxide nanoparticles have significant photocatalytic activity, there is a potential that those particles that remain in the stratum corneum, in hair follicles and/or sweat glands may produce reactive oxygen species (ROS) and result in long-term effects. The photocatalytic properties of the hypothetical nanomaterial, therefore, should be evaluated further in the testing strategy. Without data available for read-across for repeated dose toxicity following dermal exposure along with the potential concerns on the photoreactivity over time, testing may be necessary.

NIOSH (2011) summarised available repeated dose toxicity studies on titanium dioxide following inhalation exposure. These data represent both non-nanoforms (fine) as well as nanoforms (ultrafine) of titanium dioxide. In a 13-week inhalation toxicity study with ultrafine titanium dioxide (21 nm primary particle size) in female rats, mice and hamsters exposed to 0.5, 2 or 10 mg/m^3, retardation of pulmonary clearance and pulmonary inflammation following exposure to 10 mg/m^3 was observed in rats and mice, but not in hamsters (Bermudez et al., 2004). Furthermore, in rats exposed to 10 mg/m^3, epithelial and fibroproliferative changes, interstitial particle accumulation and alveolar septal fibrosis were observed. In addition, at 52 weeks post-exposure,

minimal to mild metaplastic changes and minimal to mild particle induced alveolar septal fibroplasia were seen in rats. No epithelial, metaplastic or fibroproliferative changes were observed in mice or hamsters. In a 2-year chronic inhalation toxicity study, female rats were exposed to 10 mg/m^3 of ultrafine titanium dioxide (80 % anatase, 20 % rutile; 15-40 nm primary particles size) (Heinrich et al., 1995). Following exposure for 6 months, 99/100 of the rats had developed bronchial hyperplasia and following 2 years all rats had developed slight to moderate interstitial fibrosis. Similar effects were also reported in other studies summarised in the NIOSH (2011) report for non-nanotitanium dioxide.
Based on these data, NIOSH (2011) concluded that any potential effects in the lungs following repeated exposure to titanium dioxide are likely elicited through chronic pulmonary inflammation. Because nanotitanium dioxide particles are poorly soluble, at sufficiently high particle mass or surface area dose they have the potential to remain in the lungs, resulting in pulmonary inflammation, oxidative stress and, potentially, tissue damage (NIOSH, 2011). Because the effects following repeated inhalation exposure to both nanotitanium dioxide and non-nanotitanium dioxide are similar and the proposed mechanism of action is also similar, read-across to the available data would likely be appropriate. Confirmation of particle size distribution and solubility in the testing strategy can help substantiate read-across for this endpoint.

Carcinogenicity

No standard guideline carcinogenicity assays were specifically available on nanotitanium dioxide as presented in SCCS (2014a). However, a number of non-standard tumour-promoting activity studies were conducted on nanotitanium dioxide and summarised by SCCS (2014a), including 2 two-stage skin carcinogenicity studies with mice, 2 two-stage skin carcinogenicity studies with rats and one two-stage lung study with rats. The SCCS concluded that it was difficult to draw any conclusions from the skin studies. However, based on the rat lung data, the SCCS (2014a) concluded that non-coated nanotitanium dioxide showed promoter activity.
In addition to the carcinogenicity data presented in the SCCS (2014a) report, carcinogenicity data were summarised in both NIOSH (2011) and IARC (2010). Two key studies were summarised. In the first study, rats were exposed for two years to fine titanium dioxide particles (rutile; aerodynamic mass median diameter of 1.5-1.7 µm) via inhalation at doses of 10, 50 or 250 mg/m^3 (Lee et al., 1985). An increase in lung tumours was not observed following exposure to 10 or 50 mg/m^3; however, following exposure to 250 mg/m^3, an increase in lung tumours was observed. In the second study, female rats and mice were exposed to 10 mg/m^3 ultrafine titanium dioxide (80 % anatase, 20 % rutile; 15-40 nm primary particles size) (Heinrich et al., 1995). Following exposure for 6 months, 99/100 of the rats had developed bronchial hyperplasia, and following 2 years all rats had developed slight to moderate interstitial fibrosis. Following two years of exposure and six

months post-exposure, a statistically significant increase in adenocarcinomas was observed in rats. However, an elevated tumour response was not observed in mice. Based on these data, NIOSH (2011) has concluded that "TiO_2 is not a direct-acting carcinogen, but acts through a secondary genotoxicity mechanism that is not specific to TiO_2, but primarily related to particle size and surface area". Furthermore, "on the basis of the study by Heinrich et al. (1995) and the pattern of pulmonary inflammatory responses, NIOSH has determined that exposure to ultrafine TiO_2 should be considered a potential occupational carcinogen".
Based on the availability of carcinogenicity data for nanotitanium dioxide and the proposed mechanism of action by NIOSH (2011), read-across may be warranted. Confirmation of particle size distribution and solubility can help substantiate read-across for this endpoint.

10.2.2.3 Testing Strategy

Table 22 summarises the human health endpoints for which read-across may be considered in developing a testing strategy for this hypothetical substance.

Table 22 Human health endpoints for which read-across may be considered

Endpoint	**Sufficient Data Available for Read-across**
Acute toxicity	Yes; Acute oral toxicity data are available for read-across
Skin irritation	Yes
Eye irritation	Yes
Skin sensitisation	Yes
Mutagenicity	Yes
Repeated dose toxicity	Yes
Carcinogenicity	Yes

Tier 0 Testing

The available information on the hypothetical nanotitanium dioxide material indicates that the primary particle size is up to 100 nm. But further testing is needed to confirm the specific size range of the particles. The size range of the nanoparticles should be determined at manufacture and at the potential time of exposure. As discussed in previous sections of this report and re-emphasised by the SCCS (2014a), the particle size can impact the potential deposition within the lungs, with particles >10 µm generally depositing in the extrathoracic region of the lungs. While nanoparticles also mainly deposit in the extrathoracic region, particles in the size range of 300-200 nm down to 3-2 nm may also deposit in the alveolar region.
Although nanotitanium dioxide particles are typically poorly soluble and this hypothetical nanomaterial would likely also be poorly soluble, the solubility should be confirmed.

Tier 1 testing

While the pH of a suspension of the titanium dioxide nanomaterial is not expected to be in the range that would dictate classification as a corrosive, the pH of the nanomaterial should be evaluated.

Tier 2 testing

The Tier 2 testing involves any *in vitro* assays that are required under the regulatory framework and/or may support read-across for *in vivo* data.
Because photocatalytic activity is an important property of this hypothetical nanomaterial and could impact potential toxicity, especially for the dermal route of exposure, the photocatalytic activity should be tested. However, there currently is not a standard method for the evaluation of photocatalytic activity. The SCCS (2014a) report did reference a non-standard method described by Egerton et al. (2007). But because this method is not standard, it would need to be further evaluated for acceptability under REACH. Based on their evaluation, the SCCS concluded that a photocatalytic activity of within 10 % of non-coated or non-doped reference would be acceptable with respect to the cosmetics applications of nanotitanium dioxide. While this case study reflects a different form of dermal exposure (i.e. workers applying this hypothetical product to a surface), the SCCS determination still offers a useful benchmark. Determining whether the hypothetical nanomaterial has a photocatalytic activity within 10 % of a non-doped or coated form would help determine whether local effects may be of concern upon dermal exposure and whether the hypothetical nanomaterial is substantially similar to those nanotitanium dioxide materials for which read-across data are available.
At the proposed tonnage, *in vitro* irritation and *in vitro* mutagenicity assays would be required. Skin and eye irritation data are available for read-across. The results of the Tier 0 and Tier 1 testing, along with the photocatalytic testing, should be evaluated to determine whether read-across to these data is warranted.
Genotoxicity data are also currently available for read-across. The results from these data suggest that titanium dioxide is genotoxic. While the data collected in Tiers 0 and 1 may support the use of read-across for the genotoxicity/mutagenicity endpoint, conducting *in vitro* genotoxicity assays may help provide additional weight of evidence to substantiate read-across for other endpoints. If conducting *in vitro* testing, any potential compatibility issues with nanotitanium dioxide and these types of *in vitro* assays should be considered before pursuing testing.

Tier 3 Testing

Based on the results of the Tier 2 testing, additional *in vivo* testing may be warranted. The specific *in vivo* testing should take into account the endpoints that need to be considered for the regulatory framework, as well as whether the data can be filled through read-across.

Based on the tonnage of the nanomaterial, acute toxicity testing would be required. As dictated by the regulation, acute oral toxicity testing is required and, potentially, another route based on the likely routes of exposure. Acute oral toxicity data are available for nanotitanium dioxide that could be used for read-across. The data developed in the previous Tiers can be used to support potential read-across for acute oral toxicity. Both the inhalation and dermal routes of exposure are relevant for this material. As specified under REACH, if the inhalation route of exposure is relevant, acute inhalation toxicity testing would be conducted rather than acute dermal toxicity. While some acute toxicity data are available for the inhalation route of exposure, the data do not appear to be sufficient for use under the REACH regulation. Acute inhalation toxicity data may therefore need to be developed.
Repeated dose toxicity data and carcinogenicity data for the inhalation route of exposure are available for read-across. The data generated in Tiers 0-2, particularly the particle size and solubility information, should be used to confirm that read-across is appropriate for these endpoints. Although the dermal route of exposure is relevant for this nanomaterial, significant dermal absorption is not expected. Systemic toxicity following repeated exposure to the dermal route, therefore, is not of concern. But because the hypothetical nanotitanium dioxide material is photocatalytic and there is a potential for local effects after repeated exposure, repeated dose toxicity testing for the dermal route may be warranted.

11 Summary and Conclusions

This project began with a review of the literature in order to inventory the nano-specific characteristics that may be essential in the development of read-across concepts and grouping criteria, as these may affect the kinetics and fate of nanomaterials and the hazards and risks they present for humans and the environment. The summary of literature pertaining to environmental concerns began with a discussion of environmental fate and transport, then continued with a discussion regarding toxicity to organisms in the environment and discussed what is known about the mechanisms of toxicity in general (across trophic levels) and current uncertainties. That information was presented graphically by trophic level. Similarly, the summary of human health effects and critical characteristics provided an overview of what is currently known about toxicokinetics, and then discussed toxicity with respect to port-of-entry effects and the general mechanisms of toxicity. Table 23 provides a brief summary of these findings.

The development of a read-across or grouping scheme, by its nature, requires one to make generalisations about the behaviours of nanomaterials. The research described in this report did identify some parameters common to the behaviour and effects of many nanomaterials, as summarised in Figure 17. But the importance of other parameters seems to be particular to a subset of nanomaterials. For example, many authorities note that the crystalline phase of an inorganic nanomaterial may influence its behaviour. Based on the current literature, however, that factor appears to be of primary importance for titanium dioxide, which may be used in the anatase or rutile form, rather than a generalisation that commonly applies to a broad suite of inorganic nanomaterials. Similarly, some authorities cite the rigidity of a particle as an important factor that contributes to toxicity. However, that parameter may only be important for certain needle-like particles such as carbon nanotubes.

As evident throughout this report, the body of knowledge about the behaviour of nanomaterials and the effects of exposure to nanomaterials is growing rapidly. Research may soon begin to explain some of the more puzzling findings described in this report and apparent contradictions between the results of different experiments. Research may also uncover new modes of action; for example, scientists have posited that toxic effects on the gut biome may be of concern for both fish and humans, but the data are not yet available to fully explore that concern. As research continues, views regarding the critical parameters may change.

Table 23 Synopsis of Key Findings

Phenomenon	Environment	Ecological Receptors (Algae, Daphnia, Fish)	Human Health
Transport	Particle size determines buoyancy, which keeps particles in suspension. Van der Waals forces (Hamaker constant) cause particles to agglomerate, which increases particle size and contributes to sedimentation. Particle charge can cause electrostatic repulsion that hinders agglomeration. The rate of dissolution, which can increase with decreasing particle size, can affect how the substance is transported. Biodegradation of a surface coating or biomodification of functional groups can change the behaviour of a nanomaterial.	Within higher organisms, formation of a protein corona or, more generally, sorption to biological compounds influences the fate and effects of nanomaterials. This phenomenon depends in part on the particle charge, which may reflect the identity of the substance and its coating or functionalisation. Biodegradation of a surface coating or biomodification of functional groups can change the behaviour of a nanomaterial. The rate of dissolution, which can increase with decreasing particle size, can affect how the substance is transported within the organism.	Formation of a protein corona or, more generally, sorption to biological compounds influences the fate and effects of nanomaterials. This phenomenon depends, in part, on the particle charge, which may reflect the identity of the substance and its coating or functionalisation. Biodegradation of a surface coating or biomodification of functional groups can change the behaviour of a nanomaterial. The rate of dissolution, which can increase with decreasing particle size, can affect how the substance is transported within the body. Particle size determines the zone of deposition of inhaled nanoparticles in the lungs and can influence translocation within the body. Translocation through the epithelial barrier in the lungs or gastrointestinal tract depends on particle size and surface charge, the latter of which may reflect the identity of the substance and its coating or functionalisation.

Phenomenon	Environment	Ecological Receptors (Algae, Daphnia, Fish)	Human Health
Sorption	Sorption to natural organic matter can keep particles in suspension. Sorption to soil or sediments can sequester nanomaterials	Sorption to aquatic organisms may cause algae to agglomerate or block light and thereby hinder photosynthesis; physically hinder the movement of daphnia; or in fish, hinder the functioning of the gills. These physical impairments would occur at relatively high concentrations of nanomaterials that might not occur frequently under environmental conditions. Within higher organisms, formation of a protein corona or, more generally, sorption to biological compounds influences the fate and effects of nanomaterials. This phenomenon depends, in part, on the particle charge, which may reflect the identity of the substance and its coating or functionalisation.	Formation of a protein corona or, more generally, sorption to biological compounds influences the fate and effects of nanomaterials. This phenomenon depends, in part, on the particle charge, which may reflect the identity of the substance and its coating or functionalisation.

Phenomenon	Environment	Ecological Receptors (Algae, Daphnia, Fish)	Human Health
Cell entry	---	Surface interactions with sorbed nanoparticles can pit the cell wall and may increase the size of pores in the cell wall. (Given the small size of those pores, they would otherwise block all but the smallest nanomaterials.) Needle-shaped particles can pierce the cell membrane to a degree that may depend on the diameter and the rigidity of the particle. Endocytosis depends, in part, on the particle size.	Needle-shaped particles can pierce the cell membrane to a degree that may depend on the diameter and the rigidity of the particle. Nanoparticles may damage the cell membrane to an extent that depends on particle size; particle charge influences the mechanism by which the membrane is damaged. Disruption of the cytoskeleton may occur and be influenced by the particle composition, size, shape and surface modifications. Smaller particles are more readily taken up via endocytosis. Particle charge (which can reflect the material itself, the coating or functionalisation) can also affect endocytosis; though whether a positive or negative charge enhances endocytosis depends on the type of cell.

Phenomenon	Environment	Ecological Receptors (Algae, Daphnia, Fish)	Human Health
Reactivity	The reactivity/photoreactivity of a nanomaterial may change with particle size.	The following reactions may damage DNA, interfere with cellular functions or cause cell death: ▪ Generation of reactive oxygen species (ROS) ▪ Redox reactions with biological molecules, under certain circumstances ▪ Trojan horse toxicity	Nanoparticles may be able to enter cell nuclei and bind to DNA, depending on particle size and charge. Nanoparticles may disrupt cell functions by binding to cellular proteins. Generation of ROS may cause cell damage, by one of several mechanisms: ▪ Reactivity of the particle itself or its surface coating, ▪ The presence of a particle within a cell may trigger the cell's production of ROS.

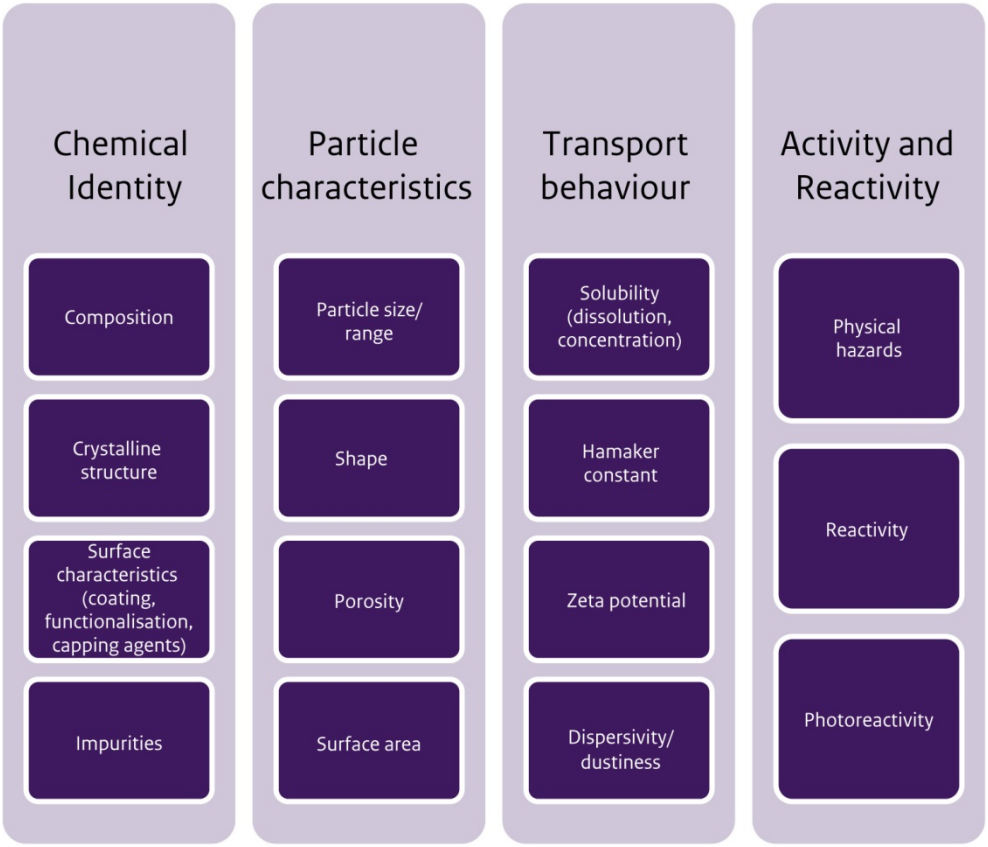


Figure 17 Critical Parameters

Efforts to develop a testing strategy from this basis soon encounter two obstacles. Firstly, standardised test methods are not available to measure certain critical parameters. OECD technical guidelines are under development for several critical parameters. Other measurements may be available on an experimental basis, e.g. in research laboratories. Secondly, few comprehensive and publicly available compilations of data on nanomaterials are available to test concepts related to read-across and grouping. The OECD Sponsorship Programme will produce comprehensive information on 11 nanomaterials, but as yet limited data are available. Several other data compilations were reviewed in this study and publicly available data compilations were utilised for two case studies.

Other parties have begun to propose schemes for read-across and the grouping of nanomaterials, although authorities concede the difficulty in arriving at definitive concepts given the state of the science. These schemes represent different concepts about how to group and test nanomaterials:

- ITS Nano focused on the functionality of nanomaterials (i.e. what they are, what they do and where they go);
- The Nano Safety Cluster Working Group 10 focused their testing scheme on effects, indicating that nanomaterials could be grouped based on (a) similar biopersistence, biokinetics and/or bioeffects, or (b) by concern.
- The Regulatory Cooperation Council (RCC) focused on chemical identity, specifically similarities in chemical composition.

- The Nanomaterial Registry determines similarity based on four specific physico-chemical properties: size, shape, material type and surface charge, as represented by the isoelectric point.

Two precedential determinations made in December 2014 under REACH are also relevant to this work. Firstly, and this is proposed but not definitive as yet, REACH may reportedly be modified in 2015 to require, among other things, that the dossier for a nanomaterial include information on the particle size distribution, surface treatment, shape, morphology and surface area. Secondly, ECHA's Member State Committee adopted the first substance evaluation decision for a nanomaterial under the Community Rolling Action Plan (CoRAP). This decision affirmed the finding by RIVM that two properties are critical to grouping silicon dioxide nanoforms: surface area and surface functionalisation (or more specifically, the level of hydroxylation).
In light of these findings, but proceeding a priori from information in the literature on the mode of action of nanomaterials, the physico-chemical characteristics of nanomaterials and the kinetic profile of nanomaterials, the project team developed a framework for testing nanomaterials and assessed the framework using the nanoforms of silver and titanium dioxide as illustrative cases.
The framework described in this report follows a logical sequence of activities: compile known information, develop a hypothesis as to whether or not to expect unique behaviour or effects as a result of nanosizing a material, perform tiered testing, and assess the results. This process might be iterative as a project team accumulates information for different endpoints and evaluates it based on the weight of evidence.
The three-tiered testing scheme described in this report reflects increasingly complex testing:

- Tier 1: basic physico-chemical properties. Tier 1 testing would provide fundamental material characterisation; data to develop the scope of Tier 2 and Tier 3 tests; and support read-across.
- Tier 2: explore the behaviour of a nanomaterial, in particular with respect to the rate or degree of dissolution in a complex and biologically or environmentally relevant medium or with respect to reactivity. Includes *in vitro* testing. Tier 2 testing would typically support read-across. For some endpoints such as mutagenicity, Tier 2 testing might provide sufficient data so that no Tier 3 testing was necessary.
- Tier 3: bioassays and animal testing.

This logical framework breaks down into two aspects of practical implementation. Firstly, while scientists have identified many of the physico-chemical parameters that affect environmental fate and transport and (eco)toxicity, that knowledge is neither complete nor quantified. One cannot yet simply compare the physico-chemical characteristics of a nanomaterial to those of its non-nanoform (for example) and draw reliable quantitative conclusions about dose response unless working at the extremes of behaviour. Such extremes of behaviour might mean, for example, that exposure to a sufficient dose of a nanoform of a highly toxic substance would result in severe effects. Another example, for which it might be relatively simple to draw conclusions, would be an effect that is related to aspect ratio resulting from inhaling needle-like particles.

The need to assess whether a nanomaterial might have unique behaviour and to relate such behaviour to (eco)toxicity leads to the inclusion of Tier 2 testing in the framework and to the second of the two aspects where the framework breaks down. In concept, Tier 2 tests would characterise the behaviour of a nanomaterial in a way that would aggregate the changes in physico-chemical parameters relating from nanosizing. The focus of Tier 2 testing in this report was on understanding effects on solubility (which influences bioavailability and biopersistence) and reactivity. But standardised methods for many of those tests do not yet exist, nor do commercial laboratories routinely offer many such tests. In this regard, the tiered framework proposed in this report is more forward-thinking than immediately implementable. Despite these limitations, the developing body of knowledge about the behaviour of nanomaterials will enable some expert judgement regarding the effects of exposure to nanomaterials. This judgement may support read-across in some cases and in others support the thoughtful adaptation of Tier 3 tests to nanomaterials.

Two case studies applied this testing framework to hypothetical products comprising nanosilver and nanotitanium dioxide. Hypothetical case studies of nanomaterials represent a paradox: without available data, one cannot test a conceptual framework; however, relatively well-studied nanomaterials are difficult to adapt in a believable way to create a hypothetical case.

The hypothetical nanosilver product was a liquid suspension of citrate-coated nanoparticles with a primary particle size in the range 20 to 40 nm. While the citrate coating was expected to limit agglomeration, the actual (aged) particle size was unknown.

With respect to ecotoxicological endpoints, the presence and availability of a coating can influence the availability of nanosilver. Read-across to data for other citrate-coated silver nanoparticles, therefore, could be appropriate. Literature reports of the acute toxicity of similarly-sized silver nanoparticles to fish and aquatic invertebrates.

The relevance of read-across to such data would depend on several factors. Under conditions and timeframes relevant to ecotoxicity testing, one should determine the agglomerated particle size/surface area of the nanomaterial to be tested and its rate of dissolution. Data on the particle shape may also be relevant to determining whether read-across is appropriate. Finally, valid read-across requires that the test media be well-documented and understood because of the strong influence of the nature of the medium on the behaviour of the nanomaterial undergoing testing.

In this hypothetical case, such additional data might support read-across to literature reports. In the case of uncertainty, ecotoxicity testing could perhaps be focused on one endpoint. Testing results obtained by Kennedy et al. (2010) indicate that *Daphnia magna* is more sensitive to citrate-coated silver nanoparticles than fish (*Pimephales promelas*). It would therefore be advisable to perform an additional study for the nanomaterial of interest, with *Daphnia magna* as the test species. If the LC_{50} value obtained from this Tier 3 testing were comparable to values reported in the literature, then a short-term toxicity test to fish should not be performed. Using a weight-of-evidence approach on all data and information gathered, read-across between the nanomaterials can then be considered.

The testing strategy for human health endpoints reflected the consideration that the main human health concern with regard to silver

is the ionic form, rather than the particulate form. Furthermore, the citrate coating itself is not anticipated to be toxic.
To elucidate read-across to the ionic form of silver, it would be important to test the agglomerated particle size/surface area of the nanomaterial to be tested and its rate of dissolution in biologically relevant fluids. Such data could contribute to the weight of evidence for read-across.
Based on the information available for the hypothetical nanomaterial, potential read-across substances reported in the literature and the testing requirements under REACH, one might also test mutagenicity *in vitro* and *in vivo* toxicokinetics. Depending on these results and the weight of evidence to support read-across, one might also test mutagenicity *in vivo* and acute toxicity.
In the second case study, nanotitanium dioxide was evaluated only with respect to human health endpoints. The hypothetical product was a liquid suspension of manganese-doped titanium dioxide particles in polyurethane that dried after coating. The primary particle size is generally up to 100 nm.
The likely routes of exposure to this substance would be by dermal contact or inhalation. The particles would be unlikely to penetrate the skin. Deposition in the lungs upon inhalation would depend on the particle size. The effects of exposure, whether on the surface of the skin or in the lungs, would depend in great part on the reactivity/photoreactivity of the particle. The anatase crystalline form might enhance reactivity and the potential for the manganese dopant to enhance the reactivity of the titanium dioxide particles would be of concern.
The testing strategy would therefore focus initially on characterising the particle size and understanding the degree of enhanced reactivity/photoreactivity; to the extent test methods are available. Understanding the solubility of the substance in physiologically relevant fluids would add to the weight of evidence regarding the availability of the dissolved substance.
At the proposed tonnage, *in vitro* irritation and *in vitro* mutagenicity assays would be required. Skin and eye irritation data are available for read-across, as are genotoxicity data that suggest that titanium dioxide is genotoxic. The physico-chemical testing described above would help to substantiate read-across. In addition, *in vitro* genotoxicity assays may help provide additional weight of evidence to substantiate read-across for other endpoints. If conducting *in vitro* testing, any potential compatibility issues with nanotitanium dioxide and these types of *in vitro* assays should be considered before pursuing testing.
Based on the results of the Tier 2 testing, additional *in vivo* testing may be warranted. The specific *in vivo* testing should take into account the endpoints that need to be considered for the regulatory framework, as well as whether the data can be filled through read-across. Based on the tonnage of the nanomaterial, acute toxicity testing would be required. Acute oral toxicity data are available for nanotitanium dioxide that could be used for read-across if supported by the data described above. Both the inhalation and dermal routes of exposure are relevant for this material. As specified under REACH, if the inhalation route of exposure is relevant, then acute inhalation toxicity testing would be conducted rather than acute dermal toxicity. While some acute toxicity data are available for the inhalation route of exposure, the data do not appear to

be sufficient for use under the REACH regulation. Acute inhalation toxicity data may therefore need to be developed.
Repeated dose toxicity data and carcinogenicity data for the inhalation route of exposure are available for read-across. The data generated as described above, particularly the particle size and solubility information, should be used to confirm that read-across is appropriate for these endpoints.
Although the dermal route of exposure is relevant for this nanomaterial, significant dermal absorption is not expected. Systemic toxicity following repeated exposure to the dermal route is therefore not of concern. But because the hypothetical nanotitanium dioxide material is photocatalytic and there is a potential for local effects after repeated exposure, repeated dose toxicity testing for the dermal route may be warranted.
In summary, and as illustrated by these case studies, grouping and read-across do offer the potential to minimise the testing needed for nanomaterials. But, as yet, the science does not allow for a straightforward algorithm for defining structure-activity relationships or grouping nanomaterials. Research to date points to certain characteristics as critical: chemical identity, particle characteristics, parameters that characterise fundamental transport behaviours, and activity and reactivity. Our ability to normalise an effect to particle size may be one key to read-across; another may be tests, some under development, that allow scientists to gauge whether a nanomaterial may behave or react differently under relevant conditions than would a read-across substance. Such data, in combination with more conventional parameters, may enable one to read-across from a nanoparticle to an ionic form (as in the case of nanosilver) or to other nanoparticles (as illustrated for nanosilver and nanotitanium dioxide) and thus minimise testing.

12 References

Allen, H.J., Impellitteri, C.A., Macke, D.A., Heckman, J.L., Poynton, H.C., Lazorchak, J.M., Govindaswamy, S., Roose, D.L., Nadagouda, M.N., 2010. Effects from filtration, capping agents, and presence/absence of food on the toxicity of silver nanoparticles to *Daphnia magna*. Environ. Toxicol. Chem. 29(12): 2742-2750.

Amiano, I., Olabarrieta, J., Vitorica, J., Zorita, S., 2012. Acute toxicity of nanosized TiO_2 to *Daphnia magna* under UVA irradiation. Environ. Toxicol. Chem. 31 (11): 2564-2566.

Arora, S., Rajwade, J.M., Paknikar, M., 2012. Nanotoxicology and *in vitro* studies: The need of the hour. Toxicol. Appl. Pharmacol. 258: 151-165.

Asghari, S., Johari, S.A., Lee, J.H., Kim, Y.S., Jeon, Y.B., Choi, H.J., Moon, M.C., Yu, I.J., 2012. Toxicity of various silver nanoparticles compared to silver ions to *Daphnia magna*. J. Nanobiotechnol. 10: 14.

ASTM International, 2006. Designation: E 2456 – 06. Standard Terminology Relating to Nanotechnology.

Auffan, M., Rose, J., Bottero, J.-Y., Lowry, G.V., Jolivet, J.-P., Wiesner, M.R., 2009. Towards a definition of inorganic nanoparticles from an environmental, health and safety perspective. Nat. Nanotechnol. 4: 634-641.

Bakand S., Hayes A., Dechsakulthorn F., 2012. Nanoparticles: a review of particle toxicology following inhalation exposure. Inhal. Toxicol. 24(2): 125-135.

Baun, A., Hartmann, N.B., Grieger, K., Kusk, K.O., 2008. Ecotoxicity of engineered nanoparticles to aquatic invertebrates: a brief review and recommendations for future toxicity testing. Ecotoxicology 17: 387-395.

Bergin, I.L., Witzmann, F.A., 2013. Nanoparticle toxicity by the gastrointestinal route: evidence and knowledge gaps. Int. J. Biomed. Nanosci. Nanotechnol. 3 (1-2): 1-44.

Bermudez, E., Mangum, J.B., Wong, B.A., Asgharian, B., Hext, P.M., Warheit, D.B., Everitt, J.I., 2004. Pulmonary responses of mice, rats and hamsters to subchronic inhalation of ultrafine titanium dioxide particles. Toxicol. Sci. *77*:347–357.

Bilberg, K., Hovgaard, M.B., Besenbacher, F., Baatrup. E. 2012. *In vivo* toxicology of silver nanoparticles and silver ions in zebrafish (*Danio rerio*). J. Toxicol. 2012: 293784.

Bleeker, E.A.J., de Jong, W.H., Geertsma, R.E., Groenewold, M., Heugens, E.H.W., Koers-Jacquemijns, M., van de Meent, D., Popma, J.R., Rietveld, A.G., Wijnhoven, S.W.P., Cassee, F.R., Oomen, A.G., 2013. Considerations on the EU definition of a nanomaterial: Science to support policy making. Regul. Toxicol. Pharmacol. 65: 119–125.

Bondarenko, O., Juganson, K., Ivask, A., Kasemets, K., Mortimer, M., Kahru, A., 2013. Toxicity of Ag, CuO and ZnO nanoparticles to selected environmentally relevant test organisms and mammalian cells *in vitro*: a critical review. Arch. Toxicol. 87: 1181-1200.

Borm, P., Klaessig, F.C., Landry, T.D., Moudgil, B., Pauluhn, J., Thomas, K., Trottier, R., Wood, S., 2006. Research Strategies for Safety Evaluation of Nanomaterials, Part V: Role of Dissolution in

Biological Fate and Effects of Nanoscale Particles. Toxicol, Sci. 90(1): 23–32.
Braakhuis, H.M., Park, M.V.D.Z., Gosens, I., De Jong, W.H., Cassee, F.R., 2014. Physico-chemical characteristics of nanomaterials that affect pulmonary inflammation. Part. Fibre Toxicol. 11:18.
Burello, E., Worth, A.P., 2011. A theoretical framework for predicting the oxidative stress potential of oxide nanoparticles. Nanotoxicology 5(2): 228-235.
Byrne, H.J., Oomen, A.G., Bos, P.M.J., Fernandes, T.F., Hund-Rinke, K., Boraschi, D., Aschberger, K., Gottardo, S. von der Kammer, F., Kühnel, D., Hristozov, D., Marcomini, A., Migliore, L., 2014. Concern-Driven Integrated Toxicity Testing Strategies for Nanomaterials-Report of the NanoSafety Cluster Working Group 10. Available at: http://arrow.dit.ie/biophonart/7 (accessed April 2014); may also be cited as:
"Concern-driven integrated toxicity testing strategies for nanomaterials - Report of the NanoSafety Cluster Working Group 10", Agnes Oomen, Peter Bos, Teresa Fernandes, Kerstin Hund-Rinke, Diana Boraschi, Hugh J. Byrne, Karin Aschberger, Stefania Gottardo, Frank von der Kammer, Dana Kühnel, Danail Hristozov, Antonio Marcomini, Lucia Migliore, Janeck Scott-Fordsmand, Peter Wick and Robert Landsiedel, Nanotoxicology, 14: 195-216 (2014) doi:10.3109/17435390.2013.802387.
CEFIC, 2013?. Experts Workshop on Read-Across Assessment organised by ECHA with the active support from Cefic-LRI (October 3, 2012) - Use of "read-across" for Chemical Safety Assessment under REACH - Workshop Report. Available at: http://www.cefic-lri.org/uploads/Events%202012/ECHA-Cefic%20LRI%20Read-across%20Workshop%20Report_171211%20FINAL.pdf (accessed 17 April 2014).
Chemical Watch, 2014. US-Canada project develops nano classification scheme. 15 January 2014.
Choi, J-Y., Ramachandran, G., Kandlikar, M., 2009. The Impact of Toxicity Testing Costs on Nanomaterial Regulation. Environ. Sci. Technol. 43(9): 3030-3034.
Davies, E., 2014. ECHA committee adopts first CoRAP decision for a nanomaterial. Chemical Watch. 18 December 2014.
Djokić, S., 2008. Synthesis and antimicrobial activity of silver citrate complexes. Bioinorg. Chem. Appl., 2008. Available at: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC2590638/
Du, J., Wang, S., You, H., Zhao, X., 2013. Understanding the toxicity of carbon nanotubes in the environment is crucial to the control of nanomaterials in producing and processing and the assessment of health risk for human: A Review. Environ. Toxicol. Pharmacol. 36(2): 451-462.
Ebert, D., 2005. Ecology, epidemiology, and evolution of parasitism in Daphnia. National Library of Medicine (US), National Center for Biotechnology Information, Bethesda, MD. http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=Books. (accessed 19 Mar 2008).
ECHA, undated. Role of animal testing in ensuring the safe use of chemical substances. ECHA-12-FS-08-EN. Available at: http://echa.europa.eu/documents/10162/13630/reach_factsheet_animal_testing_en.pdf .

ECHA, 2008. Guidance on information requirements and chemical safety assessment. Chapter R.7a: Endpoint specific guidance.
ECHA, 2009. Practical Guide 6: How to report Read-across and categories. Available at: http://echa.europa.eu/documents/10162/pg_report_readacross_en.pdf.
ECHA, 2010. Practical guide 2: How to report weight of evidence. Available at: http://echa.europa.eu/documents/10162/13655/pg_report_weight_of_evidence_en.pdf.
ECHA, 2012a. Guidance on information requirements and chemical safety assessment. Appendix R7-1 Recommendations for nanomaterials applicable to Chapter R7a Endpoint specific guidance. Available at: http://echa.europa.eu/documents/10162/13632/appendix_r7a_nanomaterials_en.pdf.
ECHA, 2012b. Best practices on physico-chemical and substance identity information for nanomaterials, 1st GAARN meeting Helsinki, 29 May 2012. ECHA-12-R-06-EN. Available at: http://echa.europa.eu/documents/10162/5399565/best_practices_physiochem_subst_id_nano_en.pdf.
ECHA, 2013a. Assessing human health and environmental hazards of nanomaterials-Best practice for REACH Registrants. Second GAARN meeting. ECHA-13-R-04-EN. Available at: http://echa.europa.eu/documents/10162/5399565/best_practices_human_health_environment_nano_en.pdf.
ECHA, 2013b. Grouping of substances and read-across approach. Part 1: Introductory note. Available at: http://echa.europa.eu/documents/10162/13628/read_across_introductory_note_en.pdf.
ECHA, 2014. Human health and environmental exposure assessment and risk characterisation of nanomaterials - Best practice for REACH registrants. Available at: http://echa.europa.eu/documents/10162/5399565/best_practices_human_health_environment_nano_3rd_en.pdf.
Eckhoff, R. K., 2003. Dust explosions in the process industries. 3rd edition, Gulf Professional Publishing, 719 pp. As cited in Pronk et al., 2009.
EPA, 2010. Nanomaterial Case Studies: Nanoscale Titanium Dioxide in Water Treatment and in Topical Sunscreen. EPA/600/R-09/057F. November.
European Commission, 2012. COMMISSION STAFF WORKING PAPER: Types and uses of nanomaterials, including safety aspects. Accompanying the Communication from the Commission to the European Parliament, the Council and the European Economic and Social Committee on the Second Regulatory Review on Nanomaterials {COM(2012) 572 final}. Available at: http://ec.europa.eu/nanotechnology/pdf/second_regulatory_review_on_nanomaterials_-_staff_working_paper_accompanying_com(2012)_572.pdf (accessed 3 November 2013).
European Commission, 2014. Titanium Dioxide, NM-100, NM-101, NM-102, NM-103, NM-104, NM-105: Characterisation and Physico-chemical Properties. JRC Repository: NM-Series of Representative Manufactured Nanomaterials.

European Commission Joint Research Centre Institute for Health and Consumer Protection, 2011. REACH Implementation Project - Substance Identification of Nanomaterials(RIP-oN 1). AA N°070307/2009/D1/534733 between DG ENV and JRC Advisory Report. March 2011. Available at: http://ec.europa.eu/environment/chemicals/nanotech/pdf/report_ripon1.pdf.

Fabrega, J., Luoma, S.N., Tyler, C.R., Galloway, T.S., Lead, J.R., 2011. Silver nanoparticles: behaviour and effects in the aquatic environment. Environment International 37:517-531.

Fauss, E., Smith, J., Swami, N., 2011. Correlating silver nanoparticle functionalization to generation of reactive oxygen species and silver ion release rates for disinfection applications. Abstract in: International Conference on the Environmental Implications of Nanotechnology & EPA Grantees Meeting, May 2011. Available at: http://www.ceint.duke.edu/sites/ceint.duke.edu/files/ICEIN2011–EPA_Nano_Grantees_AbstractBook.pdf.

Federal Institute for Risk Assessment, 2014. What mode of action do Nanomaterials have in Liver and Intestine? Available at: http://www.bfr.bund.de/en/press_information/2014/14/what_mode_of_action_do_nanomaterials_have_in_liver_and_intestine_-190452.html.

Franklin, N.M., Rogers, N.J., Apte, S.C., Batley, G.E., Gadd, G.E., Casey, P.S., 2007. Comparative toxicity of nanoparticulate ZnO, bulk ZnO, and $ZnCl_2$ to a freshwater microalga (*Pseudokirchenerialla subcaptitata*): the importance of particle solubility. Environ. Sci. Technol. 41(24): 8484-8490.

Fraunhofer, 2012. Research activities 2012: Transformation/dissolution testing of silver nanoparticles.

Fröhlich, E., 2012. The role of surface charge in cellular uptake and cytotoxicity of medical nanoparticles. Int. J. Nanomedicine 7: 5577-5591. doi: 10.2147/IJN.S36111.

Fröhlich, E., Roblegg, E., 2012. Models for oral uptake of nanoparticles in consumer products. Toxicology 291: 10–17

Geiser, M., Kreyling, W.G., 2010. Deposition and biokinetics of inhaled nanoparticles. Part. Fibre Toxicol. 7: 2. doi: 10.1186/1743-8977-7-2.

Geraets, L. Oomen, A.G., Schroeter, J.D., Coleman, V.A., Cassee, F.R., 2012. Tissue Distribution of Inhaled Micro- and Nano-sized Cerium Oxide Particles in Rats: Results From a 28-Day Exposure Study. Toxicol. Sci. 127(2): 463–473.

Gondikas, A., Reinsch, B., Lowry, G., Hsu-Kim, H., 2011. Sorption of cysteine to silver nanoparticles: Implications for aggregation, dissolution and silver speciation. Abstract in: International Conference on the Environmental Implications of Nanotechnology & EPA Grantees Meeting, May 2011. Available at: http://www.ceint.duke.edu/sites/ceint.duke.edu/files/ICEIN2011–EPA_Nano_Grantees_AbstractBook.pdf.

Grassian, V.H., O'Shaughnessy, P.T., Adamcakova-Dodd, A., Pettibone, J.M., Thorne, P.S., 2007. Inhalation exposure study of titanium dioxide nanoparticles with a primary particle size of 2 to 5 nm. Environ. Health Perspect. 115(3): 397–402.

Griffitt, R.J., Luo, J., Gao, J., Bonzongo, J.C., Barber, D.S., 2008. Effect of particle composition and species on toxicity of metallic

nanompaterials in aquatic organisms. Environ. Toxicol. Chem. 27(9): 1972-1978.
Handy, R.D., Bairuty, G.A.L., Al-Jubory, A., Ramsden, C.S., Boyle, D., Shaw, B.J., Henry, T.D., 2011. Effects of manufactured nanomaterials on fishes: a target organ and body systems physiology approach. Journal of Fish Biology: 79: 821-853.
Handy, R.D., Owen, R., Valsami-Jones, E., 2008a. The ecotoxicology of nanoparticles and nanomaterials: current status, knowledge gaps, challenges and future needs. Ecotoxicology 17(5): 315-325.
Handy, R.D., Henry T.B., Scown T.M., Johnston B.D., Tyler C.R., 2008b. Manufactured nanoparticles: their uptake and effects on fish – a mechanistic analysis. Ecotoxicology 17: 396-409.
Heinrich, U., Fuhst, R., Rittinghausen, S., Creutzenberg, O., Bellmann, B., Koch, W., Levsen, K., 1995. Chronic inhalation exposure of Wistar rats and two different strains of mice to diesel engine exhaust, carbon black, and titanium dioxide. Inhal. Toxicol. *7*(4): 533–556.
Heywood, M., 1947. The scope of particle-size analysis and standardization. In Symposium on particle-size analysis. Suppl. Trans. Inst. Chem. Eng., 25:14-24. As cited in OECD, 2010.
Hotze, E. M., 2010. Nanoparticle Aggregation: Challenges to Understanding Transport and Reactivity in the Environment. J. Environ. Qual. 39: 1909-24.
Hou, W.C., Westerhoff, P., Posner, J.D., 2013. Biological accumulation of engineered nanomaterials: a review of current knowledge. Environ. Sci. Proc. Imp. 15(1): 103-122.
Huang, Y., Risha, G.A., Yang, V., Yetter, R.A., 2007. Combustion of bimodal nano/micron-sized aluminum particle dust in air. Proc. Comb. Inst. 31(2), 2001-2009.
Hull, M.S., Kennedy, A.J., Steevens, J.A., Bednar, A.J., Weiss, C.A., Vikesland, P.J., 2009. Release of metal impurities from carbon nanomaterials influences aquatic toxicity. Environ. Sci. Technol. 43(11): 4169-4174.
IARC. 2010. Carbon Black, Titanium Dioxide, and Talc. IARC Monographs on the Evaluation of Carcinogenic Risks to Humans 93: 193-214.
Iavicoli, I., Leso, V., Bergamaschi, A., 2012. Toxicological Effects of Titanium Dioxide Nanoparticles: A Review of *In vivo* Studies. J. Nanomat. 2012: 964381.
ICON, 2014. Nano-EHS Database Analysis Tool. Available at: http://icon.rice.edu/report.cfm.
Ivask, A., Juganson, K., Bondarenko, O., Mortimer, M., Aruoj, W., Kasemets, K., Blinova, I., Heinlaan, M., Slaveykova, V., Kahru, A., 2013. Mechanisms of toxic action of Ag, ZnO and CuO nanoparticles to selected ecotoxicological test organisms and mammalian cells *in vitro*: A comparative review. Nanotoxicology 8(S1): 57-71.
Iversen, T-G., Skotland, T., Sandvig, K., 2011. Endocytosis and intracellular transport of nanoparticles: Present knowledge and need for future studies. Nanotoday 6(2):176-185.
Jackson, P., Jacobsen, N.R., Baun, A., Birkedal, R., Kühnel, D., Jensen, K.A., Vogel, U., Wallin, H., 2013. Bioaccumulation and ecotoxicity of carbon nanotubes. Chem. Cent. J. 7(1):154.

Johnston, H.J., Hutchison, G.R., Christensen, F.M., Peters, S., Hankin, S., Aschberger, K., Stone, V., 2010. A critical review of the biological mechanisms underlying the *in vivo* and *in vitro* toxicity of carbon nanotubes: The contribution of physico-chemical characteristics. Nanotoxicology 4(2): 207-246.

Joint Research Centre (JRC), 2011. REACH Implementation Project Substance Identification of Nanomaterials (RIP oN1) AA N°070307/2009/D1/534733 between DG ENV and JRC. Advisory Report. Available at: http://ec.europa.eu/environment/chemicals/nanotech/pdf/report_ripon1.pdf.

Jones, C., Grainger, D.W., 2009. *In vitro* Assessments of Nanomaterial Toxicity. Adv. Drug Deliv. Rev. 61(6): 438-456.

Jones, P.N., 2013. EU Commission under fire for slow progress on nano. Chemical Watch – Global Risk and Regulation News. 29 October 2013.

Kennedy, A., Hull, M., Bednar, A.J., Goss, J., Boulding, J.N., Vikesland, P.L., Steevens, J., 2010. Fractionating Nanosilver: importance for determining toxicity to aquatic test organisms. Environ. Sci. Technol. 44: 9571-9577.

Kennedy, A.J., Hull, M.S., Steevens, J.A., Dontsova, K.M., Chappell, M.A., Gunter, J.C., Weiss, C.A., 2008. Factors influencing the partitioning and toxicity of nanotubes in the aquatic environment. Environ. Toxicol. Chem. 27(9): 1932-1948.

Kettiger, H., Schipanski, A., Wick, P., Huwyler, J., 2013. Engineered nanomaterial uptake and tissue distribution: from cell to organism. Int. J. Nanomedicine 8:3255-69. doi: 10.2147/IJN.S49770.

Kittler, S., Greulich, C., Diendorf, J., Koller, M., Epple, M., 2010. Toxicity of silver nanoparticles increases during storage because of slow dissolution under release of silver ions. Chem. Mater. 22: 4548-4554.

Kvitek, L., Panáček, A., Soukupová, J., Kolář, M., Večeřová, R., Prucek, R. , Holecová, M., Zbořil, R., 2008. Effect of Surfactants and Polymers on Stability and Antibacterial Activity of Silver Nanoparticles (NPs). J. Phys. Chem. C 112: 5825-34.

Landsiedel, R., Fabian, E., Ma-Hock L., van Ravenzwaay, B., Wohlleben, W., Wiench, K., Oesch, F., 2012. Toxico-/biokinetics of nanomaterials. Arch Toxicol. 86(7): 1021-60. Erratum in Arch. Toxicol. 86(7): 1061.

Lankveld, D.P., Rayavarapu, R.G., Krystek, P., Oomen, A.G., Verharen, H.W., van Leeuwen, T.G., de Jong, W.H., Manohar, S., 2011. Blood clearance and tissue distribution of PEGylated and non-PEGylated gold nanorods after intravenous administration in rats. Nanomedicine 6(2): 339-49.

Lee, K.P., Trochimowicz, H.J., Reinhardt, C.F., 1985. Pulmonary response of rats exposed to titanium dioxide (TiO_2) by inhalation for two years. Toxicol. Appl. Pharmacol. 79: 179–192.

Levard, C., Michel, F.M., Wang, Y., Choi, Y., Eng, P. Brown, G.E., 2011. Probing Ag nanoparticle surface oxidation in contact with (in)organics: An x-ray scattering and fluorescence yield approach. Abstract in: International Conference on the Environmental Implications of Nanotechnology & EPA Grantees Meeting, May 2011. Available at:

http://www.ceint.duke.edu/sites/ceint.duke.edu/files/ICEIN2011–EPA_Nano_Grantees_AbstractBook.pdf.

Liang, X.W., Xu, Z.P., Grice, J., Zvyagin, A.V., Roberts, M.S., Liu, X., 2013. Penetration of Nanoparticles into Human Skin. Curr. Pharm. Des. 19(35): 6353-6366.

Linsinger, T., Roebben, G., Gilliland, D., Calzolai, L., Rossi, F., Gibson, P., Klein, C., 2012, Requirements on measurements for the implementation of the European Commission definition of the term "nanomaterial", JRC Reference Report, EUR 25404, ISBN 978-92-79-25603-5. Available at: http://publications.jrc.ec.europa.eu/repository/bitstream/111111111/26399/2/irmm_nanomaterials %20(online).pdf

Liu, I., Hurt, R.H., 2010. Ion release kinetics and particle persistence in aqueous nano-silver colloids. Environ. Sci. Technol. 44: 2169-2175.

Lopes, S., Ribeiro, F., Woinarowicz, J., Loikowski, W., Jurkschat, K., Crossley, A., Soares, A.M.W.V., Loureiro, S., 2014. Zinc oxide nanoparticles toxicity to Daphnia magna: size-dependent effects and dissolution. Environ. Toxicol. Chem. 33 (1): 190-198.

Lu, N., Zhu, Z., Zhao, X., Tao, R., Yang, X., Gao, Z., 2008. Nano titanium dioxide photocatalytic protein tyrosine nitration: A potential hazard of TiO_2 on skin. Biochem. Biophys. Res. Comm. 370: 675–680.

Ma, R., Levard, C., Marinakos, S., Chen, Y., Liu, J., Brown, G.E., Lowry, T.V., 2011. Size-controlled dissolution of silver nanoparticles. Abstract in: International Conference on the Environmental Implications of Nanotechnology & EPA Grantees Meeting, May 2011. Available at: http://www.ceint.duke.edu/sites/ceint.duke.edu/files/ICEIN2011–EPA_Nano_Grantees_AbstractBook.pdf.

Ma, S., Lin, D., 2013. The biophysico-chemical interactions at the interfaces between nanoparticles and aquatic organisms: adsorption and internalization. Environ. Sci.: Proc. Imp. 15: 145-160.

Magdolenova, Z., Collins, A., Kumar, A., Dhawan, A., Stone, V., Dusinska, M., 2014. Mechanisms of genotoxicity. A review of *in vitro* and *in vivo* studies with engineered nanoparticles. Nanotoxicology 8(3): 233-278.

Manke, A., Wang, L., Rojanasakul, Y., 2013. Mechanisms of Nanoparticle-Induced Oxidative Stress and Toxicity. BioMed Research International.

McKim, J.M., 2010. Building a tiered approach to *in vitro* predictive toxicity screening: a focus on assays with *in vivo* relevance. Comb. Chem. High Throughput Screen. 13(2): 188-206.

Metzler, D.M., Erdem, A., Tseng, Y.H., Huang, C.P., 2012. Responses of algal cells to engineered nanoparticles measured as algal cell population, chlorophyll a, and lipid peroxidation: effect of particle size and type. J. Nanotechnol. doi:10.1155/2012/237284

Nagai, H., Toyokuni, S., 2012. Differences and similarities between carbon nanotubes and asbestos fibers during mesothelial carcinogenesis: Shedding light on fiber entry mechanism. Cancer Sci. 103(8): 1378-1390.

nanoComposix. 2011. Reference Materials. Available at: http://www.nanocomposix.com/products/silver/reference-materials.

Nanotechnology Industries Association (NIA), 2014. US and Canada develop Classification Scheme for Nanomaterials. Available at: http://www.nanotechia.org/node/18580.

Navarro, E., Piccapietra, F., Wagner, B., Marconi, F., Kaegi, R., Odzak, N., Sigg, L., Behra, R., 2008. Toxicity of silver nanoparticles to Chlamydomonas reinhardtii. Environ. Sci. Technol. 42(23): 8959-8964.

Nel, A., Xia, T., Meng, H., Wang, X., Lin, S., Ji, X., Zhang, H., 2013. Nanomaterial Toxicity Testing in the 21st Century: Use of a Predictive Toxicological Approach and High Throughput Screening. Acc. Chem Res. 46(3): 607-621.

NIH, 2014. Nanomaterial Similarity. Available at: https://www.nanomaterialregistry.org/about/NanomaterialSimilarity.aspx (accessed 1 May 2014).

NIOSH. 2011. Occupational Exposure to Titanium Dioxide. Current Intelligence Bulletin 63 DHHS (NIOSH) Publication No. 2011–160. April.

Nogueira, D.R., Mitjans, M., Rolim, C.M.B., Vinardell, M.P., 2014. Mechanisms Underlying Cytotoxicity Induced by Engineered Nanomaterials: A Review of *In vitro* Studies. Nanomaterials 4: 454-484.

Nurkiewicz, T.R., Porter, D.W., Hubbs, A.F., Cumpston, J.L., Chen, B.T., Frazer, D.G., Castranova, V., 2008. Nanoparticle inhalation augments particle-dependent systemic microvascular dysfunction. Part. Fibre Toxicol. 5: 1.

OECD, 1995. OECD Guidelines for the testing of chemicals. 105. Water Solubility.

OECD, 2000. Guidance document on aquatic toxicity testing of difficult substances and mixtures. OECD series on testing and assessment No.23. ENV/JM/MONO (2000)6. Available at: http://www.oecd-ilibrary.org/docserver/download/9750231e.pdf?expires=1417776250&id=id&accname=guest&checksum=33FD9DC1CCA64E316E1EFF6060F49B54

OECD, 2001. Guidance document on transformation/dissolution of metals and metal compounds in aqueous media. ENV/JM/MONO (2009). OECD series on testing and assessment No. 29. Available at: http://www.oecd.org/officialdocuments/publicdisplaydocumentpdf/?cote=env/jm/mono(2001)9&doclanguage=en

OECD, 2004. OECD Guidelines for the testing of chemicals. 202. Daphnia sp. Acute immobilization test.

OECD, 2007. Series on Testing and Assessment Number 80 Guidance on Grouping of Chemicals. ENV/JM/MONO(2007)28. Available at: http://search.oecd.org/officialdocuments/displaydocumentpdf/?doclanguage=en&cote=env/jm/mono(2007)28.

OECD, 2008. Considerations regarding applicability of the guidance on transformation/dissolution of metals and metal compounds in aqueous media (transformation/dissolution protocol). ENV/JM/MONO(2008)25. Series on testing and assessment No. 98. Available at: http://www.oecd.org/officialdocuments/publicdisplaydocumentpdf/?cote=env/jm/mono(2008)25&doclanguage=en

OECD, 2010. Guidance manual for the testing of manufactured nanomaterials: OECD Sponsorship Programme, first revision. ENV/JM/MONO(2009)20/REV. Available at:

http://www.oecd.org/officialdocuments/publicdisplaydocumentpdf/?cote=env/jm/mono(2009)20/rev&doclanguage=en.
OECD, 2011. OECD Guidelines for the testing of chemicals. 201. Freshwater alga and cyanobacteria, Growth inhibition test.
OECD, 2012a. Guidance on Sample Preparation and Dosimetry for the Safety Testing of Manufactured Nanomaterials. Series on the Safety of Manufactured Nanomaterials No. 36. ENV/JM/MONO(2012)40. Available at: http://www.oecd.org/officialdocuments/publicdisplaydocumentpdf/?cote=env/jm/mono(2012)40&doclanguage=en
OECD, 2012b. OECD Guidelines for the testing of chemicals. 211. Daphnia magna reproduction test.
OECD, 2014a. Ecotoxicology and Environmental Fate of Manufactured Nanomaterials: Test Guidelines. Expert Meeting Report. Series on the Safety of Manufactured Nanomaterials. No. 40. Available at: http://search.oecd.org/officialdocuments/displaydocumentpdf/?cote=ENV/JM/MONO(2014)1&doclanguage=en.
OECD, 2014b. Report of the OECD Expert Meeting on the Physico-chemical Properties of Manufactured Nanomaterials and Test Guidelines. ENV/JM/MONO(2014)15. Series on the Safety of Manufactured Nanomaterials. No. 41. Available at: http://www.oecd.org/officialdocuments/publicdisplaydocumentpdf/?cote=env/jm/mono(2014)15&doclanguage=en .
Oh, S.J., Kim, H., Liu, Y., Han, H.K., Kwon, K., Chang, K.H., Park, K., Kim, Y., Shim, K., An, S.S.A., Lee, M.Y., 2014. Incompatibility of silver nanoparticles with lactate dehydrogenase leakage assay for cellular viability test is attributed to protein binding and reactive oxygen species generation. Toxicol. Lett. 225: 422-432.
ONAMI & Oregon State University, 2014. Nanomaterial-Biological Interactions Knowledgebase. Available at: http://nbi.oregonstate.edu/.
Oomen, A., Bos, P., Fernandes, T.F., Hund-Rinke, K., Borschi, D., Byrne, H.J., Aschberger, K., Gottardo, S., von der Kammer, F., Kühnel, D., Hristozov, D., Marcomini, A., Migliore, L., Scott-Fordsmand, J.J., Wick, P., Landsiedel, R., 2013. Concern-driven integrated approaches to nanomaterial testing and assessment – report to the NanoSafety Cluster Working Group 10. Nanotoxicology 8(3): 334-348.
Park, M.V.D.Z, Lankveld, D.P.K., Loveren, H.V., de Jong, W.H., 2009. The status of *in vitro* toxicity studies in the risk assessment of nanomaterials. Nanomedicine. 4(6): 669-685.
Park, T.-J., Papaefthymiou, G.C., Viescas, A.J., Moodenbaugh, A.R., Stanislaus, S.W., 2007. Size-Dependent Magnetic Properties of Single-Crystalline Multiferroic $BiFeO_3$ Nanoparticles. Nano Lett., 7(3): 766-772.
Patlewicz, G., Roberts, D.W., Aptula, A., Blackburn, K., Hubesch, B., 2013. Workshop: Use of "read-across" for chemical safety assessment under REACH. Regul. Toxicol. Pharmacol. 65(2): 226-228.
Paun, C., 2014. REACH nano registration rules coming next year, says Vella. Chemical Watch, 18 December 2014.
Pelaz, B., Charron, G., Pfeiffer, C., Zhao, Y., de la Fuente, J.M., Liang, X.J., Parak, W.J., Del Pino, P., 2013. Interfacing Engineered Nanoparticles with Biological Systems: Anticipating Adverse Nano-Bio Interactions. Small 9(9-10):1573-1584.

Perrault, F., Oukarroum, A., Melegari, S.P., Matias, W.G., Popovic, R., 2012. Polymer coating of copper oxide nanoparticles increases nanoparticles uptake and toxicity in the green alga *Chlamydomonas reinhardtii*. Chemosphere 87(11): 1388-1394.

Petersen, E.J., Zhang, L., Mattison, N.T., O'Carroll, D., Whelton, A.J., Uddin, N., Nguyen, T., Huang, Q., Henry, T.B., Holbrook, R.D., Chen, K.L., 2011. Potential release pathways, environmental fate and ecological risks of carbon nanotubes. Environ. Sci. Technol. 45: 9837-9856.

Pronk, M.E.J., Wijnhoven, S.W.P., Bleeker, E.A.J., Heugens, E.H.W., Peijnenburg, W.J.G.M., Luttik, R. Hakkert, B.C., 2009. Nanomaterials under REACH - Nanosilver as a case study. RIVM report 601780003/2009. Available at: http://www.rivm.nl/bibliotheek/rapporten/601780003.pdf.

Powers, K.W., Brown, S.C., Krishna, V.B., Wasdo, S.C., Moudgil, B.M., Roberts, S.M., 2006. Research strategies for safety evaluation of nanomaterials. Part VI. Characterization of nanoscale particles for toxicological evaluation. Toxicol. Sci. 90: 296–303.

Puzyn, T., Rasulev, B., Gajewicz, A., Hu, X., Dasari, T.P., Michalkova, A., Leszczynski, J., 2011. Using nano-QSAR to predict the cytotoxicity of metal oxide nanoparticles. Nat. Nanotechnol. 6(3): 175-178.

Regulatory Cooperation Council (RCC) Nanotechnology Initiative, 2014?. Work Element 2. Development of a classification scheme for nanomaterials regulated under the New Substances Programs of Canada and the United States. Draft.

Reidy, B., Haase, A., Luch, A., Dawson, K.A., Lynch, I., 2013. Mechanisms of Silver Nanoparticle Release, Transformation and Toxicity: A Critical Review of Current Knowledge and Recommendations for Future Studies and Applications. Materials, 6(6): 2295-2350.

Rodea-Palomares, I., Boltes, K., Fernandez-Pinas, F., Legane's, F., Garcia-Calva, E., Santiago, J., Rosal, R., 2011. Physico-chemical characterization and ecotoxicological assessment of CeO_2 nanoparticles using two aquatic microorganisms. Toxicol. Sci. 119(1): 135-145.

Roesslein, M., Hirsch, C., Kaiser, J.P., Krug, H.F., Wick, P., 2013. Comparability of *in vitro* tests for bioactive nanoparticles: a common assay to detect reactive oxygen species as an example. Int. J. Mol. Sci. 14: 24320-24337.

Saptarshi, S.R., Duschl, A., Lopata, A.L., 2013. Interaction of nanoparticles with proteins: relation to bio-reactivity of the nanoparticle. J. Nanobiotechnol. 11: 26.

SCCS (Scientific Committee on Consumer Safety). 2014a. Opinion on titanium dioxide (nanoform). COLIPA n° S75. SCCS/1516/13. Revision of 22 April 2014.

SCCS (Scientific Committee on Consumer Safety). 2014b. Opinion on carbon black (nano-form), 12 December 2013, SCCS/1515/13, revision of 27 March 2014.

Scientific Committee on Emerging and Newly Identified Health Risks (SCENIHR). 2009. Risk assessment of products of nanotechnologies. Available at: http://ec.europa.eu/health/ph_risk/committees/04_scenihr/docs/scenihr_o_023.pdf.

Scientific Committee on Emerging and Newly Identified Health Risks (SCENIHR). 2010. Scientific basis for the definition of the term "nanomaterial." Available at: http://ec.europa.eu/health/scientific_committees/emerging/docs/scenihr_o_032.pdf.

Scientific Committee on Emerging and Newly Identified Health Risk (SCENIHR), 2014. Opinion on Nanosilver: safety, health and environmental effects and role in antimicrobial resistance. Available at: http://ec.europa.eu/dgs/health_consumer/dyna/enews/enews.cfm?al_id=1494.

Sellers, K., Hassinger, C., 2012. What Defines Nanomaterials? Request A1: National Institute for Public Health and the Environment (RIVM). Available at: http://www.rivm.nl/bibliotheek/digitaaldepot/What_Defines_Nanomaterials.pdf.

Shaw, B.J., Handy, R.D., 2011. Physiological effects of nanoparticles on fish: a comparison of nanometals versus metal ions. Environ. Int. 37: 1083-1097

Shi, H., Magaye, R., Castranova, V., Zhao, J., 2013. Titanium dioxide nanoparticles: a review of current toxicological data. Part. Fibre Toxicol. 10: 15.

Skeaff, J.M., Hardy, D.J., King, P., 2008. A new approach to the hazard classification of alloys based on transformation/dissolution. Integr. Environ. Assess. Manag. 4(1): 75-93.

Soenen, S., Rivera-Gil, P., Montenegro, J.M., Parak, W., De Smedt, S., Braeckmans, K., 2011. Cellular toxicity of inorganic nanoparticles: Common aspects and guidelines for improved nanotoxicity evaluation. Nanotoday 6(5): 446-465.

Stone, V., Pozzi-Mucelli, S., Tran, L., Aschberger, K., Sabella, S., Vogel, U., Poland, C., Balharry, D., Fernandes, T., Gottardo, S., Hankin, S., Hartl, M.G.H., Hartmann, N., Hristozov, D., Hund-Rinke, K., Johnston, H., Marcomini, A., Panzer, O., Roncato, D., Saber, A.T., Wallin, H., Scott-Fordsmand, J.J., (2014). ITS-NANO-Prioritising nanosafety research to develop a stakeholder-driven intelligent testing strategy. Part. Fibre Toxicol. 11(1): 9.

Stone, V., Pozzi-Mucelli, S., Tran, C.L., Aschberger, K., Sabella, S., Vogel, U., Poland, C., Balharry, D., Fernandes, T.F., Gottardo, S., Hankin, S., Hartl, M.G., Hartmann, N., Hristozov, D., Hund-Rinke, K., Johnston, H., Marcomini, A., Panzer, O., Roncato, D., Saber, A.T., Wallin, H., Scott-Fordsmand, J., 2013. Research Prioritisation to Deliver an Intelligent Testing Strategy for the Human and Environmental Safety of Nanomaterials; 2013. Available at http://www.nano.hw.ac (accessed 17 April 2014).

Takhar, P. Mahant, S., 2011. *In vitro* methods for nanotoxicity assessment: advantages and applications. Arch. Appl. Sci. Res. 3(2): 389-403.

Tan, Y.N., Wong, C.L., Mohamed, A.R., 2011. An Overview on the Photocatalytic Activity of Nano-Doped-TiO2 in the Degradation of Organic Pollutants. International Scholarly Research Network Mater. Sci. doi:10.5402/2011/261219

Taurozzi, J.S., Hackley, V.A. Preparation of a nanoscale TiO_2 aqueous dispersion for toxicological or environmental testing. NIST Special Publication 1200-3. http://dx.doi.org/10.6028/NIST.SP.1200-3

Tejamaya, M., Römer, I., Merrifield, R.C., Lad, J.R., 2012. Stability of citrate, PVP, and PEG-coated silver nanoparticles in ecotoxicology media. Environ. Sci. Technol. 46(13): 7011-7017.

Tolaymat, T.M., El Badawy, A.M., Genaidy, A., Scheckel, K.G., Luxton, T.P., Suidan, M., 2010. An evidence-based environmental perspective of manufactured silver nanoparticle in syntheses and applications: A systematic review and critical appraisal of peer-reviewed scientific papers. Sci. Tot. Environ. 408: 999–1006.

Unrine, J.M., Hunyadi, S.E., Tsyusko, O.V., Rao, W., Schoults-Wilson, W.A., Bertsch, P.M., 2010. Evidence for bioavailability of Au nanoparticles from soil and biodistribution within earthworms (Eisenia fetida). Environ. Sci. Technol. 44: 8308-8313

U.S. Environmental Protection Agency (U.S. EPA). 2010. Nanomaterial Case Studies: Nanoscale Titanium Dioxide in Water Treatment and in Topical Sunscreen. EPA/600/R-09/057F. November.

U.S. Environmental Protection Agency (U.S. EPA). 2011. Release, fate and transport of engineered nanosilver from consumer products. Final Report. Prepared for: U.S. EPA Air Pollution Prevention and Control Division. Research Triangle Park, NC 27711.

U.S. FDA, 1977. Select Committee on GRAS Substances (SCOGS) Opinion: Sodium citrate. Available at: http://www.fda.gov/Food/IngredientsPackagingLabeling/GRAS/SCOGS/ucm260742.htm

Van Hoecke, K., 2010. *In vitro* and *in vivo* evaluations of the ecotoxicity of nanoparticles. Dissertation Ghent University, ISBN 978-90-5989-398-6.

Van Hoecke, K., De Schamphelaere, K.A.C., Ali, Z., Zhang, F., Elsaesser, A., Rivera-Gil, P., Parak, W.J., Smagghe, G., Howard, C.V., Janssen, C.R., 2013. Ecotoxicity and uptake of polymer-coated gold nanoparticles. Nanotoxicology 7(1): 37-47.

Von Moos, B., Slaverykova, V.I., 2014. Oxidative stress induced by inorganic nanoparticles in bacteria and aquatic microalgae - state of the art and knowledge gaps. Nanotoxicology 8(6): 605-630.

Wang, B., Feng, W., Zhao, Y., Chai, Z., 2013. Metallomics insights for *in vivo* studies of metal-based nanomaterials. Metallomics 5(7): 793-803.

Wang, X.Z., Yang, Y., Li, R., Mcguinnes, C., Adamson, J., Megson, I.L., Donaldson, K., 2014. Principal component and causal analysis of structural and acute *in vitro* toxicity data for nanoparticles. Nanotoxicology 8(5): 465-476.

Warheit, D.B., Hoke, R.A., Finlay, C., Donner, E.M., Reed, K.L., Sayes, C.M., 2007. Development of a base set of toxicity tests using ultrafine TiO_2 particles as a component of nanoparticle risk management. Toxicol. Lett. 171(3): 99-110.

Wick, P., Malek, A., Manser, P., Meili, D., Maeder-Althaus, X., Diener, L., Diener, P.A., Zisch, A., Krug, H.F., von Mandach, U., 2010. Barrier capacity of human placenta for nanosized materials. Environ. Health Persp. 118(3): 432.

Wijnhoven, S.W.P., Dekkers, S., Kooi, M., Jongeneel, W.P., de Jong, W.H., 2010. Nanomaterials in consumer products - Update of products on the European market in 2010. RIVM Report 340370003/2010. Available at: http://www.rivm.nl/dsresource?objectid=rivmp:25101&type=org&disposition=inline.

Worsfold, S.M., Amyotte, P.R., Khan, F.I., Dastidar, A.G., Eckhoff, R.K., 2012. Review of the explosibility of nontraditional dusts. Ind. Engin. Chem. Res. 51(22): 7651-7655.

Wu, Y.L., Putcha, N., Ng, K.W., Leong, D.T., Lim, C.T., Loo, S.C., Chen, X., 2012. Biophysical Responses upon the Interaction of Nanomaterials with Cellular Interfaces. Acc. Chem. Res. 46(3): 782-791.

Yokel, R., Grulke, E., MacPhail, R., 2013. Metal-based nanoparticle interactions with the nervous system: the challenge of brain entry and the risk of retention in the organism. Wiley Interdiscip. Rev. Nanomed. Nanobiotechnol. 5(4): 346-373.

Zaleska, A., 2008. Doped-TiO_2: A Review. Recent Patents on Engineering (2): 157-164.

Zhang, W., Yao, Y., Sullivan, N., Chen, Y., 2011a. Modelling the Primary Size Effects of Citrate-Coated Silver Nanoparticles on Their Ion Release Kinetics. Environ. Sci. Technol. 45: 4422-4428.

Zhang, W., Yao, Y., Li, K., Huang, Y., Sullivan, N., Chen, Y., 2011b. Ion release and aggregation kinetics of citrate-coated silver nanoparticles in aqueous environment. Presented at: International Conference on the Environmental Implications of Nanotechnology & EPA Grantees Meeting, May 2011. Available at: http://www.ceint.duke.edu/sites/ceint.duke.edu/files/ICEIN2011–EPA_Nano_Grantees_AbstractBook.pdf.

Zhang, H., Ji, Z., Xia, T., Meng, H., Low-Kam, C., Liu, R., and Nel, A. E., 2012. Use of metal oxide nanoparticle band gap to develop a predictive paradigm for oxidative stress and acute pulmonary inflammation. ACS Nano 6(5): 4349-4368.

Zhang, C., Zhang, Q., Zhang, Y., Yan, B., 2013. Toward a Better Understanding of Pharmacokinetics of Nanomaterials. Curr. Pharm. Des. 19(37): 6667-6680.

Zhao, C.M., Wang, W.X., 2012. Importance of surface coatings and soluble silver in silver nanoparticles toxicity to *Daphnia magna* . Nanotoxicology 6(4): 361-370.

Zhu, W., Zhu, L., Chen, Y., Tian, S., 2009. Acute toxicities of six manufactured nanomaterial suspensions to *Daphnia magna*. J. Nanopart. Res. 11: 67-75.

Zhu, M., Nie, G., Meng, H., Xia, T., Nel, A., Zhao, Y., 2013. Physico-chemical Properties Determine Nanomaterial Cellular Uptake, Transport, and Fate. Acc. Chem. Res. 46(3): 622–631.

Annex A: Summary of review papers on ecotoxicity

Some recent reviews of ecotoxicity include the following papers.

Bondarenko et al. (2013)

Summarized toxicity data of Effect and Lethal concentrations (EC_{50}/LC_{50} values) for silver, copper and zinc salts versus nanosilver, nanocopper oxide and nanozinc oxide for algae, crustaceans, fish, bacteria, yeast, nematodes, protozoa, and mammalian cell lines.
Silver nanoparticles seem to be 10-15 times less toxic than silver ions in most organisms tested.
Copper oxide nanoparticles also seem to be less toxic than copper ions in most test organisms.
Zinc oxide nanoparticles seem to be more or less equally toxic compared to zinc ions. Toxicity is assumed to be mainly due to dissolution.
Overall, daphnids or algae seem to be most sensitive. As mentioned already elsewhere in this report, the higher sensitivity of daphnids may be related to their feeding behaviour (filter feeders).
Toxicity outcomes for mammalian cells, yeast and bacteria are often different from what is expected. This most likely has something to do with the use of organic test media. Higher-than-expected toxicity may be due to increased bioavailability after formation of stable dispersions. For instance, protein coating in yeast test media may result in enhanced sorption to the cell wall and enhanced dissolution of copper at the sorption sites. On the other hand, coating with organic matter may also result in reduced bioavailability, which may explain why bacteria are among the least sensitive groups to nanoparticles. Overall, bacteria are quite resistant to intake of nanoparticles.
The effect of ***size and coating*** on toxicity to bacteria and mammalian cells *in vitro* was investigated, but interlaboratory variation hampered the ability to draw general conclusions. For mammalian cells, which can easily internalize nanoparticles, a size-correlation (the smaller the more toxic) is expected, but the correlation decreases with the amount of data. Concerning coatings, not much variation is found for copper oxide and zinc oxide nanoparticles, whereas for silver nanoparticles uncoated nanoparticles appear to be less inhibitory for bacteria (not for mammalian cells), regardless of the type of coating.

Du et al. (2013)

Summarized toxicity data for *in vitro* cell lines (human, chicken, rat, mouse, *Tetrahymena* and RWA 264.7 macrophages) and i*n vivo* data for fish, *Daphnia*, amphipods, axolotl, copepods, oligochaetes, polychaetes, insects, algae, *Drosophila* and higher plants for different (single-wall and multiwall) carbon nanotubes (SWCNT and MWCNT).
The effects of **particle size, shape, surface charge, chemical composition, coatings and surface roughness** were discussed.
Firstly, smaller sized particles in general have more opportunities to get into cells and be translocated through different cellular barriers. A study with MWCNTs indicated that well-dispersed MWCNTs induce more developmental toxicity than agglomerated MWCNTs on zebrafish embryos. Agglomeration was mainly observed for MWCNT, hampering translocation, whereas smaller particles consisting of SWCNTs were easily phagocytosed by macrophages and transported to local lymph

nodes. The reason for this difference in behaviour was not clearly stated, but may be related to size. Agglomeration and accumulation in organs could induce toxic effects.
Secondly, shape can have an influence on toxicity. Rigid or semi-rigid fibres may cause cell toxicity and death by perforating cell membranes. Furthermore, rod-shaped or fibre particles can have more contact area with the cell membrane, can more easily get through capillaries, adhere to blood vessels, stimulate platelet aggregation and block potassium ion channels, compared with spherical carbon nanoparticles such as fullerenes. CNTs in fibrous structures may be difficult to engulf by macrophages. Longer CNTs may show a higher inflammatory response. For example, dendritic-clusters of nanonickel could induce higher toxicity than spherical nanonickel in zebrafish.
Concerning surface charge, Du et al. (2013) mention that cationic nanoparticles could induce stronger toxicity than anionic nanoparticles. Coatings affect toxicity by preventing dissolution, changing surface chemistry and interactions at the nano-bio-interface.

Handy et al. (2011)

An overview is given of acute and sublethal toxicity of nanoparticles to fish, specifically with regard to copper oxide, silver, titanium dioxide and carbon nanoparticles (fullerenes, SWCNT and MWCNT). Sublethal effects are considered on the respiratory, gastrointestinal and circulatory system, on liver/kidney, spleen/immune system, brain/behaviour, fish early life stages and fish populations.
Some nanoparticle characteristics (e.g., **size, shape, aspect ratio**) were marginally discussed, not being the focus of the review. For instance, it is mentioned that there is a specific concern for the fact that high aspect ratio materials cannot easily be engulfed by macrophages and can therefore cause additional stress by mechanisms such as reactive oxygen species (ROS) or inflammation. Furthermore, with respect to the effects on fish embryos, shape may also play an important role (perforation, teratogenicity).

Jackson et al. (2013)

This review discusses a wide range of studies on the effects of CNT on various types of organisms, including microorganisms, algae, aquatic invertebrates, fish, sediment organisms, terrestrial organisms and higher plants.
For microorganisms, some relationships were discussed between toxicity and nanoparticle characteristics. In general, **surface functionalization** (e.g., –OH, –COOH) facilitates interaction with the cell wall. For SWCNTs, toxicity seems to increase with increasing **length**. In general, MWCNTs appear to be less toxic than SWCNTs, due to their higher **rigidity** and smaller **Van Der Waals forces**. For MWCNTs, toxicity increases with decreasing length and **diameter**.
For other organisms, no clear conclusions could be drawn concerning the relationship between toxicity and nanoparticle characteristics.
In general, fish appear to be less sensitive than aquatic invertebrates (similar finding as in other review papers). The most sensitive endpoint in fish is respiratory toxicity (rainbow trout).

Shaw and Handy (2011)

Here, too, an overview is given of literature on the lethal and sublethal toxicity of nanomaterials to fish. Information is given on silver, carbon, copper, iron, nickel, titanium dioxide and zinc oxide nanoparticles.
In general, poorly soluble metal oxide nanoparticles have rather low toxicity, whereas nanoparticles which show dissolution may have acutely toxic effect concentrations in the µg/L range. However, the available studies generally do not show increased toxicity of nanoparticles compared with non-nano forms.
Toxicity mediating factors such as **size and shape** are discussed.

von Moos and Slaveykova (2014)

These authors recently reviewed oxidative stress mechanisms in bacteria and aquatic microalgae. Information is reported on testing with TiO_2, CdTe/CdS quantum dots, Al_2O_3, SiO_2, ZnO, Ag, CeO_2, CuO, Au, Fe_2O_3, Fe_3O_4, FeO, CdO, MgO, ZrO_2, MWCNT, and CdSe/ZnS quantum dots.
Several factors affecting ROS generation and toxicity were discussed**: chemical composition and purity, size, specific surface area, reactivity, the presence of coatings and functionalization, surface charge, band gap energy**
Von Moos and Slaveykova (2014) indicated that size alone is not the key factor that determines toxicity. Toxicity tests performed with copper oxide, zinc oxide and titanium dioxide with the same particle size for *Pseudokirchnierella subcapitata* showed that different chemical composition results in different toxicities (Aruoja et al., 2009). However, a comparative study that ranks inorganic engineered nanoparticles according to their toxicity has apparently not been performed yet.
Several studies (as reviewed by van Moos and Slaveykova) have shown that small particulate matter is usually more toxic than the non-nanomaterial of similar chemical composition. In addition, increasing surface area leads to an increase in toxicity as particle surface area determines the particle activity and the generation of oxidants and radical activity.
With respect to surface coating and functionalization, some contradictory effects have been found. On the one hand, a study with polymer-coated copper oxide engineered nanoparticles indicated more ROS formation in the algae *Chlamydomonas reinhardtii.* On the other hand, contradictory findings were observed with quantum dots in the bacterium *Cupriavidus metallidurans* (Slaveykova et al 2009 and Perreaul et al 2012).
Inorganic engineered nanoparticles are mostly negatively charged in a physiological medium. As the biological surfaces are also negatively charged, it favours interaction with cationic engineered nanoparticles, but this does not indicate that negatively charged, engineered nanoparticles are not taken up by endocytosis. Another factor that determines toxicity is when there is direct contact between the engineered nanoparticle and the cell. A study with seven metal oxide engineered nanoparticles showed that direct contact with cell structure enhances cytotoxicity (Shi et al., 2012). Surface-bound humic acid, for example, can reduce the toxicity because it prevents the adhesion of the nanoparticle itself to the cell.
A last factor is the band gap energy. According to the 'band gap hypothesis', engineered nanoparticles that have overlapping band energy levels with the cellular redox potential or energy levels of biomolecular redox couples could induce oxidative stress.

In summary, one can conclude that, in a first step, chemical composition is a key factor (different metal nanoparticles are not comparable within the same size range). Secondly, the smaller the particle, the higher the surface area and corresponding surface energy leads to higher ROS activity. Thirdly, engineered nanoparticles associated strongly with cell surface induce more cytotoxicity. A last factor is the band gap energy. Engineered nanoparticles that have overlapping band energy levels with the cellular redox potential or energy levels of biomolecular redox couples could induce oxidative stress.

Baun et al. (2008)

This review summarized information on toxicity testing with C_{60}, TiO_2, SiO_2, ZnO, SWCNT, MWCNT, and CdTe quantum dots in aquatic invertebrates.
Several factors affecting toxicity were discussed: **size, the presence of coatings, surface properties, presence of co-contaminants, surface charge, pH**.
As in other review papers, the intake of nanoparticles in filter feeders such as daphnids is believed to occur mainly via the dietary pathway, and in aggregated form (size limitations of filter feeding apparatus). There seems to be at least some evidence for uptake, although most of the internalized nanomaterial is believed to be eliminated from the gut without actual uptake.
In daphnids, adhesion of nanoparticle agglomerates such as TiO_2 and C_{60} to the exoskeleton is frequently described and may affect behaviour and mobility.
Adverse effects (Trojan horse effects) of adsorbed co-contaminants are reported, e.g. for Cd and As on TiO_2, for diuron on carbon black, for methyl parathion, phenanthrene, and pentachlorophenol on C_{60} nanoparticles and other permutations.
Overall, it becomes clear from these review papers that the level of toxicity depends on the nanoparticle characteristics (size, charge, coatings, functional groups, shape, solubility, reactivity) and the abiotic parameters (ionic strength, ligands, (dissolved) organic material, pH, temperature, light, viscosity, dissolved oxygen concentration, other toxicants) in the medium tested.
In a review of Jackson et al. (2013), CNTs were classified according to their toxicity for invertebrate and vertebrates according to the European Union Commission guideline 93/67/EEC. SWCNTs seemed to be extremely toxic (< 0.1 mg/L), very toxic (0.1-1 mg/L), toxic (1-10 mg/L) for invertebrates. MWCNTs and DWCNT (double-wall CNT) were classified one category lower (very toxic, toxic and harmful) for aquatic invertebrates. For inorganic nanoparticles, often a lower toxicity (or as a worst case, equal toxicity) is observed compared with the dissolved forms. For example Ivask et al. (2013) give an overview of LC_{50} values of nanoparticles of silver, copper and zinc and their respective ions for bacteria, yeast, algae crustaceans and fish. From this overview, it is clear that, for example, for silver the nanoparticle has a higher $L(E)C_{50}$ compared with silver ions (Ag^+) for bacteria, yeast, algae, crustaceans and fish.
It must however be noted that many data gaps remain (e.g., less information is available on the long-term or chronic effects, less information is available on dietary toxicity, less information is available on sediment and terrestrial organisms compared with aquatic organisms, whereas the sediment compartment especially is expected to

be a ‘sink’ for nanoparticles). Furthermore, many uncertainties and methodological issues remain to be resolved (see further in Chapter 5). Overall, for a reliable pragmatic assessment of nanoparticle toxicity, it will come down to asking the most relevant questions. Most relevant will be to find out whether or not certain nanoparticle-specific effects occur that may result in increased toxicity compared with non-nanoforms of the substance under consideration. If this can be excluded, there will be not much use in extensive testing.

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