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Guidance on information requirements and chemical safety assessment
Appendix R7-1 for nanomaterials applicable to Chapter R7a Endpoint specific guidance

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Guidance on information requirements and chemical safety assessment
Appendix R7-1 for nanomaterials applicable to Chapter R7a - Endpoint specific guidance

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DOCUMENT HISTORY

Version	Changes	Date
Version 1	First edition	April 2012
Version 2	• New advisory note (section 1.1) on testing for ecotoxicity and fate, to provide overall advice for conducting ecotoxicity and environmental fate testing for nanomaterials • Update of section 1.2.1on aquatic pelagic toxicity, to clarify that high insolubility cannot be used as a waiver and to include further recommendations on the text to be performed for this endpoint • Update of section 1.2.2. on Toxicity for sediments organisms to provide advice on spiking methods and include applicability of available OECD guidelines • Update of section 1.2.3 on degradation/ biodegradation to clarify that waivers for hydrolysis and degradation simulation testing are not applicable as sole evidence, provide advice on photocatalytic degradation and general advice on performing the tests Please note that the numbering of the sections has changed, the section numbers above refer to the updated numbering of the guidance sections.	May 2017
Version 3	• Create a **Glossary section** with definitions • **Section 3.1.1. becomes 2.1.1. General advisory note on testing with nanoforms and sampling strategy and sample preparation for human health endpoints**: content updated and restructured: ○ Update Fig 1. and added a disclaimer. ○ Section 2.1.1.1. Considerations on solubility and dissolution rate ○ Section 2.1.1.2. Available methods for solubility and dissolution testing ○ Section 2.1.1.3. Test material characterisation and reporting and sample preparation ○ Section 2.1.1.4. Biological sampling ○ Section 2.1.1.5. Use of Non-Animal Testing Approaches • **Section 3.2.2. becomes 2.2.2. Repeated dose toxicity**: update regarding: ○ The mandatory status of some parameters (e.g. dissolution rate in water and relevant biological media). ○ The conditions under which an older study can be used. ○ The doses to be used in repeated dose inhalation studies. ○ The consequences of the dose on agglomeration for designing and interpreting repeated dose toxicity studies (RDT). ○ The mechanism of toxicity for fibres ○ The type of toxicokinetic investigations that can be done under RDT studies ○ Section 2.2.2.1. The concept of lung overload: updated with the most recent references. ○ Section 2.2.2.2. Indirect genotoxicity	October 2021

	• **Section 3.2.3. becomes 2.2.3. Mutagenicity and Carcinogenicity** updated and restructured: ○ 2.2.3.1. Bacterial (Ames) mutagenicity assays are not recommended ○ 2.2.3.2. Recommended approach for gathering mutagenicity information on nanomaterials according to REACH ○ 2.2.3.3. Recommendations for the *in vitro* tests ▪ 2.2.3.3.1. Nanomaterials characterization in the test medium ▪ 2.2.3.3.2. Verification of uptake into target cells ▪ 2.2.3.3.3. Recommendations to avoid interference with uptake or endpoint analysis ○ 2.2.3.4. *In vivo* test and exposure of target tissue	
Version 4	• **Update of the advisory note on testing and sampling strategy and sample preparation for ecotoxicological endpoints:** ○ Update and changing of Section 1.1 and sub sections with 1.1.1. Characterisation of test materials, 1.1.2 Sample preparation of test materials and 1.1.3 General considerations for Fate and (Eco)-Toxicological testing ○ Update of Glossary Section • **Update of the section 1 for water solubility, granulometry, K_{ow}, adsorption/desorption:** ○ Addition of sub-section for Section 1.2.1 Water solubility with waivers and dissolution rate, ○ Addition of sub-section for Section 1.2.2 Partition coefficient n-octanol/water, with waivers and dispersion stability, Addition of the sub-section for Section 1.2.4 Dustiness, ○ Addition of sub-section for Section 1.2.5 Adsorption/Desorption with waivers and alternative methods to Koc	2022

PREFACE

Three appendices (appendices to Information Requirements and Chemical Safety Assessment Guidance Chapters R7a, R7b and R7c) specifying information requirements have been developed to provide advice for registrants when preparing REACH registration dossiers that cover *nanoforms* [1].The advice provided in this document focuses on specific recommendations for testing materials that are nanoforms of substances[1]. As most of the guidelines and publications are referring to nanomaterials or nanoparticles, also the terms "*nanomaterial*" and "*nanoparticle*" are used. Annex VI of REACH defines the terms "nanoform" and "set of similar nanoforms"[2] and establishes the requirements for characterisation of the identified nanoforms of the substance. Advice on substance identification and registration of nanoforms can be found in the guidance "Appendix for nanoforms applicable to the Guidance on Registration and Substance Identification" and shall be applied when registering nanoforms of a substance under REACH [2]. A glossary is available to define the terms used in this guidance. As this appendix is specifically addressing REACH information requirements, nanoform is the preferred term and used whenever possible.

Part of the provided advice is not strictly nanoform specific and may, for instance, also be applicable to other particulate forms of substances (e.g. where dissolution rate is relevant). However, advice has been included when it is considered especially relevant for nanoforms and should therefore be part of the nanoform specific guidance. If such nanospecific advice is not available no additional guidance for the information requirement has been included in this appendix because of one of the following reasons a) the endpoint is not relevant for nanoforms, b) the parent guidance is considered equally applicable to nanoforms or c) more research is needed to develop nanospecific advice. This appendix is providing advice specific to nanoforms and does not supersede the applicability of the general principles given in Chapter R.7a [3], i.e. the parent guidance which is applicable in case of the absence of nanospecific advice in this appendix. Please note that this document and its parent guidance provides specific guidance for meeting the information requirements set out in Annexes VII to XI to the REACH Regulation. General information for meeting the information requirements such as collection and evaluation of available information, and adaptation of information requirements, is available in Chapters R.2 to R.5 of Guidance on IR&CSA. Moreover, when considering the use of data already available, "*Guidance on information requirements and chemical safety assessment – Appendix R.6-1 for nanoforms applicable to the Guidance on QSARs and Grouping of Chemicals*" [4] may be useful as it provides how to approach read-across for hazard data between nanoforms, as well as nanoforms and the non-nanoform of the same substance.

[1] See Annex VI of the REACH Regulation (EU) 1907/2006, as amended by Commission Regulation (EU) 2018/1881 on how to address nanoforms of substances.

[2] In this document the term "set of nanoforms" is used equivalent to "set of similar nanoforms" as defined in Annex VI.

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Glossary [3]

Accumulation:
In (eco)toxicology, accumulation is the gradual build-up over time of a compound, in this case nanoparticles and their metabolites, in a whole organism or a tissue or organ, also defined as bioaccumulation. In the environment accumulation is the gradual build-up over time of compounds, in this case nanoparticles and their degradation products, in a defined (or part of a) environmental compartment.

ADME: Absorption, distribution, metabolism, excretion.

Agglomerate: 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 [2], [5], [6] and [7].

Agglomeration: Process of contact and adhesion whereby dispersed particles are held together by weak physical interactions ultimately leading to enhanced sedimentation by the formation of particles (agglomerates) of larger than colloidal size [8]. The process occurs when two particles, *i* and *j*, collide and attach to one another to form an agglomerate. The agglomeration rate constant, k_{agg}^{ij},provides a quantitative description of the speed of the agglomeration process and depends on the collision rate constant, $k_{coll}^{i,j}$ and the probability for favourable attachment upon collision, described by the attachment efficiency α [9].

Aggregate: A particle comprising of strongly bound or fused particles [2]. In contrast to agglomeration, aggregation occurs where particles are held by strong bonds like sinter bridges.

Bronchoalveolar lavage (BAL): The sample containing cells, particles, and secretions, obtained by flushing the small airways and alveoli of the lungs with saline while the animal is anaesthetised.

BALF: Bronchoalveolar lavage fluid.

Bioavailability : The amount of a substance accessible to an organism for uptake or absorption across its cellular membrane. In toxicology this is measured as the proportion of a substance in the systemic circulation compared with the total amount of a substance that has been ingested or inhaled (modified from [10]). In ecotoxicology it is also measured as the amount taken up via cell surfaces (e.g. gills for fish) from the aqueous, sediment and/or soil compartment.

Biodegradation: Degradation of a substance resulting from interaction with the biological environment [11].

Biodurability: The ability to resist chemical and biochemical alteration through dissolution and enzymatic biodegradation or chemical disintegration within biological media (modified from [11]). Biodurability (dissolution and biodegradation) is measured using *in vitro* acellular and cellular tests.

Biopersistence: The ability of a material to persist in the body due to its biodurability and resistance to physiological clearance [11]. It is determined using *in vivo* methods.

Biotransformation: Alteration of a substance resulting from interaction with biological systems [11].

[3] As most of the guidelines and publications are referring to nanomaterials or nanoparticles, also the terms 'nanomaterial' and 'nanoparticle' are used in addition to "nanoform". Nanoform, is a recent REACH regulatory term which was not used in the scientific studies or authority reports mentioned in this guidance.

Clearance: (1) In (eco)toxicology, the volume of blood or plasma or mass of an organ effectively cleared of a substance by *elimination* (including *metabolism* and *excretion)* divided by the time of elimination. Total clearance is the sum of the clearances of each *eliminating* organ or tissue for a given substance. (2) In pulmonary toxicology, the volume or mass of the lung cleared divided by the time of *elimination* is used qualitatively to describe removal of any inhaled substance which deposits on the lining surface of the lung [12].

Dissolution: Dissolution, as used in this guidance, is the process by which a nanomaterial in an aqueous medium or biological environment is dissolving into their constituent ions or molecules [7].

Dissolution half-life/half-time: A time interval in the dissolution process that corresponds to a concentration decrease by a factor of 2 for the nanomaterials and a corresponding increase of its ions or molecular forms [11].

Dispersion: Microscopic multi-phase system in which discontinuities of any state (solid, liquid or gas: discontinuous phase) are dispersed in a continuous phase of a different composition or state [11]. Dispersion may also refer to the "*act of*" dispersion.

Heteroagglomeration: Agglomeration of particles (here nanomaterials) with other particles, including other nanomaterials and non-nanomaterials (synthetic or natural), that differ in composition or size, for example Suspended Particulate Matter (SPM) [11], [8] and [13].

α_{hetero}**:** Heteroagglomeration attachment efficiency.

Homoagglomeration: A form of agglomeration describing the agglomeration of the same type of particles, e.g. the nanoparticles with each other [13] and [14].

Impaired clearance: A continuously increasing prolongation of lung clearance of poorly soluble particles or fibres when the retained lung burden exceeds a certain threshold (modified from [12]). It can be caused by toxicity (impairment of alveolar macrophages function or cytotoxicity), or by overload of alveolar macrophages.

Lung burden: The amount of test chemical that can be analytically measured in the lung at a given time point (modified from [12]).

Lung overload: A phenomenon of impaired clearance in which the deposited dose of inhaled poorly soluble particles of low toxicity (PSLT) in the lung overwhelms clearance from the alveolar region leading to a reduction in the ability of the lung to remove particles. Lung particle overload results in an accumulation of particles greater than that expected under normal physiological clearance. This definition is relevant for all species (not just rat). This definition is independent of the underlying mechanism(s) (e.g. macrophage mobility impairment). A key issue is that increased particle retention due to large lung burdens needs to be differentiated from that due to high cytotoxicity particles (e.g. quartz) [15].

Nanoform: A form of a natural or manufactured substance 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, including also by derogation fullerenes, graphene flakes and single wall carbon nanotubes with one or more external dimensions below 1 nm [2].

Nanomaterial: A natural, incidental or manufactured material consisting of solid particles that are present, either on their own or as identifiable constituent particles in aggregates or agglomerates, and where 50% or more of these particles in the number-based size distribution fulfil at least one of the following conditions:
(a) one or more external dimensions of the particle are in the size range 1 nm to 100 nm;

(b) the particle has an elongated shape, such as a rod, fibre or tube, where two external dimensions are smaller than 1 nm and the other dimension is larger than 100 nm;
(c) the particle has a plate-like shape, where one external dimension is smaller than 1 nm and the other dimensions are larger than 100 nm.
In the determination of the particle number-based size distribution, particles with at least two orthogonal external dimensions larger than 100 µm need not be considered. A material with a specific surface area by volume of <6 m^2/cm^3 shall not be considered a nanomaterial [5].

Nanoparticle: A particle with one or more external dimensions in the size range of 1 nm to 100 nm. This covers all particles with any external dimension on the nanoscale including "nanofibres" (two external dimensions in the nanoscale) and "nanoplates" (one external dimension in the nanoscale).

NOM: Natural Organic Matter.

Particle: A minute piece of matter with defined physical boundaries [2].

PEO: Post-exposure observation.

Poorly soluble particle (PSP): Solid aerosol particles deposited in the lung that do not undergo rapid dissolution and clearance [12]. The definition is restricted to lung and to aerosols. A PSP is generally understood as having a solubility of less than 0.1 g dissolved in 100 ml dissolvent within 24 hours [12]. Examples of solvents are the simulated biofluids which include artificial lung lining fluid that contains salts and proteins or in an acidic environment that mimics the lysosomal fluid of macrophages. Specific criteria determining a PSP were recently elaborated [9].

Poorly soluble particles of low toxicity (PSLT): A PSP which does "*not cause more than minimal and transient granulocytic inflammation up to a lung burden causing overload in the rat"* [12].

QSAR: Quantitative structure–activity relationship.

Set of similar nanoforms: Under the REACH Regulation, it is a group of nanoforms characterised in accordance with section 2.4 of REACH where the clearly defined boundaries in the parameters in the points 2.4.2 to 2.4.5 of the individual nanoforms within the set still allow to conclude that the hazard assessment, exposure assessment and risk assessment of these nanoforms can be performed jointly[4] [2].

Simulated body fluid: A solution with an ion concentration close to that of a physiological fluid.

Solubility: The proportion of a solute that dissolves in a given quantity of solvent, at a given temperature under equilibrium conditions, *i.e.* in a saturated state [16].

[4] A justification shall be provided to demonstrate that a variation within these boundaries does not affect the hazard assessment, exposure assessment and risk assessment of the similar nanoforms in the set. A nanoform can only belong to one set of similar nanoforms.

1. RECOMMENDATIONS FOR PHYSICO-CHEMICAL PROPERTIES

1.1 General remarks

This guidance provides advice on the testing of physico-chemical properties of the nanoforms of a substance as per REACH information requirements. This information is relevant to fulfill the information requirements set in REACH and are applicable to nanoforms.

Many of these physico-chemical properties of the nanoforms will depend on the test conditions, i.e. they may present themselves distinctively variable depending on the specific surrounding, e.g. a nanoform may easily dissolve in one medium, while it remains a particle in another medium. This is reflected, for instance, in the requirement that for nanoforms apart from water solubility, information on the dissolution rate in relevant biological and environmental media is required.

The information requirements addressed in R.7a are therefore also highly relevant for the design and justification of the environmental and toxicological testing required under Annex VII to X of REACH, including characterisation of the test material and sample preparation to allow toxicity testing. Further guidance is provided below on the relevance of the determination of these physico-chemical properties and methods for (eco)toxicological and fate testing.

Moreover, Appendix R6-1 for nanoforms applicable to the Guidance on QSARs and Grouping of Chemicals [4] provides an approach on how to justify the use of hazard data between nanoforms (and the non-nanoform) of the same substance. The Guidance is describing additional parameters that may be required for assessing whether the available hazard and fate data are valid for different nanoforms of a substance or not. The registrant will have to characterise the test material according to these parameters to be able to follow the above-mentioned guidance. For example, baseline information on solubility, dissolution rate and dispersion stability of the nanoforms to be grouped is needed for the development of grouping hypothesis [4].

1.1.1 Characterisation of test materials

The fate and hazard posed by all possible forms of the substance, including nanoforms, covered by a registration, must be addressed with the provided toxicological, ecotoxicological and environmental fate information in the registration dossier. In order to show that the test material(s) are representative for the assessed nanoforms, specific information has to be reported in the endpoint study record under the test material information field in IUCLID (see below).

Recital 12 of the REACH amended Regulation (EU 2018/1881) [17] for nanoforms stipulates: "*to allow for adequate assessment of the relevance of any physicochemical, toxicological and ecotoxicological information for the different nanoforms, the test material should be appropriately characterised. For the same reasons, test conditions documented and a scientific justification for the relevance and adequacy of the utilised test material as well as documentation for the relevance and adequacy of the information obtained from means other than testing for the different nanoforms should be provided.*"
Consequently, the following parameters have to be provided in line with Annex VI Section 2.4 requirements for the tested nanoform:

- Name(s) or other identifiers of the nanoform of the substance.
- Number based particle size distribution with indication of the number fraction of constituent particles in the size range within 1 nm – 100 nm.

- Description of surface functionalisation or treatment and identification of each agent including IUPAC name and CAS or EC number.

- Shape, aspect ratio and other morphological characterisation: crystallinity, information on assembly structure including, e.g. shell-like structures or hollow structures, if appropriate.

- Surface area (specific surface area by volume, specific surface area by mass or both).

Besides this information data from the test material including purity and, if technically feasible, quantities of identified contaminants and impurities must be analysed prior to the start of a study and provided in the registration dossier, in order to prevent errors in the interpretation of results due to impurities of the particles.

1.1.2 Sample preparation of test materials

The following section focuses on the preparation of the sample for testing. Sample preparation is widely recognised as one of the most critical steps towards reliable testing of nanoforms and requires monitoring of the nanoforms stability. There are many variables to consider when establishing a method for sample preparation. Common issues to be considered include storage (see for instance ACEnano [18]), colloidal and chemical stability of the tested nanoform, the media composition, characterisation of stock dispersions and characterisation of samples (prepared from stock dispersions) prior to administration/testing [19].
When considering aqueous media, it can be difficult to distinguish between a *dispersed* and a *dissolved* nanoform due to its small particle size in aqueous media. It is important to recognise that solubility and dispersibility are two distinct phenomena. Solubility is the degree to which a material (the solute) can be dissolved in another material (the solvent) resulting in a single, homogeneous, stable phase and it is relevant for solids, liquids and gases. Dispersibility is the degree to which a particulate material can be uniformly distributed in another material (the dispersing medium or continuous phase). Historically, the term "*dissolved*" was defined as the [i]part of a liquid sample that had passed through a 0.45 µm filter. However, dispersions of nanoparticles will pass through such filters. Therefore, "*dispersed*" is the term to use when particles are present in the aqueous media, while the use of the term "*dissolved*" is restricted to solutions in the strict sense of the definition ([19], [20]). In the context of sample preparation next to information on dissolution, knowledge about the dispersion of a nanoform and how stable it is under given conditions is important.

To avoid misinterpretation of the results of a dissolution study by not distinguishing between dispersed and truly dissolved fractions of the nanoform, a thorough characterisation of all used stock dispersions and their stability, is required, i.e. the original dispersion, as received from a supplier or as prepared from a powder prior to testing, the nanoform stock dispersion prepared in the respective test medium and all potential intermediate nanoform dispersions required for a dilution series to reach the desired test concentration. In this context, an adequate analytical method should be applied for particle detection and the choice of method should be justified.

Information on the stability of the dispersion of a nanoform is central for the interpretation of the characterisation results and the actual testing of nanoforms ("*as received", "as used", "as dosed/as exposed*") and is a key factor during sample preparation. It is further noted that the stability of the dispersion and the level of agglomeration may be different for the different doses/concentrations and is affected by dilution. Thus, detailed information on the dilution factors ha to be provided.

The stability of a dispersion is determined by intermolecular forces involving particle-particle interactions as well as those between the particles and their surrounding matrix. Due to attractive forces (e.g. van der Waals interactions) particles tend to agglomerate unless stabilised

by surface charge or steric effects. In liquid media, modifications in pH, ionic strength and concentrations of molecular constituents can significantly alter the particle dispersion.

The stability of a dispersion is also typically assessed using comparative particle size measurements and requires a reliable method of measuring the baseline particle size distribution of the material. To assess the initial agglomeration and/or aggregation state a number of parameters are recommended including the effective cross section and can be measured via aerodynamic/light scattering or by electron microscopy ([21], [22]).
For example, by comparing changes in particle size distribution, a qualitative assessment or proxy measure of the state of dispersion can be made. Examples of methods applicable for spherical particles are Zeta potential measurement, combined with Dynamic Light Scattering (DLS) or Ultra-violet visible (UV-VIS) spectroscopy to monitor the stability of nanoparticle dispersions in stock or test media and to gain a qualitative understanding of the agglomeration process. Other methods [23], when suitable, e.g. particle tracking analysis, can also be used [24]. For aquatic and sediment toxicity testing, the Guidance Document (GD) OECD GD 317 also includes analytical techniques to characterize the *as-produced* or *as-received* test material, as well as the test material in stock and test dispersions.

Several documents are available to assist registrants in the sample preparation process of test material for nanomaterial testing. Initially the following reports were provided as guiding instructions:

- Sample Preparation and Dosimetry for the Safety Testing of Manufactured Nanomaterials OECD GD 36 [19]
- Ecotoxicology and Environmental Fate of Manufactured Nanomaterials: Test Guidelines [25]

However, most recently the advice provided in guidance document OECD GD 317 [26] has to be followed to the extent possible.

To sum up the following important aspects need to be considered for sample preparation of nanoforms:

- Characterization of the physicochemical properties of nanoforms (e.g. particle size distribution, shape, composition, specific surface area, surface chemistry and impurities) as produced and as present in the test medium (degree of agglomeration/sedimentation/dissolution where applicable).

Sample preparation needs to be controlled, consistent, relevant, reliable and robust. The different test preparation stages may include e.g. the use of powder and/or dispersions depending on the respective endpoint and required exposure concentrations which carries the risk that the test item undergoes distinct physico-chemical changes already during these different preparation stages. Monitoring of changes is necessary for an accurate description of the actual test item instead of the assumptive, pristine nanoform.

- The preparation of the nanoform dispersion (including stability) and composition of the test medium (such as pH, organic matter, salts etc.) are key characteristics and interactions between the nanoform and the test medium influence their physico-chemical properties ultimately determining their fate and behaviour and consequently potential adverse (eco)toxicological effects.

- The applied sample preparation protocol detailing procedures for controlled, reliable, and robust processes need to be scientifically justified and reported in the study summary.

ISO 14887:2000 [27] outlines procedures for the preparation of good dispersions from various powder/liquid combinations for particle size analysis of substances in general. Suggested dispersion procedures for a range of nanoforms are also emerging in the scientific literature e.g.,

[28] [29].

In relation to sample preparation, it is necessary to be aware that aggregates and agglomerates of nanomaterials can be formed in the dispersion (powder and aerosol forms) and that their presence is influenced by several factors including method of synthesis, storage and handling conditions. For aerosolised powders, the situation can be even more complex as the concentration and diffusion characteristics of the aerosol can cause the state of dispersion to change over time.

The procedures should be carefully assessed to determine if they are adequate for the respective test material. Modifications may be required for different materials in the context of (eco)toxicological testing. For example, for testing inhalation toxicity, standards are available that outline procedures for the generation of metal nanoparticles using the evaporation/condensation method (ISO 10801:2010 [29] and support the characterisation of nanoforms in inhalation exposure chambers [29].

Another important component of sample preparation is the reliability of the sampling itself which means that the test aliquot represents the physical and chemical characteristics of the entire sample. The characterisation of particle properties like size, form and specific surface area requires very careful sampling and sample aliquoting, as well as considerate storage if the sample is not processed immediately to prevent transformation of the nanoform in question. ISO 14887:2000 [27] specifies methods for obtaining a representative test aliquot with a specified confidence level from a defined sample of particulate material (powder, paste, suspension or dust) for the measurement of particle size, size distribution and surface area.

1.1.3 General considerations for Fate and (Eco)-Toxicological testing

The interactions between nanoforms and their surroundings need to be accounted for, when testing fate and (eco-)toxicity, as this will influence the overall (eco)toxicity and fate processes. In particular, the interaction of nanomaterials with the environmental or biological media has to be considered in case dissolution occurs. The test design and more specifically the test conditions (e.g. composition of test media, agitation, light, exposure method, temperature) influence the dissolution of nanomaterials affecting then the test results [19] [30].
If a nanoform is well soluble in water (see Sections 1.2.1 and 2.1.1 for thresholds) and has a high dissolution rate in relevant biological (see Section 2.1.1) or environmental (see Section 1.2.1) media, then it is likely that the nanoform is rapidly, increasingly and finally completely present in its molecular or ionic form. Therefore, it can be expected to behave similarly and elicit the same response as the non-nanoform of the substance. This could be the case for the metal ions released from the nanoform, where e.g. the metal salts ("*ionic form*") can be used as a positive control, compared to the nanoform. If, however, the nanoform under investigation is poorly or only partially soluble with a low dissolution rate in biologically or environmentally relevant test media, then it will likely be present in the test system in a particulate form. In this case, the advice provided in *Appendices for nanoforms applicable to Chapters R.7a* (this document)*, R.7b and R.7c* would apply including the considerations for partially soluble nanoforms where both the dissolved and particulate forms need to be accounted for.

In addition to Section 1.1.2. (Sample preparation of test materials) the following requirements for (eco)toxicological and fate testing must be fulfilled:

- Any (eco)toxicological and fate testing must be accompanied with suitable characterisation methods to monitor the exposure situation, such as exposure concentration and form of the tested nanoform (dissolved and/or particulate form). Concerning dose metrics for nanoforms using exclusively chemical analysis to determine mass based concentrations/metrics is not sufficient as further explained in the last bullet point, (for aquatic and sediment toxicity testing see OECD GD 317 [26], Section 4).

- Monitoring of dissolution and dispersion stability must be conducted at least at the beginning and at the end of the test but depending on the results additional intermediate monitoring may be required if a high dissolution rate or reduced stability of the test dispersion is observed.

- Since the most appropriate dose metrics may not be known, the use of other dose metrics than mass-based, such as surface area and particle counts, are a favourable addition to the mass metrics. These measurements will allow for a conversion from mass to particle counts and/or to surface area and are considered essential. While diminishing the uncertainty related to the conversion when the metrics are used independently it will also consequently reduce the amount of required testing.

As fate and behaviour of a nanoform can differ from that of another nanoform and/or their non-nanoform, appropriate analytical methods are needed for fate and (eco-) toxicity testing of nanomaterials. These also need to consider how the dissolution and the stability of the dispersion of the investigated nanomaterial influence each other. With respect to aquatic and sediment toxicity testing OECD flowcharts outlining strategies for preparing, controlling and maintaining test dispersions are given in OECD GD 317 [26], Section 5. OECD GD 318 [8] Sections 2 to 4 are advising how solubility, dissolution rate and dispersion stability testing informs further fate and ecotoxicological testing. Characterising the dissolution rate and dispersion stability in the respective test medium does not only help to find the appropriate testing strategy and test conditions, but also amplifies the correct interpretation of the results. General information on dissolution and dispersion stability for nanoforms is beneficial when building grouping and read across hypothesis [31].

1.1.3.1 Considerations of impurities and contaminants for (eco)toxicological testing

Complementary to the considerations listed in Section 1.1.2 (Sample preparation) and 1.1.3 (Fate and (eco)toxicological testing) the influence of contaminants (including biological contaminants) and impurities on (eco)toxicological test results need to be taken into account. For example, metallic impurities such as Cobalt and Nickel catalysts used in the production process of certain nanomaterials were shown to inhibit hatching in zebrafish embryos (e.g. [32]). Likewise metal impurities of MWCNT can contribute to their inhalation toxicity in rodents (e.g. [33] and [34]).

Also of particular concern is the adverse impact on test results of endotoxin contamination in nanomaterial samples. Endotoxin (lipopolysaccharide) is a constituent of the outer cell wall of gram-negative bacteria and as such is found ubiquitously within the environment. Endotoxin can however generate a range of toxic effects either at the whole organism level causing responses such as fever, "endotoxin shock" and death, or at the cellular level via the triggering of inflammatory cascades leading to the secretion of pro-inflammatory mediators.

Due to this potent response an endotoxin contaminated test sample may lead to confounding results (including a potential false positive) in biological assays. Therefore, establishing the presence and levels of endotoxin in a test sample is an important step during the sample preparation for (eco)toxicological testing. Endotoxin can be measured using *in vitro* methods such as the macrophage activation test (MAT), validated by European Committee on Validation of Alternative Test Methods [35] or the Recombinant Factor C (rFC) test [36]. Both rFC assay and MAT assay have been adopted by the European Pharmacopoeia [37], [38] and [39]. With ISO 29701:2010 an international standard, the limulus amebocyte lysate (LAL) test, for analysing endotoxin contamination in nanomaterial samples is available [40]. Although issues regarding contamination are not nanospecific, the increased relative surface area of nanosized systems compared to other particles means that the possible amounts of adsorbed endotoxin (e.g. grams adsorbed endotoxin per gram of material) are significant and deserve therefore special attention [41].

1.2 Specific advice for endpoints

1.2.1 Water solubility

Annex VII, Section 7.7 of REACH requires the determination of solubility and, additionally for nanoforms, the determination of the dissolution rate in relevant media.

Water solubility is covered in Section R.7.1.7 of the parent guidance. There is no specific test guideline to test water solubility of nanomaterials. However, OECD GD 318 [8] describes applicability and limitations of OECD TG 105 [42] and OECD GD 29 [43] for testing solubility and dissolution of nanomaterials in simulated environmental media.

For water solubility, both OECD TG 105 and GD 29 adapted for nanomaterial testing [8] are applicable.
The flask method presented in OECD TG 105 states to measure solubility after a 24 hour equilibration period. Analysis is to be done after a separation step. However, the recommended method in OECD TG 105 is only considered to be adequate when using ultra-filtration.
OECD GD 29 [43] allows to test dissolution and transformation of metals with test durations varying between 1 up to 28 days (common duration of 7 days). The OECD GD 29 protocol provides advice on how to determine the transformation or dissolution and provides knowledge to which extent metals and poorly soluble metal compounds can produce soluble ionic forms and other metal bearing species in aqueous media, at different pHs. It should be noted that if the recommendations provided under OECD GD 318 are not considered, the method as described in OECD GD 29 is not applicable to nanoform testing (e.g. 0.20 µm filtration is not adequate). Nevertheless, with appropriate adaptations (in particular for the filtration step) the method can be applied for nanoform testing. Ultra-filtration using a 3-10 kDa cut-off membrane is considered an applicable separation method for nanomaterials [16].
OECD GD 318 provides further guidance on the application of OECD GD 29 and other TGs for measuring water solubility and dissolution rate of nanomaterials in environmental relevant conditions, including the applicability of the static batch mode (screening test, adapted from OECD GD 29) and the dynamic mode (based on ISO Technical Report (TR) 19057:2017 [44]).

Overall, solubility measurements should be performed after 24 hours, or up to equilibrium, and must consider different pHs, as per OECD GD 29 recommendations, and an appropriate separation method *i.e*. ultrafiltration with 3-10 kDa cut-off membrane.
Under these conditions, a nanoform is considered highly soluble *i.e.* no detectable particles are present, if its water solubility is above 100 mg/L (see **Figure 1**). The threshold of 100 mg/L for solubility in water is set based on the work done in support of OECD GD 29 [43] and considered as a conservative approach with prioritising environmental safety. The duration of solubility studies to identify highly soluble nanoforms should be set to 24 hours, longer tests will not improve significance and value of ecotoxicological test design, their results or their interpretation. The analytical method should be sufficient to measure reliably the water solubility hence, the limit of quantification (LOQ) of the analytical method must be justified.

For nanoforms REACH regulation (Section 7.7 of REACH Annex VII) requires in addition to solubility also the determination of the dissolution rate in water and relevant media. Based on this guidance, this must be considered for nanoforms with a water solubility of 100 mg/L or below.

For nanoforms, it is necessary to consider that the dissolution rate may be affected by the specific properties of the nano-sized materials. Properties such as particle size and shape, impact the materials' surface area and the specific surface area and surface coating, will influence the nanoform-solute interactions. Therefore, the impact of such properties on the determination of the dissolution rate must be considered. Further information regarding the dissolution rate can be found in Section 1.2.1.1 below.

Providing information on dissolution is needed to determine the potential release to the aquatic environment and therefore the aquatic exposure.
The fate and behaviour of nanoforms is affected by their dissolution rate and their degree of dispersion. Distinction between a dispersed nanoform (where both solid and liquid fractions are present) and a dissolved nanoform (where no detectable solid fraction is present) is important as it has implications on the testing and characterisation strategies, as illustrated in **Figure 1**.

This situation is not unique to nanoforms, and indeed the parent guidance already highlights that *"measurement of the solubility of sparingly soluble compounds requires extreme care to generate saturated solutions of the material without the introduction of dispersed material"*. However, this problem is further amplified in the case of sparingly soluble nanoforms. It is important to ensure that no undissolved material contributes to what is being measured as dissolved material, meaning that an appropriate separation method is used.

For environmental endpoints covering fate and ecotoxicity the choice of nanospecific testing and/or following the parent guidance must be duly justified and documented based on the presence of soluble and/or solid fraction. Where partial dissolution is observed, the impact of each fraction must be considered (*i.e.* data on non-nanoforms of the substance may inform on the hazards of the solubilised fraction).

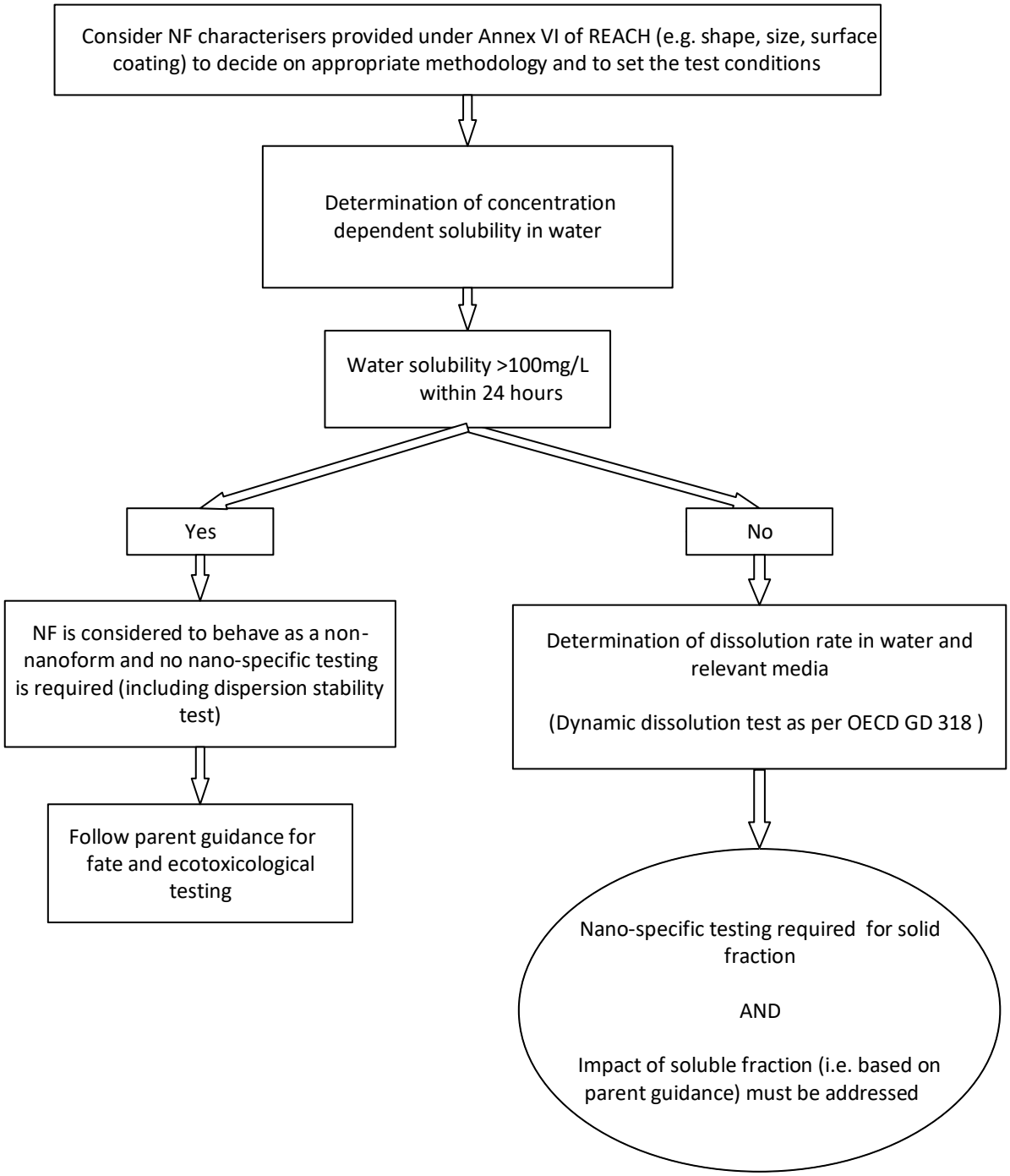

Figure 1: Scheme of the testing required to fulfill Annex VII, Section 7.7 information requirement of nanoforms of a substance and their impact on testing strategies with respect to environmental fate and ecotoxicity. Solubility in water defines whether determination of dissolution rate is required and subsequently nanospecific testing has to be performed for fate and ecotoxicity assessment of those nanoforms with a solubility limit ≤100 mg/L within 24 hours or whether the nanoform is considered to behave as a soluble substance and the parent guidance applies.

With respect to solublity testing that can inform on human toxicity testing, information can be found under section 2.1.1.

1.2.1.1 Dissolution rate

Annex VII, section 7.7 of REACH states that: "*For nanoforms, in addition the testing of dissolution rate in water as well as in relevant biological and environmental media shall be considered.".* Thus, information on the dissolution rate has to be provided as part of the solubility endpoint and should be investigated in relevant media, such as the ones used in the (eco)toxicological tests. For nanoforms with a water solubility above 100 mg/L this information requirement is considered of low value.

Dissolution rates in the different biological and environmental media used for (eco)toxicological and fate tests are variable and may affect the bioavailability of substances. Knowledge on dissolution rates will also help to predict the toxicokinetic behaviour of particles.

Currently, no test guideline is available for determining the dissolution rate of nanomaterials although some projects are working on developing a specific TG for dissolution rate of nanomaterials in water and biological media (OECD Working Group of the National Coordinators for the Test Guidelines Programme(WNT) 1.5 project on "*Determination of Solubility and Dissolution Rate of Nanomaterials in Water and Relevant Synthetic Biological Media*") and in environmental media (OECD WNT projects 3.10 "*Dissolution rate of nanomaterials in the aquatic environment*").

Nevertheless, OECD GD 318 [8] provides guidance on how to determine dissolution, dispersion and agglomeration of certain nanoforms in environmental media, based on available TGs. Such methods are also considered relevant to determine nanoform dissolution rates in water as required under Section 7.7 of REACH. Where the nanoform is outside the applicability domain of the available TGs and GDs, the registrant is required to adapt such an information requirement with a robust technical and scientific justification for the inapplicability of the used test methods, and the lack of an adopted relevant guidance and/or test guideline.

Parameters such as pH, ionic strength or suspended particulate matter influence the dissolution rate of nanoforms and should be carefully evaluated and reported.
The following paragraphs are mainly focussed on environmental testing. Nevertheless, many of the statements may be relevant for water and biological media as well.

To test dissolution, a static batch mode (screening test, adapted from OECD GD 29) and a dynamic flow-through mode (based on ISO TR 19057) for environmental relevant conditions are described in OECD GD 318.

As discussed above the determination of the dissolution rates depends on an appropriate separation of particulates from the dissolved fraction and suitable time resolution. However, this can be a drawback for a screening test based on the static batch mode, especially for rapidly and fully dissolving nanoforms. Information on appropriate separation techniques for nanomaterials can be found in ISO TR 19057. Based on OECD GD 318 the recommended method is centrifugal ultrafiltration as it promotes a suitably rapid separation allowing for calculation of dissolution rates. Still, care should be taken, when choosing filter cut-off to ensure no passage of smaller solid fractions below the filter cut-off value. Also, build-up of ions should be considered when small filter cut-off values are chosen and similarly caution should be taken when using NOM as it can block filters with smaller cut-off sizes.

For slowly dissolving nanoforms, the applicability of the batch method is mostly dependent on the analytical power as the solubility limit may not be within the resolution of the analytical method, i.e. staying under the limit of quantification or even limit of detection.

The dynamic method is based on a flow-through system and comprises the use of a test medium delivered at constant flow rate through a compartment which entraps the nanoparticles, *i.e.*

using ultrafiltration membranes. Sensitivity can be partially compensated by adapting the low flow rate and nanoform concentration.

For both methods, static batch mode and dynamic flow through mode, their applicability for testing non-metallic nanoforms depends on the accuracy of the analytical method. In general, most of the nanoforms tested are inorganic materials (mainly metal and metal oxide forms). Other nanoforms such as organic or organometallic and carbon-based nanoforms have been less tested and analytical methods may lack precision for quantification. The development of various analytical methods is ongoing (including methods for organic nanoforms) to increase the accuracy and sensitivity of the measurements and their applicability for measuring dissolution rates of nanoforms in water. Further specific considerations to the adaptation of analytical methods to nanoform testing (e.g. quantification of solid or dissolved fractions) is not yet fully developed.

OECD GD 318 also provides a formula to calculate the dissolution rate.

Dissolution rate can be determined from a graph showing solubility (as ionic concentration in mg/L) as a function of time. Most nanomaterials follow (pseudo-) first order kinetics and can be determined as the loss of solid material over time. The parameters impacting the results are the initial mass of nanomaterial in the test, its specific surface area and solubility, and the test conditions (e.g. shaken, stirred, not agitated). For comparison purpose, the dissolution rate should be normalised by surface area and can be calculated based on Noyes-Whitney equation:

$$\text{Dissolution rate } = \frac{dm}{dt} = \left(D \cdot \frac{A}{h}\right) \cdot (c_S - c)$$

where D is the diffusion coefficient of the dissolved species in the medium, A is the surface area of the nanomaterial, h is the thickness of the diffusion layer, c_s is the saturation concentration (solubility limit), and c is the concentration of dissolved ions in the test medium.

OECD GD 318 also provides advice on how both static batch and dynamic methods can be adapted to measure dissolution rate in natural or artificial environmental media. According to OECD GD 318, the applicability of the dynamic method based on ISO TR 19057 for toxicological test media, i.e. in lung and gastro fluids, has already been demonstrated in [45] and [46]. In addition, the OECD WNT 1.5. project will provide a specific guidance document to determine dissolution in biological media and water [47]. While this is ongoing work, further toxicological considerations and advice on information regarding solubility and dissolution of nanoforms in biological media are described under Section 2.1.1 of this guidance and are to be followed.

Alternatively, OECD TG 105 is also considered to be potentially adaptable to determine dissolution rates. The use of the column elution method with continuous measurements of the dissolved fraction would allow the determination of a nanoform dissolution rate. To do so, nanoform adsorption to the substrate must be warranted.

With respect to dissolution rate testing that can inform on human toxicity testing, information can be found under section 2.1.1.1.

1.2.1.2 Waiving of water solubility

Annex VII, Section 7.7 of REACH states in column 2 : "*The study does not need to be conducted if — the substance is hydrolytically unstable at pH 4, 7 and 9 (half-life less than 12 hours), or — the substance is readily oxidisable in water. If the substance appears "insoluble" in water, a limit test up to the detection limit of the analytical method shall be performed. For nanoforms the potential confounding effect of dispersion shall be assessed when conducting the study.*"

In the parent guidance Section R.7.1.7.1, it is noted that water insolubility is used as a regulatory trigger for waiving certain physicochemical and ecotoxicological endpoints.

Considering the nanospecific properties and constraints in assessing the solubility of nanoforms, waiving the information requirement based on their apparent insolubility as quoted in Annex VII, Section 7.7, column 2: "must always be accompanied with robust technical and scientific justification comprising information on dissolution and dispersion stability of the nanoform(s)" (see Section 1.2.1.1 & 1.2.2.2).

1.2.2 Partition coefficient n-octanol/water

1.2.2.1 Applicability of partition coefficient n-octanol/water

The n-octanol/water partition coefficient (K_{ow}) is defined as the ratio of the equilibrium concentrations of a dissolved substance in a two-phase system consisting of the largely immiscible solvents n-octanol and water. In a two-phase system, nanoparticles behave differently from organic molecules. Particles do not form solutions but colloidal dispersions, which are multiphase systems and thermodynamically unstable. Therefore, the fate of undissolved nanomaterials cannot be predicted by equilibrium partitioning [48], [49] as nanomaterials cannot reach thermodynamic equilibrium by distributing between two phases, water and n-octanol, due to their particulate nature.

OECD TGs recommended in the Section R.7.1.8.3 of the parent ECHA Guidance for partition coefficient n-octanol/water, i.e. OECD TG 107 [50], OECD TG 117 [51] and OECD TG 123 [52], are in most cases not applicable to nanoforms [21], [20], [53]. Results will be impacted by the presence of a colloidal suspension, which could be present if the manufactured nanomaterial does not completely dissolve [19], [20], [31].

If it is shown that the nanoform has a water solubility above 100 mg/L, as explained under Section 1.2.1, the impact of particles may be neglected, and the parent guidance applies.

If on the other hand, it is shown that the nanoform has a water solubility equal or below 100 mg/L, dispersion stability is an information requirement under Annex VII, Section 7.8 of REACH. Hence, nanospecific testing is needed for the fate and ecotoxicity assessment of the solid fraction. Information on K_{ow} may still be of value for the dissolved fraction of organic nanoforms and nanoforms with an organic coating. This is illustrated in **Figure 2** and further detailed under section 1.2.2.2 and 1.2.2.3.

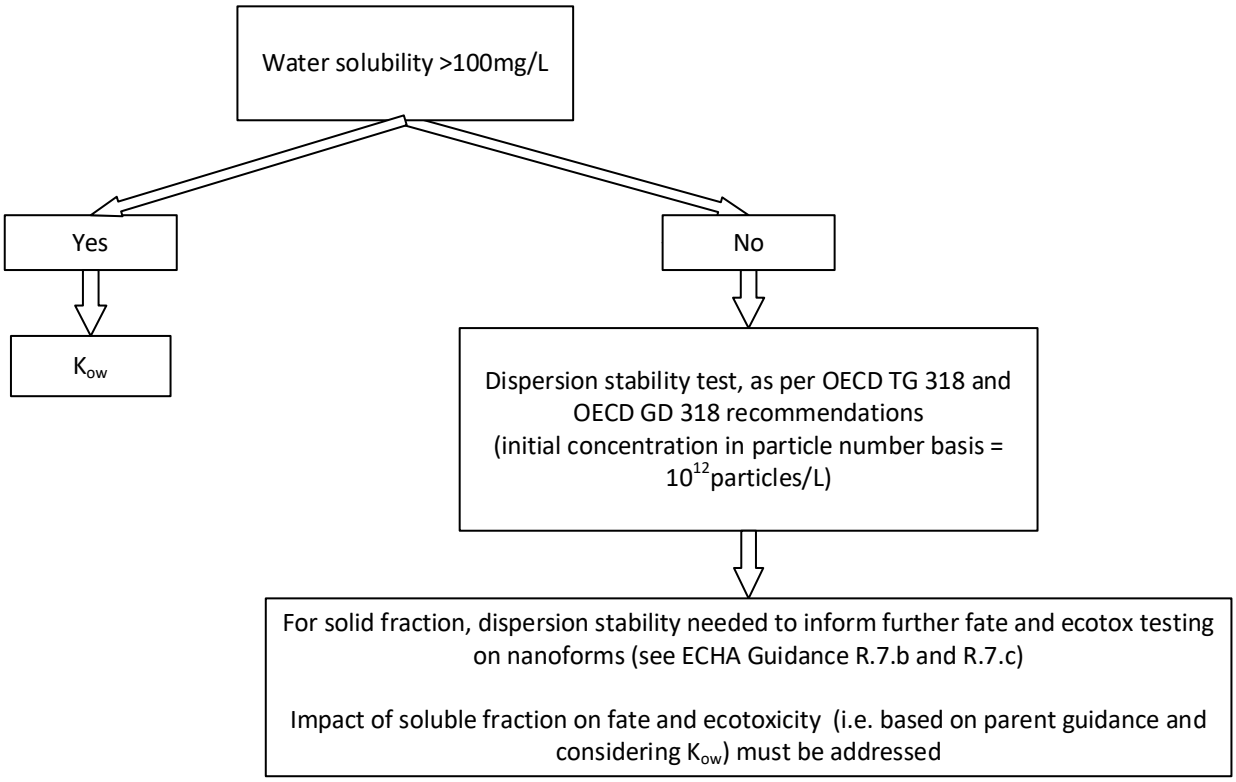

Figure 2: Scheme of the testing required to fulfill Annex VII, Section 7.8 information requirement of nanoforms of a substance and their impact on testing strategy.

Where the water solubility is below or equal to 100 mg/L determining the dispersion stability is required for assessing the solid fraction, along with the Kow for assessing the dissolved fraction.

1.2.2.2 Dispersion stability

According to Annex VII, section 7.8, determination of dispersion stability in water is required for nanoforms.

Furthermore, information on dispersion stability in relevant environmental media is needed to help predict fate and transport of the nanoforms in the environment, as illustrated in **Figure 2**. However, information on dispersion stability can not be seen as a one-to-one replacement of K_{OW} and additional properties need to be considered as well.
Dissolution (rate), agglomeration, aggregation, deposition and attachment are considered informative properties to predict the environmental behaviour of the nanomaterials in the environment and organisms [48], [54], [55], [56].
In this line, OECD GD 318 [8] includes recent developments on dispersion stability measurements, considering both homoagglomeration, as per OECD TG 318 [57], and heteroagglomeration. These agglomeration parameters can be provided in addition to dispersion stability in order to justify behaviour and fate of the nanoforms and consequently some choice in the testing strategy.

Dispersion stability is a relevant fate descriptor to also inform on further testing strategies related to aquatic fate and hazard testing while adsorption/desorption informs on test strategy on soil and sediment. Providing information on dispersion stability (as per OECD TG/GD 318) is needed to determine the potential exposure to the aquatic environment. Adsorption/desorption (mobility potential in soil) as per OECD GD 342 [58]/TG 312 [59] will complement the information on the environmental behaviour of nanomaterials.

Dispersion stability based on homoagglomeration

Agglomeration is the process by which two particles interact. In case the two particles are of the same kind, this process is called homoagglomeration. The OECD TG 318 [57] defines dispersion stability, accounting for agglomeration and sedimentation. As such, it can be used to design a testing strategy to determine homoagglomeration; i.e. particle-particle attachment of nanomaterials in ecotoxicological test media.

OECD TG 318 proposes (screening) testing for dispersion stability based on homoagglomeration (nanoparticle-nanoparticle interaction) under environmental conditions by a screening test and an extended test. For the screening test, the media is stabilised with NOM and, for comparison purposes, the tests are performed on particle number concentration basis. The test guideline provides information on the quantity or relative percentage of the nanomaterial that remains dispersed in the aquatic medium tested in a given time frame.

Testing in other environmental conditions is described in OECD GD 318 [8] where feasibility of testing in other test media, such as natural waters, is also explained.

The dispersion stability test follows a 2-tiered approach based on the particles remaining in the water phase after homoagglomeration and sedimentation during a given time frame (described in OECD TG 318 and under Section 3 of OECD GD 318):

- Tier 1 (screening test): measurements undertaken at two time points (0 and 6h, which represents test start and end) at relevant pH conditions, with natural organic matter (10 mg/L dissolved organic matter) and a range of electrolyte concentrations (0, 1 and 10 mM Ca(NO3)2). An additional measurement is done at the end of the test, after centrifugation of the sample. The centrifugation parameter is calculated in order to achieve a particle cut off value of 1 µm.

- Tier 2 (extended test): in addition to the condition range described in the screening test *i.e.* pH and electrolyte range, presence and absence of natural organic matter is tested. When no organic matter is added, sodium bicarbonate (5 mM) has to be added as a buffering agent. The stability of the dispersion is measured hourly with sub-sampling over the test duration of 6 hours).

Based on the results of the screening test, *i.e.* the percentage of nanoforms remaining in dispersion under all test conditions, nanoforms can be qualified as of:

- High stability - if ≥90% of the initial test concentration remains in dispersion for all test conditions or

- Low stability – if ≤10% of the initial test concentration remains in dispersion for all test conditions.

Where the measured concentration is within 10 and 90% of the initial test concentration, *i.e.* intermediate stability, at any of the test conditions, the extended test (Tier 2) needs to be performed. The extended test allows a differentiation of settling behaviour (which is due to certain nanomaterial properties, e.g. density), over time. As shown in **Figure 3**, the red line represents a nanoparticle agglomerating but not settling due to low density whereas the green line may represent either high density nanoparticles that agglomerate and settle quickly or a mixed sample composed of an unstable, high density, fraction and a stable one.

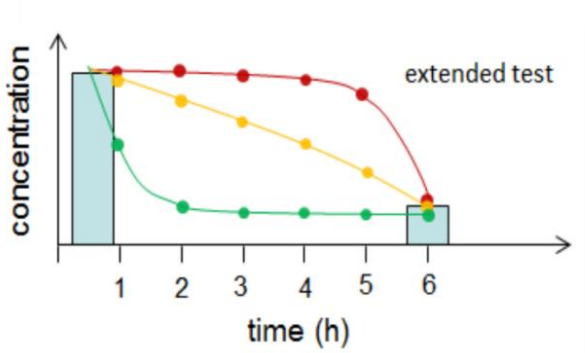

Figure 3: Schematic representation of exemplified dispersion stability results from an extended test (extracted from OECD TG 318). The hourly measurements revealing the sedimentation behaviour of the nanomaterial.

Overall, the applicability of OECD TG 318 for nanoforms should be considered based on:

- Material density (i.e. >1g/cm3).
- Sensitivity of the analytical method allowing quantification of the nanoforms from 100 to 10% of the particles present in the water column, *i.e.* LOQ lower or equal to 10% of initial mass concentration.

When applying the test with natural waters, careful removal of natural particles/colloids and microorganisms via filtration over a membrane of pore size ≤ 0.1 µm or via ultracentrifugation should be pursued in order not to compromise the test. Complex samples such as natural waters are considered to have a very specific hydro-chemical composition and if no pre-filtration or centrifugation is performed the results of the test will be rather representative of hetero-agglomeration (see heteroagglomeration considerations below).
To ensure data comparability media constituents interfering with agglomeration have to be reported (e.g. divalent cations and anions, pH and type of organic matter). The elements to be characterised are further discriminated under Section 3.0 and 3.1 of OECD GD 318.

OECD GD 318 provides further consideration when testing nanoform dispersion stability namely:

- Although NOM-nanoparticle interactions vary with particle properties (e.g. surface charge), NOM is known to form nanoparticle "coronas", i.e. surface coverage, which highly influences the dispersion behaviour of the test material.
- Use of stabiliser aids dispersing extremely hydrophobic materials will impair the evaluation of the stability of the pristine material.
- A 6-hour long test performed with a fixed particle number concentration (e.g. 10^{12} particles/L, as per OECD TG 318) is recommended, to generate comparable results. It is advised to take size distribution (instead of simple average diameter) into account, in addition to density for the conversion of nanomaterial mass concentration to particle number concentration. Nevertheless, average diameter should still produce acceptable data, *i.e.* within the methods sensitivity range (e.g. one order magnitude difference).
- The sedimentation velocity of particles depends on several factors such as the relative density of the starting material and its agglomerates, the 3D structure of the agglomerate, its surface chemistry and therefore interaction with the surrounding medium. To reduce the impact of density on determining agglomeration, it is required to proceed with sample centrifugation, as described in OECD TG/GD 318.

Regarding quantification of dispersion stability, it is noted that the applicable analytical methods for nanoforms are limited only by their sensitivity. Inductively coupled plasma mass spectrometry (ICP-MS) and optical emission spectroscopy (ICP-OES) are recommended in the test guideline however limitations (e.g. with regards to the presence of dissolved material or non-detectable elements) are also noted there.

Furthermore, the OECD GD 318 provides further considerations on alternative methods (including qualitative and (semi)quantitative ones). UV/VIS photometry, for instance, can be used as a quantitative method. However, careful considerations of its applicability based on material properties is needed. This method provides indication of the maintenance of particle size and number in suspension therefore, nanoform stability can to some extent be monitored. Additionally, non-quantitative measurements such as electrophoretic mobility, *i.e*. Zeta potential, can also be used as an indicator of particle (in)stability. Further information on methods applicability can be found in "NanoDefine Technical Report D7.6" [23].

Dispersion stability based on heteroagglomeration

Heteroagglomeration, *i.e*. the interaction between two particles of different nature, is considered the most relevant type of agglomeration process to be investigated for fate evaluation, as it is performed under test conditions closer to natural settings.
A test guideline to determine attachment efficiency of heteroagglomeration is not yet available but OECD GD 318 provides guidance on how a heteroagglomeration test could be performed. The heteroagglomeration kinetics depend on number ratios of nanoparticles and suspended particulate matter (SPM) in the system, as well as the collision rate constant, which depends on particle size, density and velocity gradient. Agglomeration rate, sedimentation or attachment efficiency provide information on the heteroagglomeration behaviour of nanomaterials. Actually, heteroagglomeration attachment efficiency has been shown to be the most suitable measurement aiding in fate evaluation, as it measures the fraction of collisions resulting in attachment. Therefore it provides a better proxy to set the study design for fate (e.g. bioaccumulation) and ecotoxicological test conditions.

1.2.2.3 Waiving of partition coefficient n-octanol/water for nanoforms

Annex VII, Section 7.8 of REACH states in column 2 that: "*For nanoforms, whether of inorganic or organic substances, for which the partition coefficient n-octanol/water is not applicable the study of dispersion stability shall be considered instead".*

There are currently constraints in assessing the n-octanol/water partition coefficient (K_{ow}) of the nanoforms, as explained in Section 1.2.2.1. Therefore, waiving n-octanol/water partition coefficient information requirement should always be accompanied by a robust technical and scientific justification on the applicability of the used test method (e.g. showing absence of water solubility or a low dissolution rate of the nanomaterial).
In case of partial solubility and dissolution rate of the nanoforms, K_{ow} can be considered and measured for the soluble fraction, at the same time than dispersion stability for the particulate fraction (see **Figure 2**).

As explained in the parent guidance (Section R7.1.8.1.) the n-octanol/water partition coefficient (K_{OW}) is used in numerous estimation models and algorithms for environmental partitioning, sorption, bioavailability, bioaccumulation and also human toxicity and ecotoxicity. To prevent erroneous interpretation of such models, where the nanoforms is not fully dissolved information on the dispersion stability may provide useful information instead of K_{ow} (see Section 1.2.2.2 for information on dispersion stability). Nevertheless, information on dispersion stability should not be seen as a one-to-one replacement of K_{OW}. Furthermore, the use of K_{ow} is not sufficient to waive the generation of further data on nanoform fate (e.g. column 2 adaptation of bioaccumulation information requirement) on its own (see ECHA Guidance R7c).

Currently there are no standardised methods for fate descriptors to predict the behaviour and transport of nanoforms in the environment and biological media as alternatives to n-octanol/water partition coefficient [48], [49]. Environmental fate descriptors for nanoforms are further discussed in Section 1.2.5 on adsorption/desorption, where state-of-the-art on attachment efficiency of nanomaterials is reported (e.g. OECD GD 342 [58]). Also, there is a list of the models and specific parameters under development, suggested as alternative methods to K_{ow} and K_{oc} as alternative fate descriptors, but sufficient validation is still pending (available in **Appendix 1**).

1.2.3 Granulometry

The data requirement in accordance with REACH Annex VI section 2.4.2 for "*number based particle size distribution with indication of the number fraction of constituent particles in the size range within 1 nm – 100 nm"* applies for each registrant of a joint submission whereas the data requirement in accordance with REACH Annex VII section 7.14 for "granulometry" applies jointly for the members of the joint submission. As REACH Annex VI requires that "*information shall be reported in such a manner that it is clear which information in the joint submission pertains to which nanoform of the substance"*, in practice the registrant submitting the Annex VII-X dataset corresponding to a nanoform or a set of nanoforms submits the granulometry data.

The size distribution of constituent particles as well as aggregates and agglomerates may have an impact on the selection of the most appropriate route of exposure, on the intake of the particles within cells or organisms and on sample preparation. Therefore, the granulometry data should provide any additional information on the particle size distribution of the nanoform necessary to carry out the hazard assessment on the actual test material ("as received", "as used", "as dosed/as exposed"). Thus, it is recommended to provide as a minimum the granulometry information of (all) the test material(s) used in tests to fulfil Annex VII-X information requirements.

The different characterisation parameters of nanoforms, such as the constituent particle size, the shape of the particles and the surface treatment, may have a significant impact on the granulometry of those nanoforms. Therefore, when the granulometry data generated on one nanoform is used to fulfil the data requirement for another nanoform, the differences in the characterisation parameters must be addressed in the read-across justification. The same applies equally to read-across between a nanoform and a non-nanoform.

As with non-nanoforms, information on granulometry is relevant for the assessment of exposure to airborne particles/dusts, as well as for the performance of toxicity studies via the inhalation route. For the purpose of performing inhalation toxicity studies, the most relevant parameter to measure and to report is the aerodynamic diameter of the particles. The aerodynamic diameter is defined as diameter of a spherical particle with a density of 1000 kg/m^3 that has the same settling velocity as the particle in question. The mass median aerodynamic diameter (MMAD) is generally reported, and is the aerodynamic diameter where 50% of the particles by mass are below that size, and 50% of the particles are above that diameter. Note that this guidance is not intended to describe how to generate or characterise exposure conditions for the purpose of inhalation toxicity studies for nanomaterials. Some guidance on this subject can be found in ISO/TR (Technical Report) 19601:2017 [60]: The generation of aerosols for the purpose of inhalation toxicity studies.

For reaching a conclusion on granulometry, it has to be taken into account that the potential release of particles into the workplace or environment is an important consideration in the design and operation of many industrial processes and safe handling of substances. Release of particles may present hazard and could cause adverse health effects to humans and affect the environment. It is therefore important to obtain data about the propensity of substances to be released as particles, allowing risks to be evaluated, controlled and minimised. Measurement of

the release of particles from powdered substances has similarities to the conventional measurement of the dustiness of a powder, but with significant differences in the methods and instrumentations suited to different particle size ranges.
A number of methods are available for determining the particle size fractions (Section 1.2.3.1) which are then used to assess the possible health effects resulting from inhalation of airborne particles in the workplace. A number of methods covering different ranges of particle sizes are available though none of them is applicable to the entire size range. Multiple techniques should be used where possible in order to formulate a complete understanding of the particle properties, and the optimum set of required techniques should be selected based on the specific substance and form under investigation. Furthermore, also the other characterisation parameters of the particles such as shape may impact the applicability of a method for the particle size distribution measurement.

1.2.3.1 Test methods for granulometry

The characterisation of particles requires very careful sampling and sample fractionation practises to be followed. ISO 14488:2007 [61] specifies methods for obtaining a test aliquot from a defined sample of particulate material (powder, paste, suspension or dust) that can be considered to be representative with a defined confidence level. Further information is available in Section 1.1.2 of this appendix on Sample Preparation.

The methods to measure the particle size distribution of the constituent particles, aggregates and agglomerates and/or mass median aerodynamic diameter must be such that they are applicable for nanoforms. The methods specified in the OECD TG 125 for particle size and particle size distribution of nanomaterials [62] and the method listed in **Table 1** can be used to measure the particle size and size distribution of nanoforms to fulfil the Granulometry endpoint. Also **Table 2** in Section 1.2.4 can be consulted for some methods to measure particles and fibres.

Table 1: Methods of measuring airborne dispersed or nebulised particles

Method and details	Material and size range	Data type
Scanning Mobility Particle Sizer (SMPS) (ISO 15900:2020 [63]**; ISO 10808:2010** [29]**; ISO 28439:2011** [64]**)** SMPS operates by charging particles and fractionating them based on their mobility when passing between electrodes. This method combines a Differential Mobility Analyser (DMA) and an Optical Particle Counter (OPC). SMPS detects and counts particles, and enables measurement of the particle size distribution and count median diameter of nano aerosols, up to 10^8 particles/cm³. This method also allows evaluation of particle surface area, mass dose, composition and dispersion to support effective analysis of inhalation toxicity testing results. SMPS also has useful application in relation to exposure estimation. Measurement with SMPS is the only currently available method that meets all of the following requirements in the size range below 100 nm: i) measurement of particle size distribution during particle exposures in a continuous manner with time resolution appropriate to check stability of particle size distribution and concentration; ii) measurement range of particle sizes and concentrations covers those of the particle aerosols exposed to the test system during the toxicity test; iii) particle size and concentration measurements are sufficiently accurate for particle toxicity testing and can be validated by ways such as calibration against appropriate reference standards; iv) resolution of particle sizing is sufficiently accurate to allow conversion from number-weighted distribution to surface area-weighted or volume-weighted distribution. However, SMPS is relatively slow and requires a scanning approach to measure different size intervals in series. This method is restricted to ambient temperatures below 35 °C (due to evaporation of butanol in the CPC) and requires aerosolisation of the sample. SMPS cannot distinguish between agglomerates and primary particles. For non-spherical particles (e.g. high	Particles in an aerosol Size range: ~1 nm – 1 µm	Size distribution based on number counted (number count per size interval). From the distribution, MMAD can be calculated, with knowledge of the density of the particles.

Method and details	Material and size range	Data type
aspect ratio nanomaterials), estimation of diameter and mass concentration by SMPS can result in significant error. Assembling data of measurements from SPMS and OPC to provide a whole picture of particle size distribution is not appropriate, due to the different principles employed by the two methods [65]. It is important to know the stability of the source, since rapid changes of the size distribution, particle concentration, or both, can affect measurement of the size distribution. This is relevant to consider for nanomaterials, which have a high tendency to agglomerate in the atmosphere		
Fast Mobility Particle Sizer (FMPS) FMPS enables determination of the size distribution of sub-micrometre aerosol particles, up to 10^7 particles/cm³ (depending on particle size). Measurements can be made with a time resolution of one second or less, enabling visualization of particle size distributions in real time. However, FMPS is typically less sensitive than the SMPS at low particle concentrations.	Particles in an aerosol Size range: ~5 - 560 nm	Size distribution based on number counted (number count per size interval). From the distribution, MMAD can be calculated, with knowledge of the density of the particles
Diffusion batteries The operation of diffusion batteries is based on the Brownian motion of the aerosol particles. Depositional losses through diffusion are a function of particle diameter. By measuring diffusion based deposition rates through systems with varying geometries, it is possible to determine particle size distribution. The deposition systems are usually placed together in series to form a diffusion battery. The diffusion battery can be designed for determination of particle sizes as low as 2 nm depending upon instrument setup. This method has useful application in relation to exposure	Particles in an aerosol Size range: 0.005 – 0.1 µm	Particle number in intervals according to diffusion diameter, from which the median diffusion diameter can be determined with knowledge of the density of the particles.

Method and details	Material and size range	Data type
estimation. The primary property measured is the diffusion coefficient of the particles and this has to be converted to particle diameter. The instrument needs to be operated with a particle counter (typically a continuous flow Condensation Particle Counter) in order to determine the number concentration before and after each diffusion stage. Inversion of the raw data to real size distribution is complex and the solutions of the equations do not give unambiguous results in the case of polydisperse aerosol size distributions. ISO/TR 27628:2007 [66] provides an informative description of this method.		
Optical Particle Counter (OPC) OPC is a widely used method for detecting and counting aerosolised particles, and operates across a wide temperature range (0 – 120 °C). Enables agglomerates/aggregates of primary particles to be measured and counted. OPC has useful application in relation to exposure estimation. However, OPC is insensitive to particles smaller than approximately 100-300 nm in diameter and provides insufficient coverage of potential primary particle. Assembling data of measurements from SPMS and OPC to provide a whole picture of particle size distribution is not appropriate, due to the different principles employed by the two methods [65]. ISO/TR 27628:2007 [66] provides an informative description of this method.	Particles in an aerosol Size range: 0.3 – 17 µm	Particle number concentration
Laser scattering/diffraction In general, the scattering of the incident light gives distinct pattern which are measured by a detector. This technique is particle property dependent – i.e. material has unique scattering and	Particles of all kind Size range: 0.06 - 100 µm	Particle size/size distribution, from which mass median diameter can be

Method and details	Material and size range	Data type
diffraction properties which are also particle size dependent. It is important to calibrate the instrument with similar material (of the same size range as the material to be measured). Laser scattering techniques are suitable for geometric particles, viz spheres, cubes and monocrystals. Particle size will be established optically. The MMAD can be calculated by means of a calculation correction. The method is suitable to determine the distribution of particles of respirable and inhalable size. Laser diffraction assumes a spherical particle shape. Test products should therefore have no extreme aspect ratios, with a restriction of 3:1 for non-spherical particles. This method has limited applicability really suitable in the sub-100 nm range. In the range below several microns, results strongly depend on optical constants of particles.		calculated, with knowledge of the density of the particles.
Light scattering aerosol spectrometer (LSAS) LSAS is a type of light scattering instrument, applicable for measuring the size, number concentration and number/size distribution of particles suspended in a gas. LSAS can be used for the determination of the particle size distribution and particle number concentration at relatively high concentrations of up to 10^{11} particles/m^3. The large measurement range of LSAS may result in high uncertainty in nanoscale measurements. Measurements may be dependent on the reflectivity of particles. Laser diffraction assumes a spherical particle shape. Test products should therefore have no extreme aspect ratios, with a restriction of 1:3 for non-spherical particles. This method has limited applicability really suitable in the sub-100 nm range. In the range below several microns, results strongly depend on optical constants of particles.	Particles in an aerosol Size range: 0.06 - 45 µm	Particle size/size distribution, from which mass median diameter can be calculated, with knowledge of the density of the particles

The particle detection methods in **Table 1** can be used to characterise the distribution of aerosolised particles. These methods are preferred since they measure particles in the air and as such the mass median aerodynamic diameter (MMAD) and geometric standard deviation (GSD), but are subject to limitations. All instruments to determine particle size are limited to specific ranges of particle size depending on the principle of operation. Therefore, more than one type of instrument is often used with overlapping size ranges. Often depending on the material, these size distributions may not match exactly, because different measuring principles deliver different equivalent diameters. Moreover, the lower sizes of 1nm to 3 nm cannot be accurately measured in aerosol measurement instrumentation because of diffusion losses in tubes or at the inlet of the instruments. Aerosolisation of substances for particle size distribution characterisation also results in a degree of artificiality if the engineering set-up introduces an upper limit on the aerosol size as a result of the operational conditions (e.g. flow rate and exit orifice). The upper size limit can be predicted using Stoke's equation. Other methods that measure inhalable fractions only or that give no detailed distributions are detailed in **Table 2**.

1.2.3.2 Published data on granulometry

Information on granulometry of nanoforms has been published in peer-reviewed literature. Some electronic databases exist collecting published information on properties of specific nanomaterials, including information on granulometry. However, registrants need to ensure that the data available is relevant for the specific nanoforms in their dossiers before using this for the purpose of REACH registrations. This includes ensuring that the test material is identified and well characterised and that the used method is described in detail and, where applicable, the test is carried out in accordance with the test guidelines. It should be also clear if the measured particle size corresponds to the constituent particle size and/or agglomerates and aggregates.

1.2.4 Dustiness

Annex VII , Section 7.14 bis of REACH includes "dustiness" as an information requirement only for nanoforms.

1.2.4.1 Type of property

Dustiness may be defined as the propensity of a powder to form airborne dust by a prescribed mechanical stimulus. It depends on a number of factors such as physicochemical properties of the particles (e.g. size, shape, relevant density, type of coating), the environment (e.g. moisture, temperature), the type of process (e.g. energy applied), the interaction between particles during agitation (e.g. friction shearing) and the sampling and measurement configuration.

Dustiness is of considerable importance for the exposure and risk assessment of particulate materials as:

- It is important when considering the potential workplace exposure;
- It is used as an input parameter for control banding and exposure modelling tools for nanomaterials;
- Knowledge on dustiness can be used to improve the product characteristics (e.g. create less dusty products) and help users of the products to choose products that potentially may lead to lower exposures for consumers.

1.2.4.2 Test methods for dustiness

There are currently no standardized methods for dustiness at OECD level. However, the OECD WNT project 1.8. [47] will provide a specific TG/GD: "Determination of the dustiness of manufactured nanomaterials". While this is ongoing work, CEN has published 5 standards (EN 17199: 1-5) for the testing of dustiness of materials that release or contain nanomaterials. EN 17199-1 [67] gives advice on the methodology and provides guidance to choose the most adequate test method. The other 4 standards, EN 17199-2 to 5 [68], [69], [70], [71], provide further details on the different test methods.

- Rotating drum (EN 15051-2 and also included in EN 17199-2) [68]
- Small rotating drum (EN 17199-4) [70]
- Continuous drop (EN 15051-3 and also included in EN 17199-3) [69]
- Vortex shaker (EN 17199-5) [71]

Table 2: Test methods to measure dustiness of bulk materials that contain or release nano-objects or submicrometer particles

Method and details	Material and size range	MMAD
Rotating drum method (EN 15051-2; EN 17199-2) [72] **, [65] and small rotating drum method (EN 17199-4) [67]** The rotating drum and small rotating drum methods involve the continuous multiple dropping of a sample of the bulk material in a slow horizontal winnowing current of air. The dust released from dropping bulk material is conducted by the airflow to a sampling section where aerosol real-time instruments measure time-resolved particle concentrations and time-resolved size distributon of the aerosol generated. In addition, airborne nano-objects and strutures can be collected for off-line (analytical) electron microscopy analysis. For the small rotating drum, a respirable dust cyclone collects the dust fractions onto a suitable media for gravimetric analysis. For the rotating drum, the determination of the inhalable, thoracic and respirable mass fractions of the released dust is carried out sparetlely according to EN 15051-1 [73] and EN 15051-2 [72]. The small rotating drum requires smaller amounts of bulk material for testing (2 to 6 g) compared to the rotaling drum method.	Dry powders/granulates/friable products Size range: 0.01-10,000 µm	MMAD (mass median aerodynamic diameter) can be determined via an appropriate coupled analytical technique.
Continuous drop method (EN 15051-3) [74] This method is based on the size selective sampling of an airborne dust cloud produced by the continuous single dropping of material in a slow vertical air current. The dust released by dropping material is conducted by the airflow to a sampling section where it is separated into the inhalable and respirable fractions. This method is suitable to determine the distribution of particles of respirable or inhalable size. The continuous single-drop method requires a total amount of 500 g for the required five single test runs. It has been highlighted that such large amounts of test material may not be practical if very toxic and/or costly materials are to be tested and there is a need for test systems that can be operated under controlled atmospheric environments using much smaller amounts of material [75].	Dry powders/granulates/friable products Size range: 0.5-10,000 µm	MMAD can be determined via an appropriate coupled analytical technique.

Method and details	Material and size range	MMAD
Vortex shaker (EN 17199-5) [71] The vortex shaker method consists of especially designed cylindrical container that is continuously shaken according to a circular orbital motion, and in which a small volume (0.5 mL) of the test sample is placed. The released aerosol is transferred to the sampling and measurement section. The aerosol real time instruments measures time-resolved particle concentrations and the time-resolved size distribution of the aerosol generated within the vortex shaker. In addition, airborne nano-objects[5] and airborne nano-structures can be collected for off-line (analytical) electron microscopy analysis. A respirable cyclone collects the dust fractions onto a suitable media for gravimetric analysis.	Dry Powders Size range: 10 nm-1000 nm	

It is recommended to choose the methods most relevant to simulate the operations/tasks expected to be performed. The first three methods are intended to simulate workplace scenarios where handling involves dropping and differ in the intensity and duration of the treatment for the material. The vortex shaker intends to simulate a worst case scenario, where the higher energy is applied to the material.

Each of the standards details the methodology for dustiness testing including sample preparation, determination of moisture content etc. The standards propose a number of measurands of dustiness and in addition, they establish test methods that characterise the aerosol from its particle size distribution and the morphology and chemical composition of its particles. It is recommended to test one of the following measurands:

- Respirable dustiness mass fraction (mg of airborne respirable particles/per of kg tested materials)
- Number based dustiness index from 10 nm to 1 µm (particles per milligram)
- Number based emission rate (particles per milligram/s)

[5] Nano-object: material with one, two or three external dimensions in the nano scale [source EN 17199-1:2019]

1.2.4.3 Exposure based waiver for dustiness

Annex VII of REACH provides in column 2 the following specific rules for adaptation of the standard information requirement for dustiness: *"The study does not need to be conducted if exposure to granular form of the substance during its life-cycle can be excluded."*

Granular can be interpreted to mean "*particles*". All nanoforms, by definition, will have at least 50% of their particles by number below 100 nm, and all nanoforms will be "*granular*" when available as a dry powder. However, some nanoforms are available only in suspensions, or are incorporated into a matrix throughout their entire lifecycle. In this case, there may be no exposure to the dry powder, or the granular form in general.

Exposure based waiving should be accompanied by scientific support to show that exposure to the dry powder or the granular form can be excluded during the entire lifecycle of the nanoforms.

1.2.5 Adsorption/desorption

In the parent guidance, the methods for determining this endpoint are shown in Table R.7.1-14 "*Methods for the measurement of adsorption*". Adsorption/desorption measurements are used in fate modelling to indicate which compartment in the environment will be exposed the most or might need to be considered in hazard and risk assessment. These measurements help to determine in which environmental compartment *i.e.* soil, sediment or water, the substance is most likely to end up and whether it is likely to be mobile or immobile. For instance, high adsorption to soil would show that both soil and sediment are highly relevant environmental compartments to be considered in hazard assessment.

Adsorption is temporary (reversible) or permanent bonding between the substance and a surface. With regards to nanoforms, the distribution coefficient between solid phase and a liquid phase (K_d) very often must be based on actual testing since estimations of K_d derived from the organic carbon-water partition coefficient (K_{oc}) and the octanol-water partition coefficient (K_{ow}) is not applicable for most nanoforms, *i.e.* non-highly dissolving nanoforms (see Section 1.2.1). K_d measurement is based on the assumption of a thermodynamic equilibrium between a liquid and a solid phase. Equilibrium partitioning does not apply to undissolved nanomaterials [53], [48], [49], [21] as described in Section 1.2.2. Nanomaterial dispersions can be kinetically stable for a long period of time (typically through electrostatic or steric stabilization) but they will never reach thermodynamic equilibrium and consequently cannot be equilibrated with an additional phase [49], [76] [77].

Therefore, nanomaterials strive to reduce their surface energy by attaching to other particles in the system. This attachment can be:

- homoagglomeration/aggregation between the particles of the same nanomaterial/nanoform, or
- heteroagglomeration/aggregation with other particles, or
- to the interface between phases (deposition or attachment).

Because of the inability to accurately quantify the physico-chemical forces contributing to particle attachment, this step is typically described by an empirical parameter termed the particle attachment efficiency (α) that needs to be determined in agglomeration (heteroagglomeration) or deposition experiments [48], [77].

OECD TG 106 Adsorption – Desorption Using Batch Equilibrium Method [78] is partially inadequate when the substance in question has a low dissolution rate, i.e. is present as a dispersion, because it is currently not possible to differentiate between adsorbed or

aggregated/agglomerated particles settled during the centrifugation step, and a new TG needs to be developed ([20], [79]). However, if it is shown that a nanomaterial has a high dissolution rate, it can be assessed in the same way as non-nanoforms of substances and the parent guidance will apply (as explained under Section 1.1.2.1 and Section 1.2.1).

1.2.5.1 Relevant method to measure adsorption/desorption of nanoforms

OECD TG 312 Leaching in Soil Columns [59] allows to study the mobility and leaching of the test substance into deeper soil layers or ground water. While it is agreed that the OECD TG 312 is generally applicable for the testing of nanoforms, a GD using this TG to test nanomaterials was established and published on 28 July 2021 OECD GD 342 [58].

This guidance provides the information and methods to measure the soil adsorption behaviour of nanoforms (mobility and retention) so that such tests can be applied to assess quantitively the mobility of nanomaterials in soils and deduce attachment efficiencies.

OECD TG 312 implies thermodynamic processes where non-nanoforms of substances often will reach an equilibrium [59]. As such processes are not applicable for nanoforms, attachment efficiency for heteroagglomeration (αhetero) can be calculated instead ([58]).

α_{hetero} expresses the probability that nanomaterials will attach when they collide with the soil grain surface and takes into account random effects caused by the way the soil matrix happens to be structured ([80]). A quantitative estimation of α can be obtained where a continuous nanomaterial input is applied into the column transport test and the nanomaterial concentration is monitored over time in the eluate as it is the case within TG 312. However, it needs to be noted that the determination of αhetero is based on the "clean bed" assumption, which is valid only during the early stages of the deposition process, when low particle loadings are applied and no significant repulsion between particles and the porous medium is present. Outside these settings more complex mechanisms can influence the particle transport (e.g. blocking, ripening) and with αhetero not being able to accurately describe the system anymore, leading to misinterpretation and misuse of the data.

To reliably report on nanomaterial transport in soils using OECD GD 342, the overall recovery (mass balance) of the nanomaterial should be reported. In accordance with GD 342 on the OECD TG 312, a recovery (for non-labelled nanomaterials) of at least 70% should be considered, but it is acknowledged that this strongly depends on many different variables. Thus, the test set up needs to be reviewed individually considering all the parameters and test set up (such as particle type, the choice of application, spiked amount and used soil), when the suggested recovery of 70% is not reached.

The selection of test soils has to relate to environmental relevance rather than to properties, and at least two soils differing either in pH, organic carbon content, clay content and/or texture should be considered. Generally, soils with high clay content are to be avoided because here particle transport occurs predominantly in macropores [81] making experiments with saturated, stacked soil cores unrepresentative for nanoform transport rates.
This reduction of number of soils to two from the parent TG is based on reasons of practicability. Because soils with high clay content (soil 1 in OECD TG 312) tend to block during leaching (nanoforms strongly attach to the clay minerals preventing break through [82], [83], [84]) and sandy soils with high carbon content (soil 5 in OECD TG 312) are only of limited availability. In case natural soils are used a control experiment with soils not previously exposed to nanomaterials has to be conducted to determine the background of naturally occurring nanoforms.

To account for more realistic conditions of nanomaterial mobility in soils for which a considerably longer residence time is expected, flow rates of 2-3 $L \cdot m^{-2} \cdot h^{-1}$ are to be used, avoiding an artificial break through and posing as a realistic worst-case scenario. As a leaching solution mimicking

artificial rain fall, aqueous solutions of 0.005 M KCl or NaCl should be used. For nanoforms reacting with chloride (e.g. silver that would precipitate and render them unavailable for the chemical analysis), other suitable anions such as NO_3 (KNO_3) have to be used. Furthermore, the use of divalent salts such as $CaCl_2$ will not provide a worst case scenario test as nanomaterials homoaggregates are generally less mobile in the presence of Ca^{2+}. The chosen monovalent salt should not react with the nanomaterial, e.g. accelerating its dissolution and the stability of the nanomaterial suspension should be measured prior to the column test. To mimic conditions where the soil pore water is dominated by divalent cations a solution of 0.005 M $CaCl_2$ can be used according to GD 342. However, it has to be taken into account that this will not work for nanomaterials reacting with chloride such as silver. Furthermore, it does not present a worst case with respect to mobility.

The advice provided here is not applicable to nanomaterials with high dissolution rates (see Section 1.1.2., 1.2.1). For those nanomaterials, it might result in testing the adsorption behaviour of the dissolved fraction and not the nanoform. The advice is also not applicable for nanomaterials with low dispersion stability in aqueous media (with definitions given in OECD TG 318 (2017) [57]). Testing of such nanomaterials will result in a not-representative and not-reproducible addition of test material to the soil column. Guidance on how to determine dispersion stability/dissolution rate of the nanoforms in environmentally relevant conditions can be gained from OECD TG 318. Information on the use of the data for further environmental testing and assessment strategies can be found in GD 318 [8]. Testing of these parameters has to be performed before applying OECD TG 312, GD 342.

1.2.5.2 Alternative fate descriptors to adsorption/desorption of nanoforms

A list of available models to predict alternative fate descriptors for nanomaterials is available in Error! Reference source not found.. The OECD published results of a study where 10 nanospecific environmental exposure tools and models were tested for functionality and accuracy. Three of the models mentioned in Appendix 1 – namely nanoFATE, simplexbox4nano and nanoDUFLOW are part of this report and details on the functional assessment as well as the outcome of the statistical analysis can be found there [85].

These models are still under development and further validation is needed, in particular with regard to the uncertainties and applications. Such validation is especially important when these models are used for exposure of the environmental compartment and organisms. When they are thoroughly validated models, they will be recommended as an option to provide suitable alternative information on the sorption and agglomeration/aggregation of nanomaterials.

1.2.5.3 Waiving of adsorption/desorption to nanoforms

Annex VIII of REACH, Section 9.3.1. states in column 2: " *For nanoforms, use of any physicochemical property (e.g. octanol-water partition coefficient) as a reason for waiving the study shall include adequate justification of its relevance to low potential for adsorption."*

It is necessary to take into account the nanoform specific properties and constraints in assessing the adsorption/desorption properties of nanoforms by currently available methods, based on K_d derived from the organic carbon-water partition coefficient (K_{oc}) and the octanol-water partition coefficient (K_{ow}), such as OECD TG 106 or with the use of particle attachment efficiency (α) specifically developed for nanomaterials. Consequently, waiving the information requirement based on low adsorption/desorption similarly to K_{ow} and dissolution rate should always be accompanied by a robust technical and scientific justification on the applicability of the used test method with further justifications on nanoforms behaviour in soil and sediment (e.g. nanoform being water soluble or having a high dissolution rate as detailed under Section 1.2.1 or not being dispersed as detailed under Section 1.2.2).

2. RECOMMENDATIONS FOR TOXICOLOGICAL INFORMATION REQUIREMENTS for NANOMATERIALS

2.1 General advisory notes

2.1.1 General advisory note on testing with nanoforms and sampling strategy and sample preparation for human health endpoints

These advisory notes do not describe any detailed protocol but, instead, aim to provide useful advice with regard to specific aspects that are particularly important for nanoform testing, and references to relevant resources. For a test material fulfilling the definition of a nanoform, the testing strategy (Fig. 5) is dependent on its solubility and/or dissolution rate in water. Dissolution rate in relevant biological fluids is an important parameter in toxicokinetics evaluation, and may also be used for grouping and building of the read-across or to substantiate the weight of evidence[6] in the cases where existing studies with nanoforms are available.

The figure below shows a decision tree that can be used to determine whether nanospecific advice should be used, or, due to the conclusions on the nanoform's properties, the advice provided by the parent guidance can be followed instead.

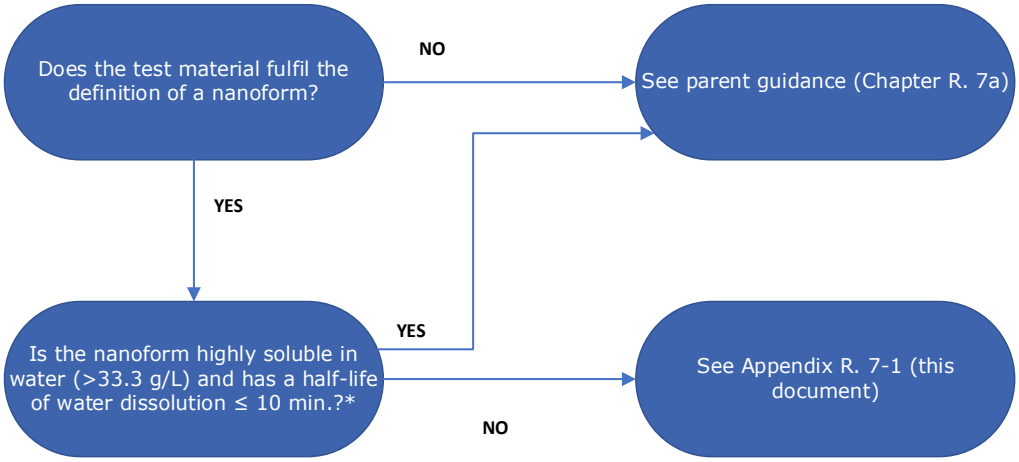

Figure 4: Decision process for selecting the applicable guidance document
*The given values [16], [86], may be updated when more validated information becomes available with regard to testing in relevant biological media.

2.1.1.1 Considerations on solubility and dissolution rate

The Scientific Committee on Emerging and Newly Identified Health Risks (SCENIHR) has stated that many nanomaterials have considerable solubility and that for "*these materials the interaction with living systems remains close enough to the bulk chemical agent to justify the use of well-established toxicological testing procedures and approaches*" [87]. Water solubility may give a first indication on the (none) biopersistence of a nanomaterial [88]. EFSA Guidance on Technical Requirements [16] considers, a solubility equal to or higher than 33.3 g/L as a high solubility in water, in line also with the SCCS Guidance (2019) [89] and with the categories for degrees of solubility proposed by JECFA [86] and the European and US Pharmacopoeias [16]. Based on a pragmatic and consistent approach among different regulatory bodies, a similar value

6 https://echa.europa.eu/documents/10162/17250/practical_guide_how_to_use_alternatives_en.pdf/148b30c7-c186-463c-a898-522a888a4404

for water solubility is recommended also here to decide whether this guidance is applicable (see **Figure 4**). In the context of risk assessment in occupational settings, BAuA [90] proposed that nanomaterials with a water solubility above 100 mg/L could be considered as soluble[7] and thus not biopersistent.

The latest approaches for the risk assessment of nanomaterials recommend a strategy in which the dissolution rate and equilibrium in water is a first key element [91], [16]. Dissolution rate is a kinetic process which informs not only whether a substance dissolves but also on how long it takes to dissolve. Dissolution rate depends on the size and surface properties of the particles and fibres, and also on the nature of media where the release of ions occurs, on the temperature, the pH and the presence of substances interacting with the nanoform's surface. The pH and temperature should be specified and relate to biologically relevant conditions. Another important factor in the dissolution of nanoparticles is aggregation [92]. Aggregation decreases the available external surface area thereby reducing the extent of dissolution [93]. It has been observed that the dissolution rate decreases as the aggregation state of nanoparticles increases.

To determine the dissolution rate in water, the protocol proposed by EFSA [16] is recommended. In the EFSA Guidance on Technical Requirements [16] a high dissolution rate in water is defined as a half-life of 10 min or less corresponding to a dissolved fraction equal to or higher than 88% in 30 min [16]. In this context EFSA Guidance on Technical Requirements refers to '*concentration corresponding to exposure at maximum use level*'. A similar threshold for dissolution rate in water is proposed in this guidance (see Fig. 5).

Annex VII, section 7.7. column 1, of the REACH Regulation specifies, that (beside the water solubility testing) "*for nanoforms, in addition the testing of dissolution rate in water as well as in relevant biological and environmental media shall be considered*" (see section 1.2.1).

The determination of the dissolution rate (*k*) as well as the dissolution half-life in biological fluids provides an insight on how a certain particle may interact with its biological environment [92] and allows an informed decision for the design of the repeated dose studies. The dissolution rates are important parameters in toxicokinetics evaluation, which can also be used for grouping and read-across. Recently a method and thresholds [45] have been proposed for grouping nanoparticles based on dissolution rates in phagolysosomal simulant fluid.

Until dissolution occurs, the kinetics of nanomaterials are governed by their particulate nature, whereas after dissolution the (dissolved) ions or molecules determine the toxicokinetics [94]. Thus, if the dissolution rate is not very high, uptake of particles may occur. A very high dissolution rate is considered close to instantly dissolved [91], and means that the material dissolves quickly at the site of entry, so that cellular uptake in the lung or gastrointestinal tract occurs almost exclusively as the dissolved species. When particles remain present for a longer duration, they can be taken up by macrophages or translocated across the epithelium as particles. Effects due to release of ions or molecules after distribution to cells/tissues (i.e. the so-called "Trojan-horse" mechanism, in which nanoparticles are internalized within cells, then release high levels of toxic ions), should be investigated, as the local dissolution in the macrophages and in tissues may lead to a different toxicity profile as compared to the solute itself. For example, although the toxicity of ZnO nanoforms is predominantly driven by the release of Zn^{2+} ions, *in vivo* studies showed that ZnO nanoparticles, but not the soluble ions, triggered the recruitment of eosinophils to the lungs [95]. In addition, the exposure to ZnO nanoparticles caused chronic effects that lasted up to four weeks. No aqueous extract caused such sustained inflammation, probably because soluble ions are rapidly cleared from the lungs [96]. Most probably the observed toxicity with ZnO nanoparticles is the result of the combined effects of the nanoparticles and of Zn^{2+} ions [97].

[7] Please note this value is only used as an indication for (non) biopersistence and should not be used as a threshold for solubility/insolubility in other contexts (such as triggering a waiver for insolubility for environmental endpoints)

The dissolution of a nanomaterial is a time-dependent process (depending on the rate of solubilisation and the surface area) and is directly related to its *in vitro* or *in vivo* biopersistence and biodurability that decreases with increasing dissolution rate [88]. In contact with a biological environment, the nanoparticles may undergo changes involving dissolution, re-precipitation, protein coating and agglomeration. Such events are part of biotransformation. Biotransformation could impact clearance and can also contribute to the biokinetics of nanoparticles [98] and potentially to their toxicity [99]. Therefore, biotransformation is an important element to investigate in the context of toxicokinetics. A method to assess biotransformation was recently described [98]. However, as no standard method applicable to all nanoforms is currently available, the method used must be justified and thoroughly documented. The information on biotransformation is not a mandatory requirement but it is considered important for the implementation of grouping and read-across using dissolution rates [98].

Dissolution studies in relevant biological fluids can also be performed to substantiate the weight of evidence[6] in cases were studies are already available and fulfill the criteria described in Section 2.2.2. OECD [100] lists as examples of biological media the artificial pulmonary interstitial fluid (Gamble's) balanced electrolyte solution (neutral) and alveolar lysosomal fluid (ALF) (acidic) as well as gastric (acidic) and intestinal (neutral) fluids. Different biological media may influence both the kinetics of dissolution and the saturation concentration depending in particular on pH and/or enzymatic action [101]. In addition, some water insoluble nanomaterials may be non-biodurable in biological fluids and this can be assessed from data on the dissolution rate. In general, the most appropriate route of administration, having regard to the likely route of human exposure, determines which biological media are relevant [91].

The OECD Guidance documents on inhalation toxicity studies [102] states that "*In order to decide whether your material is poorly soluble, the solubility of a solid material may be assessed by measuring solubility in a simulated biofluid. A poorly soluble material is generally understood as having a solubility of less than 0.1 g dissolved in 100 ml dissolvent within 24 hours*." Examples of dissolvent are the simulated biofluids. For the inhalation route of exposure, the simulated lung lining fluid and (macrophage) phagolysosomal simulant fluid are relevant for the assessment of dissolution via this route of exposure [102]. The data in the simulated lung lining fluid are important as this medium simulates the extracellular environment. Assessment of the stability in lysosomal conditions is important for the investigation of biopersistence and intracellular accumulation [92]). Within the cell, the nanomaterials generally distribute to lysosomes, where degradation can occur due to the acidic conditions and the presence of enzymes. Using an optimized abiotic flow-through method for 24 (nano)forms of 6 substances, Koltermann-Jülly *et al*. 2018 [45] proposed a grouping based on the dissolution rates in phagolysosomal fluid (pH 4.5). For example, a high dissolution rate (k>100 ng/cm^2/h) for a non-persistent nanoform corresponds to an abiotic dissolution ranging from 30% to 100% after 7 days. Indeed, a dissolution rate of 30-100% within 7 days is in line with a complete clearance during 21 days post-exposure.

It is important to assess the dissolution rate in both, simulated lung lining fluid and phagolysosomal fluid. For example, if dissolution only occurs in the lysosomal fluid but not in the simulated lung lining fluid, uptake of particles by lung macrophages as well as translocation across the lung epithelium can occur, as the lung lining fluid has been reported to stimulate the nanoparticle uptake by human alveolar epithelial cells [103]. Following translocation, the nanomaterials may relocate at a different location than the lung. In this case the read-across to the solute is not possible, since the dissolution of the nanoform (and release of the solute) takes place at a different location, which may lead to a different distribution profile. Comparisons of the distribution profiles for particles and solutes would help to assess whether read-across to the soluble form is possible.

For the oral route of exposure, dissolution of nanomaterials in simulated gastric fluid and macrophage phagolysosomal fluid is relevant. The macrophage phagolysosomal fluid may provide insight on the persistence of the nanoform after cellular uptake. A nanomaterial is

considered to dissolve quickly or to have a high dissolution rate if 12% or less of the material (mass based) remain as particles after 30 min of *in vitro* digestion in gastrointestinal fluid compared to the particulate concentration at the beginning of the dissolution test [104]. If the nanoform does not dissolve quickly in the gastrointestinal fluid, then dissolution in lysosomal fluid should be performed. A half-life of ca. 24 hours is considered indicative of high dissolution rate in lysosomal fluid. This would result in 12% or less of the material (mass based) remaining at 72 hours compared to the particulate concentration at the beginning of the dissolution test [104].
For dermal exposure, the dissolution rate in artificial sweat could be used.

2.1.1.2 Available methods for solubility and dissolution testing

Different analytical techniques/methods and their suitability to measure solubility of nanomaterials have been reviewed [105]. The determination of the solubility of nanomaterials in water should be done according to OECD TG 105 with specific considerations for nanoforms [106]. The pH and temperature should be specified for solubility testing and relate to biologically relevant conditions [107].

In the solubility/dissolution kinetics experiments, the solid phase needs to be separated from the liquid phase in order to avoid artefactual high solubility values by also analysing solid materials. Presence of particles can be checked qualitatively by e.g. Tyndall analysis and solid nanoparticles can be removed by ultracentrifugation or ultrafiltration [105].

In water, so far, no specific OECD testing guideline (TG) is available for determination of solubility and dissolution rate for nanomaterials but some may be adapted e.g. OECD TG 105 [42], [101], [105] and ISO 19057 [108].

Dissolution studies on particle systems are often carried out by inductively coupled plasma mass spectrometry (ICP-MS), inductively coupled plasma atomic emission spectroscopy (ICP-AES) and atomic absorption spectroscopy (AAS), which are a few of the most employed methods for quantitative analysis. Real-time dissolution kinetics measurements can be performed using Dynamic Light Scattering (DLS), UV/vis spectrometry, fluorescence spectroscopy and confirmed using inductively coupled plasma mass spectrometry (ICP-MS) [109].

In a complex matrix, it is acknowledged that measuring dissolution is highly challenging. At present there is no universal robust, rapid test method for regulatory testing that is applicable for all types of nanomaterials and all types of matrices [110]. During the NANoREG project, several methods have been identified for future use (e.g. ultrafiltration UF-ICP-MS/AES, ultracentrifugation UC-ICP-MS/AES, single particle sp-ICP-MS, and colorimetry). UF-ICP-MS/AES was considered a relatively easy and highly robust method that can be used for a rather broad range of nanomaterials if no nanomaterial-matrix interactions take place. If these interactions do take place, sp-ICP-MS is considered a good choice for measurement, given that the material is not below the size detection limit. Nevertheless, to select the best suitable method, knowledge of physical-chemical properties of the nanomaterial is crucial and it is recommended to use a combination of techniques [110].
Due to the fact that the assessment of dissolution in biological media is currently performed only by non-validated methods, the used protocols need to be well documented and justified.

Albeit not validated for nanomaterials, information from bioelution studies could be useful for read-across for metallic compounds. An ESAC Opinion on Scientific Validity of the Bioelution Test Method was finalised in July 2020 [111].

ENV/JM/MONO(2018)11 [112] compiled the available information on the determination of the biodurability (through dissolution) of different NMs included in the OECD Sponsorship Programme for the Testing of Manufactured Nanomaterials as indicator for their toxicity. With cellular and acellular *in vitro* tests, the dissolution of NMs is determined using static and dynamic

(such as flow-through) protocols in the presence of different biological and environmental media with different pH values and chemical compositions. New studies found that dynamic abiotic dissolution systems adequately predicted the overall pulmonary biopersistence of several nanoforms [45], [98]. A recent OECD Guidance document [106] provides guidance for the methods addressing dissolution rate and dispersion stability for nanomaterials, in particular with the development of the dynamic testing or flow through system that could be applied to estimate the dissolution in biological media. There is also ongoing work on the WNT project 1.5 on "Determination of Solubility and Dissolution Rate of Nanomaterials in Water and Relevant Synthetic Biological Media" aiming to provide harmonised approaches for testing solubility and dissolution rate of nanomaterials. In addition, development of an OECD Guidance document on "Integrated *in vitro* approach for intestinal fate of orally ingested nanomaterials" is under developement. It is based on a two-step approach in which the dissolution behavior of nanoform in simulated digestion environment is coupled with a biological evaluation of nanoparticles uptake/passage in a cellular model of intestinal barrier.

As long as no standard methods are available, detailed description and justification of the method including adequate controls (and reference materials, if possible) is required.

2.1.1.3 Test material characterization and reporting and sample preparation

The introductory text of Annexes VII-X of the REACH Regulation (EC) No 1907/2006 amended for nanomaterials, provides that: "*Without prejudice to the information submitted for other forms, any relevant physicochemical, toxicological and ecotoxicological information shall include characterisation of the nanoform tested and test conditions. A justification shall be provided where QSARs are used or evidence is obtained by means other than testing, as well as a description of the range of the characteristics/properties of the nanoforms to which the evidence can be applied*."

Prior to toxicological testing, the sample characterization and preparation including special considerations on dispersion and dosimetry, should be performed, as advised in the OECD Guidance on Sample Preparation and Dosimetry for the Safety Testing of Manufactured Nanomaterials [19], and as specified in Sections 1.1.1 and 1.1.2 of this Appendix. Additional useful information can be found in the report of the OECD expert meeting on the physical chemical properties of manufactured nanomaterials and test guidelines [113]. A harmonized preparation of the test sample will enable the comparison of the data and their further use. Information on the characterisation of test material serves multiple purposes:

a) enables linking to the identity of the nanoform/set of nanoforms being covered in the dossier and therefore supports data relevance.
b) provides general information on the material's properties 'as test sample' to support handling/storage and repeatability/reproducibility of results, and
c) facilitates the comparability and use of toxicological data for grouping of the nanoforms of a substance or justifying read-across between nanoforms, and between nanoforms and the non-nanoforms (For further information see *Appendix R.6-1 for nanoforms applicable to the Guidance on QSARs and Grouping* [31]).

Sections 1.1.2 and 1.1.3 of this Appendix explain in detail the importance of these physico chemical parameters for toxicological testing and also gives information on how these parameters can be determined. A critical review of the methods used to characterize the manufactured nanomaterials to assess their health and safety risks, describing the limitations and accessibility of each method is also available [114].

The chemical composition, the physicochemical properties, and the interaction of the nanomaterials with biological systems may in some cases influence aspects of exposure, deposition (lung), systemic uptake and bioaccumulation, dissolution, toxicokinetics and toxicity [114]. The hazards posed by all possible forms of the substance covered by a registration, including nanoforms/set of nanoforms, must be addressed by the toxicological and

ecotoxicological information provided in the registration dossier. In order to demonstrate that the results of a study are relevant for the hazard of a specific nanoform or set of nanorforms in the dossier, some information on the tested substance should be reported in the endpoint study record under the test material information field in IUCLID (please see more under "How to prepare registration dossiers for substances with nanoforms" [115] (https://echa.europa.eu/documents/10162/22308542/howto_prepare_reg_dossiers_nano_en.pdf/5e994573-6bf9-7040-054e-7ab753bd7fd6) in https://echa.europa.eu/manuals). The following parameters must be provided:

- Chemical composition (as described in the ECHA *Guidance for identification and naming of substances under REACH and CLP*);
- Characterisation parameters of the test material (as described in the ECHA Guidance *Appendix for nanoforms applicable to the Guidance on Registration and Substance Identification*
    - Number based particle size distribution with indication of the number fraction of constituent particles in the size range within 1 nm – 100 nm;
    - Shape and aspect ratio;
    - Description of surface functionalisation or treatment;
    - Specific surface area.

Moreover, *Appendix R6-1 for nanoforms applicable to the guidance on QSARs and Grouping of Chemicals* [31] provides an approach on how to justify the use of hazard data between nanoforms (and the non-nanoform(s)) of the same substance. The Guidance details some (additional) parameters that may be required to be able to assess whether the available hazard data are applicable for different nanoforms of a substance. The registrant may wish to consider taking into account such parameters when characterising the test material, in order to be able to follow the above-mentioned guidance. For example, the dissolution rate, surface chemistry and dispersability have been reported as a founding basis for the grouping of the nanomaterials ([31], [116]).

Nanoforms characterization (e.g. size, dissolved ion fraction, shape) in the exposure system at least at the start and end of the *in vitro* experiments is important to confirm their presence in the test system and to observe potential changes that they may undergo. This characterisation should also include the assessment of the state of agglomeration. This is an important parameter to investigate also for *in vivo* studies, and characterisation of aerosols is recommended in OECD 412/413. These TGs also state that "*scanning and/or transmission electron microscopy should always be used periodically (e.g., monthly) for monitoring and qualitative confirmation of particle size and shape for all particulates, not just nanomaterials*."

Suitable suspension-based techniques include Dynamic Light Scattering (DLS), analytical ultracentrifugation (AUC) or asymmetrical flow field-flow fractionation (AF4) coupled with inductively coupled plasma mass spectrometry (AF4/ICP-MS).

DLS has been used in many European projects (e.g. NANoREG, NanoReg2) in order to characterize nanomaterials in the medium. However, DLS is suitable to investigate the particle size distribution and works well with monodisperse, non-agglomerated and spheroidal nanomaterials. DLS is therefore, more appropriate in studies with more simple environments such as the ones used in dissolution studies. DLS cannot detect the presence of small aggregates and it is not able to resolve small modifications of the particle size distributions (PSDs) happening under physiological conditions [117].

Analytical ultracentrifugation (AUC) is one of the most accurate techniques for the characterization of nanoparticles in the liquid phase because it can resolve PSDs with high resolution and detail also in the sub-nm range [118]. AUC combines separation, concentration and detection steps into one single measurement improving total analysis times and reducing experiment complexity [117].

AF4 is the most commercialized flow field-flow (FFF) fractionation technique and by far the most widely used FFF technique for nanomaterials. It shows good selectivity and size resolution. By showing differences in PSDs, AF4 is also very useful for monitoring particle modifications, such as the adsorption of macromolecules on the surface, or dissolution or aggregation processes [119]. The online coupling of AF4 with multiangle light scattering (MALS), UV, differential refractive index (dRI) or fluorescence detection, or offline combination with TEM or UV, allows for detailed characterization of the physicochemical properties of different populations within a polydisperse sample with respect to size, molar mass or concentration, shape, conformation, structure, and aggregation state [120].

The capability of multi-element analysis is an added benefit when coupling AF4 with mass spectrometry. AF4/ICP-MS offer substantial advantages for detecting nanoparticles and assessing many parameters in complex matrices over traditional characterization methods such as microscopy, light scattering, and filtration [121]. AF4-ICP-MS has a good size resolution and can detect a very small nanoparticle size (up to 2 nm).

ICP-MS is a suitable method to quantify the dissolved fraction in the culture media, especially for metal nanomaterials. Single particle spICP-MS is a new promising method derived from using the inductively coupled plasma mass spectrometer (ICP-MS) in the time-resolved mode. The coupling of FFF to spICP-MS, allows nanoparticle dimensions to be determined at very low concentration levels, by choosing one or two specific chemical elements with the common instruments, or multi-elements with fast scanning quadrupoles or TOF (time-of-flight) mass analyzers [119]. Within the work alongside OECD projects to support and accelerate the development of Test Guidelines and Guidance Documents related to nanomaterial test methods, NanoHarmony project Task 1.4 (Determination of concentrations of ENMs in biological samples using spICP-MS) focuses on the optimisation and standardisation of spICP-MS but other techniques will also be addressed.

An integrated assessment of residual solids with respect to transformations of shape, size distribution, and crystallinity by protocols for preparation, analysis and statistical image analysis, using TEM, optionally supported by XPS and EDX is available [98]. However, XPS is currently not widely available. SEM and TEM are the "gold standard" techniques for nanoparticle size-, shape-, crystallinity- and composition- characterisation. While TEM is a highly valuable technique for the size analysis of nanomaterials, simple TEM sample preparation methods (by evaporation of the solution) do not distinguish between agglomerates formed during the drying of the sample on the support (grid) and aggregates already present in the NP suspension. Cryo-TEM might help to overcome this drawback by an ultra-fast conversion of the NP suspension into a vitrified film on the grid, allowing direct morphological visualization of nanomaterials at near native state, and thus, reducing the risk of aggregation and of the introduction of artefacts due to sample preparation [122]. However, the technique needs laborious sample preparation and particles or aggregates larger than 300 nm cannot be imaged.

The information on transformation is not a mandatory requirement but it is considered important for the implementation of grouping and read-across.

An important step in the sample preparation for *in vitro* or *in vivo* studies is dispersion. A generally applicable standard operation procedure (SOP) is currently not available for the dispersion of a dry powder in liquids. Since the use of a certain protocol or analytical technique depends on the type of nanomaterial and medium, the dispersion efficiency of the applied protocol and the stability of the dispersion should be tested and properly documented. A dispersion protocol can be considered effective if it yields samples which consist as much as possible of non-agglomerated/non-aggregated particles [16]. Reference materials or self-generated and properly characterised and documented test materials are essential for controlling and comparing the performance of analytical methods used for nanomaterial characterisation and in their validation [104]. Dispersion stability must be verified periodically. ISO/TR

13097:2013 (Guideline for the characterization of dispersion stability) provides technical guidance on the characterisation of dispersion stability [123].

Several dispersion protocols have been developed and published [106], [124], [125], [126], [127], [128], [129], [130], [131]. Dispersion protocols for toxicological studies have been developed by different international organisations and in various research projects, in particular EU NanoGenoTox[8], EU NANoREG[9]. While dispersion protocols are available, their applicability domain for toxicological testing needs to be considered. For example, the NanoDefine project [132], developed an integrated empirical approach, which allows identifying a material as a nanomaterial or not a nanomaterial according to the EC Recommendation. Nevertheless, care should be taken as the additives added to the dispersion medium may only be conditionally/not compatible with toxicological testing.
As long as no test guidelines are available, detailed description and justification of the method including adequate controls (and reference materials, if possible) is required.

Before *in vivo* administration of a nanoform, the possible interactions with the administration vehicle need also to be determined and reported. For example, the nanoforms may adsorb on the walls of the delivery system and may no longer be available, thus affecting the delivered dose [133]. The check of lab-ware retention capability is essential in order to make experiments reliable and improve the reproducibility of *in vivo* studies.

The "aging" of nanoforms during the storage may also influence the outcome of a toxicity study. For example, the biological action of freshly prepared and aged silver nanoparticles is strongly different due to the different amounts of released ions [134].

To help to determine the concentration of nanoforms in biological samples, data from the OECD WPMN project "Guidance on the determination of concentrations of NPs in biological samples for (eco)toxicity studies" is expected to become available by the end of 2021.

2.1.1.4 Biological Sampling

Two OECD test guidelines, OECD TG 412 (Subacute Inhalation Toxicity: 28-day Study) and OECD TG 413 (Sub chronic Inhalation Toxicity: 90-day Study) have been updated to enable the testing and characterisation of effects of nanomaterials and are currently available. The OECD guidance document GD 39 on inhalation toxicity testing has also been updated and validated for nanomaterial testing and should be consulted in the design of the studies [102].
The biological samples (e.g.organs and tissues, blood cells, body fluids) to be collected in the *in vivo* toxicological studies are specified in the relevant test guidelines. However, if there is an indication that the nanomaterials would be distributed in other tissues not listed in the OECD TGs, then the collection of these additional tissues (including the sampling method) is recommended.
It is advised to handle and store the samples adequately to allow the performance of later analysis (e.g. storage by chemical or physical tissue fixation for microscopy [135], freezing for burden analysis ([136], [137]. Caution should be exercised to use the most appropriate storage method for specific tissues as the available information is based on a limited number of nanomaterials and tissues.

2.1.1.5 Use of Non-Animal Testing Approaches[10]

Article 25 of the REACH regulation specifies that testing on vertebrate animals should be

[8] http://www.nanogenotox.eu

[9] https://www.rivm.nl/en/international-projects/nanoreg

[10] This advice is applicable for all endpoints relevant for human health, i.e., not only to those having a nanospecific entry in this document.

conducted only as a last resort, i.e. only when all other scientific available methods have been evaluated. Therefore, in order to fill any data gaps, there is a legal obligation to evaluate all the existing data and data from non-animal methods of hazard assessment before considering any new tests using vertebrates. Registrants are advised to keep informed about ongoing developments and validation efforts of the OECD and the European Union Reference Laboratory for alternatives to animal testing (EURL ECVAM), as well as on the regulatory acceptance of new methods by ECHA [138]. Implementation of non-animal approaches for nanomaterials requires the prior consideration of all available information, including nanomaterial characterisation, which is a critical requirement for grouping and read-across and quantitative structure–activity relationships (QSARs). In addition, relevant and reproducible *in vitro* systems may be used. Adverse Outcome Pathways (AOPs) relevant to nanomaterials are under development at the OECD and offer new approaches to integrated assessment (https://aopwiki.org and and OECD EAGMST https://www.oecd.org/chemicalsafety/testing/adverse-outcome-pathways-molecular-screening-and-toxicogenomics.htm).

In silico models and read-across

Regarding the use of non-testing data for nanomaterials, it is necessary to take into account that:

- The use of *in silico* models (e.g. QSARs) for nanomaterials still needs to be validated. Thus, the use of such models for nanomaterials in deriving an assessment of hazard for humans must be scientifically justified and applied on a case-by-case basis only. However, results from non-testing methods can be useful information in the context of weight of evidence[6] or can provide essential information for the planning of an animal test. A range of *in silico* models, such as those used to determine nanomaterial kinetics, QSARs and physiologically based pharmacokinetic (PBPK) models have been developed for nanomaterials [139], [140], [141] [142], [143]. *In silico* modeling could be also useful to provide an initial prediction of a deposited and retained dose in the lung (e.g. the Multi-Path Particle Dosimetry (MPPD) model). As with any *in silico* model, care must be employed with regard to the applicability domains of these models.
- The use of grouping and read-across approaches is another step to consider before performing animal testing. Annex VI of the REACH Regulation as amended stipulates that "*where technically and scientifically justified, the methodologies set out in Annex XI.1.5 shall be used within a registration dossier when two or more forms of a substance are "grouped" for the purposes of one, more or possibly all the information requirements*". In this respect, it is advised to consider the ECHA guidance *Appendix R.6-1 for nanoforms applicable to the Guidance on QSARs and Grouping of the Chemicals* [31] when data on other (nano)forms[11] of the same substance are available. Regarding read-across and/or grouping between (nano)forms of different substances the advice provided in the ECHA Guidance Chapter R.6 on QSARs and Grouping of the Chemicals [4] and its nanospecific appendix [31] must be considered. Lamon *et al*. 2018 reviewed the available approaches and case studies on the grouping of NMs to read-across hazard endpoints. The grouping frameworks identified hazard classes depending on PC properties, hazard classification modules in control banding (CB) approaches, and computational methods, that can be used for grouping for read-across [144]. Grouping frameworks are currently being developed, e.g. by the EU project GRACIOUS, to substantiate nanospecific read-across and grouping also for regulatory purposes (https://www.h2020gracious.eu/).

In vitro studies

This section discusses the possible contribution of *in vitro* data to the adaptation possibilities.

In accordance with Article 13(1) of the REACH regulation, "*Information on intrinsic properties of*

[11] The term (nano)form intends to cover nanoforms and non-nanoforms of the substance

substances may be generated by means other than tests, provided that the conditions set out in Annex XI are met". The information from *in vitro* tests should always be considered before performing an animal test.
It has been shown that *in vitro* assays (e.g. [145], [146], [147]) that are not yet formally validated but can be demonstrated to be scientifically valid, may be useful on a case-by-case-basis for the assessment of nanomaterials. They can be used as part of a weight of evidence[6] approach that involves thorough physicochemical characterisation of NMs, *in vitro* screening tests, '-omics', the use of non-testing approaches (*in silico* models, read across) and the use of OECD and EURL ECVAM validated/ approved *in vitro* methods. References [148], [89], [149] include provisions for the acceptance of data from *in vitro* studies.

According to OECD [150] for *in vitro* testing the "*Characterisation of the materials should be undertaken in the cell culture medium used both at the beginning of treatment and, where methodologies exist, after treatment. The intent when applying nanomaterials to a cell culture medium is to create conditions that are comparable, to the extent possible, with the biological and physiological conditions within the in vivo system*". Considerations for *in vitro* testing of NMs, which should lead to increased reliability and relevance are given in the scientific literature [151]. Drasler *et al*. 2017 [151], concluded that the most important criteria to produce reliable and robust data from *in vitro* nanotoxicological assays are: (i) detailed NM characterization, including physicochemical properties before, during and after the testing, (ii) use of comparable and realistic dose metrics and test conditions, and (iii) implementation of chemical positive and negative controls and reference NMs allowing for comparison between studies, both intra- and interlaboratory.

An analysis of the applicability of *in vitro* methods to NMs and recommendations for the adaptations needed is available [152].

2.1.2 Advisory note on the consideration of assay interference

Various nanomaterials have on occasion been found to interfere with several commonly used assays for determining their cellular or toxic effects. For example, some nanomaterials may contribute to the absorbance or fluorescence of colorimetric or fluorometric assays. In addition, due to their large surface area, nanomaterials may bind to assay components including the substrates (e.g. CNT with the reagent in MTT 2-(4,5-dimethyl-2-thiazolyl)-3,5-diphenyl-2H-tetrazolium bromide assays; [153], Fe_3O_4 with WST-1, 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulphophenyl)-2H-tetrazolium) [154] or the biomarker being measured, (e.g. lactate dehydrogenase (LDH) and cytokine proteins; see for example [155]). Please note that other factors such as coatings or impurities may also have an influence on the assay.
A summary list of potential sources of nanomaterial interference with commonly used *in vitro* assays has been developed by Kroll *et al.* [156] and is reproduced in the table below.

Table 3: Potential sources of nanomaterials interference with commonly used assays

Cytotoxicity assay	Detection principle	Nanomaterials interference	Altered readout	Nanomaterials type
Cell viability				
MTT/WST-1	Colorimetric detection of mitochondrial activity	Adsorption of substrate	Reduced indication of cell viability	Carbon nanomaterials
LDH	Colorimetric detection of LDH release	Inhibition of LDH	Reduced indication of necrosis	Trace metal-containing nanomaterials
Annexin V/ Propidium iodide	Fluorimetric detection of phosphatidylserine exposure (apoptosis marker) Propidium iodide staining of DNA (necrosis marker)	Ca^{2+}-depletion Dye adsorption	Reduced indication of apoptosis Reduced indication of necrosis	Carbon nanomaterials
Neutral red	Colorimetric detection of intact lysosomes	Dye adsorption	Reduced indication of cell viability	Carbon nanomaterials
Caspase	Fluorimetric detection of Caspase-3 activity (apoptosis marker)	Inhibition of Caspase-3	Reduced indication of oxidative stress	Carbon nanomaterials
Stress response				
Dichlorofluorescein (DCF)	Fluorimetric detection of ROS production	Fluorescence quenching	Reduced indication of oxidative stress	Carbon nanoparticles
Inflammatory response				
ELISA (enzyme-linked immunosorbent assay)	Colorimetric detection of cytokine secretion	Cytokine adsorption	Reduced indication of cytokine concentration	Carbon nanoparticles Metal oxide nanoparticles

It should be noted that the above list is not exhaustive and the potential for inhibition or enhancement of test results should always be investigated. The agglomeration, dispersion and/or dose may influence the outcome of the test.

Assay interference is not limited to colorimetric assays. Interference with medium components such as medium depletion or corona formation by adsorption of essential nutrients or interaction with buffer salts, resulting in increased disssolution of nanoparticles was reported [109], [157]. Within some standard methodologies, the method requires the use of spiked samples (addition of a known reference/control sample) to test for inhibition or enhancement of the spiked control. This is evaluated by assessing the measured value against the expected value, which should be a cumulative value of the spike and of the sample.

Assay interference should always be investigated wherever possible, irrespective of standard method requirements. In case it would not be possible, this has to be justified. Furthermore, for many of the studies reported, it is not possible to ascertain whether the assays were adequately controlled to assess for interferences. Thus, if other methods for assessing interferences are not available, as a general precaution, it is reccommended to use more than one assay to assess the studied endpoint or effect, as for example advised by Landsiedel *et al*. [158] for genotoxicity studies. The potential for inhibition or enhancement of the test result may impact the validity of relevant *in vitro* test methods. For example carbon nanotubes are suspected to interfere with the MTT assay [159] or WST-1 assay [154] and this may cause issues with tests such as OECD TG 431/EU B.40 bis Human Skin Model tests (EPISKIN™, EpiDerm™) which uses the MTT assay. However, knowledge on nanomaterial assay interference is incomplete and so precautions to ensure the validity of an assay, such as the above-mentioned use of control spikes should be used.

Due to the potential for interference resulting in misleading results in numerous assays, utmost care should be taken in testing for such interference and appropriate controls must be used for the assays.

2.2 Specific advice for individual endpoints

2.2.1 Acute toxicity

The parent guidance Chapter R.7a Section 7.4 [3] provides the general testing strategy for acute toxicity. The advice provided in the parent guidance should be followed together with the recommendations given in this section.
According to the revised REACH Annex VII, Section 8.5.1 Column 2: *"For nanoforms, a study by the oral route shall be replaced by a study by the inhalation route (8.5.2), unless exposure of humans via inhalation is unlikely, taking into account the possibility of exposure to aerosols, particles or droplets of inhalable size"*.

Hence, when the dossier covers nanoforms, testing by a route other than inhalation needs to be justified. The inhalation route is appropriate if exposure of humans via inhalation is likely taking into account the possibility of exposure to aerosols, particles or droplets of an inhalable size. Guidance on the interpretation of physico-chemical data regarding respiratory absorption can be found in Table R.7.12-2 in parent guidance Chapter R.7c [160]. The information derived from *in vitro* models ranging from cell lines grown in monoculture to lung-on-a chip (LOC) microphysiological systems and *in vitro* airway models aiming to characterise the cellular and molecular mechanisms responsible for the effects of inhaled toxicant exposure [161] could be useful in a WoE approach and provide an initial insight in the toxicity of the substance.

In the event there is no exposure of humans via the inhalation route then the oral route (as for most substances) is the default first choice of administration at Annex VII.

The current acute inhalation test guidelines employ death as an endpoint (OECD TG 403 and TG 436) or replace lethality as an endpoint with evident toxicity as fixed concentration procedure (FCP) (OECD TG 433). It is however, acknowledged that none of these test guidelines contain provisions specific for nanomaterials. Nevertheless, the recently updated OECD GD 39 provides some advice applicable for the acute inhalation in case of testing nanomaterials [102]. As sometimes for low density materials it is not feasible to reach the top dose, the physico-chemical properties including tested material density should be taken into account when dosing for the acute studies. The OECD GD 39 emphasizes that "*Knowledge of dustiness and particle size for solid test chemicals will allow for selection of the ideal testing approach and starting concentration that will enhance respirability (e.g., through the use of micronization)*" and that "*The particle size distribution should be determined at least once during a single exposure study for each concentration level using an appropriate method of measurement*". OECD GD 39 also states that "*Aerosol testing at greater than 2 mg/L should only be attempted if a respirable particle size can be achieved*." For nanomaterials, the degree of agglomeration increases with dose. The degree of agglomeration affects the aerodynamic diameter and thereby the deposition in the lung. For oral exposure, a higher degree of agglomeration can reduce the absorption. The consequences of the dose on agglomeration should therefore be taken into account when designing and interpreting acute toxicity tests.

2.2.2 Repeated dose toxicity

Annexes VIII-X, Section 8.6.1. and Annexes IX-X, Section 8.6.2., Column 2 of REACH Regulation (EC) No 1907/2006 amended (underlined text) for nanoforms' testing, provide that:
"*For nanoforms toxicokinetics shall be considered including recovery period and, where relevant, lung clearance*."

and under "*Further studies shall be proposed by the registrant or may be required by the Agency in accordance with Articles 40 or 41 in case of*:" third indent:

"*indications of an effect for which the available evidence is inadequate for toxicological and/or risk characterisation. In such cases it may also be more appropriate to perform specific toxicological studies that are designed to investigate these effects (e.g. immunotoxicity, neurotoxicity, and in particular for nanoforms indirect genotoxicity),*".

In contrast to acute toxicity testing, the information requirements for repeated dose testing of nanoforms do not prescribe inhalation as default route. However, Column 2 of Annex VIII, Section 8.6.1. and of Annex IX, Section 8.6.2. specify that "*Testing by the inhalation route is appropriate if exposure of humans via inhalation is likely taking into account the vapour pressure of the substance and/or the possibility of exposure to aerosols, particles or droplets of an inhalable size".* Therefore, inhalation can, in most situations, be seen as the most likely route of exposure to nanomaterials, and thus the following focuses on inhalation testing.

The testing protocols to be followed in repeated dose testing by inhalation are OECD TG 412 [162] and TG 413 [163] together with the guidance provided in OECD GD 39 [102]. When testing poorly soluble solid aerosols that are likely to be retained in the lungs, Option B in the Annex of OECD TG 412/413 should be used. OECD GD 39 defines the poorly soluble particles (PSPs) as "*Solid aerosol particles deposited in the lung that do not undergo rapid dissolution and clearance*" and states that "*A poorly soluble material is generally understood as having a solubility of less than 0.1 g dissolved in 100 ml dissolvent within 24 hours*." [102]. A recent expert group [15] on the hazards and risks of poorly soluble particles of low toxicity, concluded that PSPs can be defined as particles for which their alveolar macrophage-mediated clearance rate is not shortened by their dissolution rate in the lung. For chemicals that accumulate in the lung or translocate into specific accumulating organs following repeated exposures, toxicokinetic shall be considered, as the accumulated dose is partly a function of clearance. Therefore, for these nanoforms the lung clearance investigation is considered relevant. This is also in agreement with

OECD TG 412/413, which require the measurements of lung burden when a range-finding study or other information demonstrates that a poorly soluble aerosol is likely to be retained in the lung. For PSPs, the rat lung burden is an important issue to consider in the toxicological outcome and therefore a special chapter within this section is included.

For fibre-like particles, in addition to the overload of macrophages, it should be considered that the pathogenicity of the fibres may play a role in their toxicity [164] and that they may also cause impaired lung clearance below overload. Fibre pathogenicity is characterised by frustrated phagocytosis and/or migration of fibres into the pleural, leading to activated macrophages, which stimulate an amplified proinflammatory cytokine response from the adjacent pleural mesothelial cells [165]. This mechanism of toxicity is not addressed further in this guidance. The OECD GD 39 specifies that for chemicals with fibre geometry, additional specific observations, such as interstitial and pleural detection and possibly quantification, depending on fibre dimensions, may be needed when performing an OECD TG 412/413 study [102].
When considering the testing strategy for repeated dose toxicity (Section 7.5.6. of REACH Guidance R.7.a. [3]) of nanoforms it should be noted that:

- Especially for workers (and in some cases for consumers, e.g. in case of sprayable products) inhalation is the most likely route of exposure to nano(particles), nano aerosols and dust. Hence, the repeated dose toxicity studies should be performed via inhalation, unless there is convincing information (e.g. uses, dissolution rate) that justifies another route. Any modification of the protocols described in OECD TG 412 and 413 ([162] and [163]) should be justified;

- When dose range finding studies or repeated dose studies are performed, for PSPs, it is recommended to collect additional toxicokinetic data as described in *Appendix R7-2 for nanomaterials applicable to Chapter R7c Endpoint specific guidance*). In addition, to make full use of the test, if there is a particular concern it is recommended to address it within the study design (e.g. accumulation, specific organ toxicity, etc.).

- When testing PSPs, it may be necessary for a range-finding study to be longer than 14 days to allow for a robust assessment of test chemical solubility and lung burden [102]. Dissolution data in simulated lung fluid and lysosomal fluid are important for an informed decision before starting the animal studies. Annex VII, section 7.7. column 1, of the REACH Regulation specifies, beside the water solubility data requirement, that "*for nanoforms, in addition the testing of dissolution rate in water as well as in relevant biological and environmental media shall be considered.*". Therefore the information on dissolution must in principle be available before the *in vivo* testing.

- Exposure concentrations should be selected to cover the entire range of lung burdens, i.e., concentrations ranging from those which do not delay clearance to those which do delay clearance. Similarly, post-exposure periods should not be markedly shorter than one elimination half-time of the particulate test material [102].

- The doses to be used in repeated dose inhalation studies should not exceed the maximum tolerated concentration. However, the doses in hazard tests needs to cover a range of doses to be able to detect any potential hazard. This means the inhalation studies with PSPs should include an exposure concentration high enough to produce a toxic effect or an overload of particle clearance inducing lung inflammation, as well as exposures not causing lung particle overload. A similar approach was proposed for the design of chronic inhalation studies [15]. OECD TG 412 [162] and TG 413 [163] provide advice on doses to be used. This includes the provision that the highest dose should elicit unequivocal toxicity without causing undue stress to animals or affecting their longevity.

- For nanomaterials, the degree of agglomeration increases with dose. The degree of agglomeration affects the aerodynamic diameter and thereby the deposition in the lung. For oral exposure, a higher degree of agglomeration can reduce the absorption. The consequences of the dose on agglomeration should therefore be taken into account for designing and interpreting repeated dose toxicity tests. *In silico* modeling could be useful to provide an initial prediction of deposited and retained dose in the lung. For instance, the Multi-Path Particle Dosimetry (MPPD) model can be used to assess the influence that the characteristics of the aerosols (MMAD, GSD, solubility, density, hygroscopicity) can have on the dose [166].

- Information from the dose–range finding studies and lung burden data are useful for the interpretation of a study outcome. Knowing the retained lung burden during exposure and at different time points post-exposure, the following can be determined: nanomaterials lung clearance and retention kinetics, critical dosimetric comparisons to clearance and retention data of well-established benchmark materials (allowing comparative Dose - Response relationships to be analysed) [167]. The selection of PSP benchmark materials should be justified. Humans and rodents differ significantly in their biokinetic functions so the effects of nanomaterials observed in rodents do not directly translate to humans. Nevertheless, the lung burden data cannot be used to exclude the relevance of toxicological findings in the experimental animal for human risk assessment [162], [163]. A detailed discussion on the concept of rat lung burden of PSPs and its impact in the toxicological assessment is provided in section 2.2.2.1.

- Although lung burden measurement is mandatory at only one post-exposure observation (PEO) period in Option B (at PEO-1) of OECD TGs 412/413, the TGs provide that a minimum of two lung burden measurements are necessary when investigating clearance kinetics. If the use of two post-exposure time points is considered sufficient, lung burden measurements may be performed at PEO-1 (main study) and at PEO-2 (recovery group) only, if timing for evaluation of recovery and lung clearance can be aligned. Lung burden should be measured for all concentrations [102]. Lung burden measurement at three time points allow curve fitting on post-exposure clearance kinetics. The need for additional post-exposure observations, the duration of the post-exposure interval and the timing of the post-exposure observations (PEOs) are determined by the study director based upon results from, among others, the range-finding study.

- The data on lung burden and clearance are also important in the context of read-across of the hazard data from studies using different nanoforms of the same substance. Incorporating measurement of retained lung burden in the study design allows a coupling of subchronic and subacute inhalation data with other appropriate test data (from shorter-term inhalation,and cellular and acellular assays) which can facilitate grouping and may result in saving animals [167]. For example for nanoforms with different particle sizes, the same external concentrations can result in differences in retained dose [167]. Conversely, different external concentrations can result in the same retained dose for different particle sizes.

- Assessment of organ burden in potential target tissues other than lung should be considered to estimate the systemic availability. As a first indication of translocation, detection and burden measurements in local lymph nodes may be determined in addition to lung burden measurements. To monitor the fate and effects of PSPs in the body it is recommended to collect the samples at several time points and/or from different organs and tissues. Data from range-finding studies, if proven robust, could be used to determine the appropriate sampling times in order to investigate these toxicokinetic parameters (i.e. nanomaterials burden in secondary organs). If satellite animals are used for lung burden, the same animals should be used for any other organ burden assessment deemed necessary by the study director. In ISO TR 22019 [94] the liver, spleen, lung, brain, kidney, lymph nodes at the organ of entry, and bone marrow are considered

relevant organs for the toxicokinetics of NPs. Other relevant tissues include reproductive organs (uterus and testis) and adipose tissue [168]. The issue of bioaccumulation and organ burden is further discussed in section 2.1.3. of Appendix R7-2 to Chapter R.7c [169].

- Since the lower respiratory tract (i.e., the alveoli) is the primary site of deposition (depending on agglomerate size) and retention for inhaled nanoparticles, bronchoalveolar lavage (BAL) analysis is a useful complementary technique to histopathology predict and quantitatively estimate pulmonary inflammation and damage (for further information on BAL parameters please see OECD TGs 412 and 413). BAL analysis allows dose-response and time-course changes of alveolar injury to be suitably investigated. Therefore, for nanomaterials testing, it is mandatory to include BAL analysis (further details are given in Section R.7.5. (repeated dose toxicity) of Chapter R7.a of the parent guidance [3]. BAL fluid should be analysed for all the concentrations [102].

- It is strongly advised to use more than one dose-describing metric and to include the mass metric. The choice of method(s) selected should be justified, as also explained in Section 2.2.2.1.1.

OECD TG 412, TG 413, and GD 39 should be consulted when designing developmental and reproductive toxicity studies using TG 422, TG 421, TG 443 and TG 414 with exposure by the inhalation route.

With regard to the 'old' studies performed with nanoforms prior to the update of OECD TG 412/413, several conditions need to be fulfilled in order for these studies to be considered appropriate:

- The test material is well characterised (*i.e*. size distribution, shape and aspect ratio, surface treatment and specific surface area is known) and ensure the representativeness for the registered NF/set of NF, and
- The parameters from the testing guidelines valid at the time when the study was performed are covered. In case that only the respiratory system has been investigated and not all the organs listed for histopathology by the testing guidelines, the study cannot be seen as equivalent to an OECD 412 or 413.

If the study fulfils the conditions above, then it can be considered for the use under WoE. The updated testing guidelines specify for the substances likely to be retained in the lung that BAL and lung burden measurements at specified PEO should be performed. For the studies performed prior this update, for which this information is missing, the information must be substantiated using weight of evidence[6] approach with further data:

- dissolution studies in artificial lung lining fluid and lysosomal fluid.
- in cases where no toxicity is seen in an old repeated dose study, the lack of toxicity seen *in vivo* can be substantiated with data from studies assesing cytotoxicity and inflammation markers with the registered nanoform in relevant cell lines. Overall, the cytotoxicity assays should always consider the cell type and nanomaterials used, therefore a generalized recommendation cannot be given [151]. Various factors could have contributed to the "no effects" outcome seen in the old *in vivo* study such as agglomeration, dispersion protocol, adherence of the tested substance to the delivery system etc.

For the oral studies (OEC 407, OECD 408, OECD 421/422) the same principles apply. If a proper characterisation of the test material (nanoform) is provided (i.e. size distribution, shape and aspect ratio, surface treatment and specific surface) and ensure the representativeness for the registered NF/set of NF, and if the study has been conducted according to the testing guidelines (including analysis of all the organs listed in the TG) then the study can be considered for the

use of WoE. The information from such a study must be substantiated with further data such as dissolution studies in artificial gastrointestinal fluid and lysosomal fluid. In cases where no toxicity is seen in the repeated dose study, cytotoxicity studies in relevant mammalian cell lines could substantiate the lack of intrinsic toxicity seen *in vivo*. If an available oral study complies with all the criteria listed above, it can be used only for the relevant route of exposure, *i.e*. oral.

2.2.2.1 Advisory note on the consideration of lung burden within inhalation toxicity assessment

This section describes the concept of rat lung burden of PSPs and the associated effects, the differences between species and the extrapolation of the results to humans. Care should be taken when interpreting lung burden in the context of human risk assessment. Lung effects observed in animals exposed to PSPs by inhalation should be considered relevant for the hazard in humans unless it can be clearly substantiated otherwise.

Results from inhalation studies in rats have shown that the PSPs can induce serious adverse pulmonary effects if inhaled in high concentrations due to material accumulation, as lung clearance mechanisms are not able to remove materials at the same time or at a higher rate as the dose is delivered. This condition named "lung overload", occurs when the retained particle burden in the lung exceeds a certain threshold [170]. A recent expert group [15] on the hazards and risks of poorly soluble particles with low toxicity (PSLT) proposed the following expert consensus definition "*Lung Particle Overload is a phenomenon of impaired clearance in which the deposited dose of inhaled PSLT in the lung overwhelms clearance from the alveolar region leading to a reduction in the ability of the lung to remove particles. Lung Particle Overload results in an accumulation of particles greater than that expected under normal physiological clearance. This definition is relevant for all species (not just rat). This definition is independent of the underlying mechanism(s) (e.g. macrophage mobility impairment). A key issue is that increased particle retention due to large lung burdens needs to be differentiated from that due to high cytotoxicity particles (e.g. quartz).*"During prolonged exposure of rats to PSPs, the lung burden of particles increases until equilibrium is reached between deposition and clearance of particles [171] as shown by the curves A, B and C in **Figure 5** This equilibrium can be reached very fast or may take up to many days. Below the lung overload threshold, particles are cleared via normal mechanisms at a constant clearance rate, in general generating little or only a minor or reversible response (exposure concentrations in curves A and B).

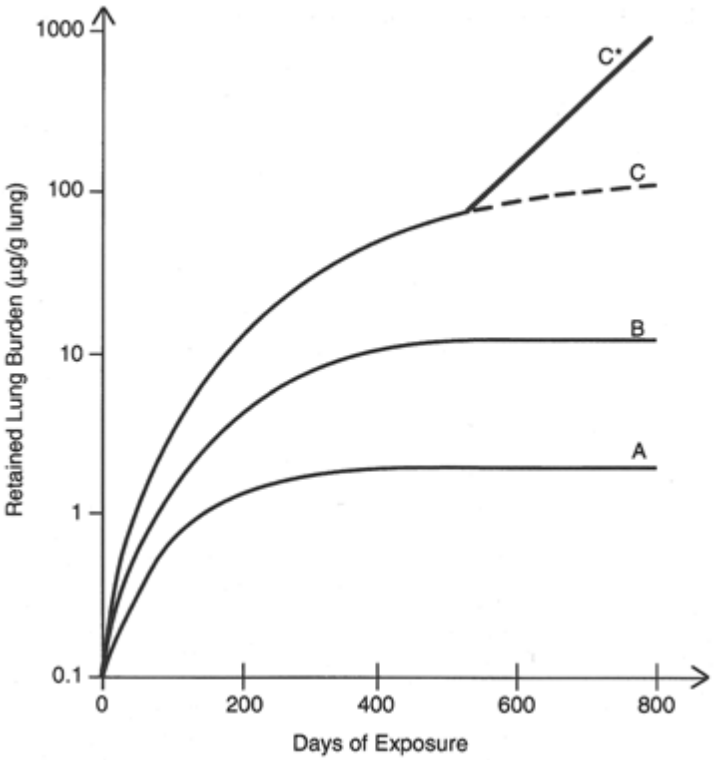

Figure 5: Schematic representation of the relationship between retained lung burden and duration of exposure leading to the phenomenon of lung overload.

Curves A, B, and C are associated with progressively increasing exposure doses. If the exposure level is sufficiently high and the duration of exposure sufficiently long, alveolar macrophage-mediated clearance of particles can be overwhelmed. When this occurs, the retained lung burden increases linearly with further exposure (curve C*), reproduced from [171].
Once the threshold has been reached, the clearance mechanisms of the lung become overloaded. This is typified by a progressive impaired particle clearance from the deep lung, reflecting a breakdown in alveolar macrophage (AM)-mediated dust removal due to the loss of AM mobility [170]. This is shown in the C* curve of **Figure 5** whereby at the point of threshold, particle retention increases rather than an equilibrium being established (as demonstrated by the dashed line).
The result of this net increase in particle accumulation is persisting lung inflammation, cessation of alveolar-mediated clearance and an increase in accumulation of particle laden macrophages and/or free (non-phagocyted) particles within the lung alveoli. The potential progression of inflammatory reactions toward a granulomatous type in rats was found to depend on the exposure duration and the level of the particle (surface) burden in the lung [172] as well as of the volumetric load of alveolar macrophages [173].

The situation of lung burden is most commonly associated with repeated inhalation exposure of rats to PSPs but it can also occur after single or repeated instillation of PSPs into the lung (due to a high deposition fraction as a result of direct instillation) or possibly as a result of a single massive inhalation exposure [174]. Since this phenomenon occurs at relatively high exposure levels of respirable PSPs it is often argued that the observed adverse effects are a product of the lung burden caused by experimental conditions and not always a true reflection on the intrinsic toxic potential of the particles to cause inflammation, fibrosis and cancer. Exposure to highly reactive or toxic particles may cause inflammation, fibrosis and cancer at lower exposure levels (non-overload conditions) due to intrinsic properties of the particles themselves.

Care should be taken when interpreting lung burden in the context of human risk assessment. Lung effects observed in animals exposed to PSPs by inhalation should be considered relevant

for the hazard in humans unless it can be clearly substantiated otherwise. When designing a new study, the doses to be used in repeated dose inhalation studies should not exceed the maximum tolerated dose. However, the doses in hazard tests needs to cover both lower doses (seen as a valid approach to determine the lung overload together with the time-course measurement of actual lung particle burdens [15]) as well as higher doses which enable to detect any potential hazard so that the study can fulfill its regulatory purposes. OECD TGs 412 [162] and 413 [163] provide advice on dosages to be used. This includes the provision that the highest dose should elicit unequivocal toxicity without causing undue stress to animals or affecting their longevity.

In the studies performed with PSPs the measurement of changes in lung burden over post-exposure time(s) provides essential information on lung clearance and allows clarification of the deposited vs the exposed particle amount. Different imaging techniques may also be used for a semi-quantitative assessment of the PSPs in the tissue [175]. The assessment of the dissolution potential as an indicator for biopersistence can also be done using *in vitro* systems [92].
Information on clearance and biopersistance is important in the context of toxicokinetics, read-across and weight of evidence.

Rat is currently considered the most sensitive species for inhalation toxicity testing for nanoparticles [176]. However, as it can be difficult to interpret the findings of overload of alveolar macrophages in rat studies, a better understanding of the rat lung burden and its relevance to humans is needed. A recent study that analysed the issues of impaired clearance and the relevance for human health hazard and risk assessment [177] pointed out that while rats and humans have a clear difference in lung clearance kinetics of PSPs, the fact that the appropriate dose metric often is unknown and the level of clearance impairment is seldom assessed makes it difficult to relate the effects observed to an impaired clearance. Several studies have assessed the responses to lung overload in different species, and the relevance of this data for humans. For instance, in a comparative study assessing the long-term pulmonary response of rats, mice and hamsters to inhalation of ultrafine grade titanium dioxide [137], the same air concentrations caused overload effects in rats and mice but not in hamsters. Also, the inflammatory and pathological responses were less severe in mice than in rats and they diminished with time irrespective of the similar lung burdens [137], [178]. However, in relation to the relevance of animal data for humans, other studies have pointed out that the lung responses to high lung burdens of PSP of low toxicity can be qualitatively similar in rats and humans [179]. Based on experience with exposure of coal miners, a primate-specific interstitial particle sequestration compartment is hypothesed [180]. Borm *et al* [173] discuss whether this mechanism could explain why humans, in contrast to rats, seem not to have an increased risk of lung cancer under lung overload conditions [173]. Nevertheless, there seems to be some conditional evidence for particle overload associated with impaired clearance in coal miners [173]. While lung inflammation after PSPs exposure does not necessarily lead to tumour formation in humans and carcinogencity [15], fibrosis is however, a response seen upon exposure to PSPs in both rats and humans, though not in mice [177]. Independent of the underlying mechanism(s) (e.g. macrophage mobility impairment), the concept of lung overload is relevant for all species (not just rat) and under overload conditions, the rat was considered "*not to be unique in its inflammatory, hyperplastic, and fibrotic responses to PSLT*" albeit the rat is more sensitive [15].

Therefore, the use of existing rodent data, obtained after exposure to high doses of PSPs, cannot automatically be dismissed as irrelevant for humans in the context of risk assessment and the interpretation of such data should be approached with caution. In the case of adverse effects observed in animals under overload conditions, the relevance for humans has to be assumed *a priori*; any claimed non-relevance for humans must be supported by data.
For further information, Bos *et al*. 2019 [177] and Driscol and Borm, 2020 [15] provide a detailed analysis on the pulmonary toxicity in rats following inhalation exposure to PSPs and discuss the relevance of rat hazard studies for human health and risk assessment. There are also several other review articles covering the subject of lung overload such as Miller [171], who provided

an in-depth discussion of particle deposition, clearance and lung overload. Borm *et al* [181] discussed the importance of overload in the context of risk assessment whereas in an editorial of Borm *et al*, [173] the state of the art concerning lung particle overload concepts is summarized. These reviews also present different views on how to assess lung overload and how to interpret the data and emphasize the fact that the topic is still under debate.
In conclusion, based on the current understanding, lung effects observed in experimental animals exposed to PSPs by inhalation should be considered relevant for humans. When designing a new study, the doses in hazard tests needs to cover both, lower doses as well as higher doses to enable to detect any potential hazard and to fulfill its regulatory purposes.

2.2.2.1.1 Metrics

The question of which dose metric best describes the association between deposited dose in the lung, and subsequent inflammation and impaired clearance function is particularly relevant. There have been several suggested metrics, but volumetric load of AM and surface area appear to be the most relevant [173] in interpreting lung overload-related as well as other adverse effects and in establishing limit concentrations. Morrow *et al*. [170] hypothesised that overload begins when the particulate volume exceeds approximately 60 μm^3/AM (which produces a 6% increase in the average alveolar macrophage volume) and that total cessation of AM-mediated clearance occurs when the particulate volume exceeds 600 μm^3/AM (producing a 60% increase in the average alveolar macrophage volume). Extending the Morrow concept, Pauluhn ([182], [183]) modelled a generic particle displacement volume threshold for agglomerated PSPs.
Oberdoerster *et al.* [184] suggested that the particle surface area better correlates the overload with impaired clearance. Several studies suggest that, particle surface area correlates well with induced pathogenic events in lung ([181], [185], [186]). In a study by Tran *et al*. [186] data from a series of chronic inhalation experiments on rats with two poorly soluble dusts (titanium dioxide and barium sulphate) was analysed. The results indicated that when lung burden was expressed as particle surface area, there was a clear relationship with the level of inflammation and translocation to the lymph nodes. Most usefully, based on the shape of the statistical relationship for lung response to particles, the authors suggested the presence of a threshold at approximately 200–300 cm^2 of lung burden for "low-toxicity dusts" in rats.

Whilst some studies indicate mass as a less sensitive indicator of lung overload [187], the mass concentration is still important because there is already a large body of data and research on the exposure to and toxicity of particles using the mass-based metric. Therefore, for the sake of comparison(s), the mass concentration should always be reported.

Other studies ([188]) found that the particle number or the number of functional groups in the surface of nanoparticles ([189], [190]) was the best dose metric.

The most relevant dose metric seems to vary depending on the specific nanoparticle in question. Surface areas, mass, and the particle number should be reported in order to establish the dose metric that best describes the association between deposited dose in the lung, overload conditions and the subsequent pathogenic effects and in order to establish the dose metric most relevant for risk assessment.

It is therefore vital to fully characterise test materials, so that the measured response can be retrospectively correlated with multiple-dose metrics, without the need for repeat testing. In general, the more metrics are reported the better.
In conclusion, it is strongly advised to use more than one different dose-describing metric and to justify the choice of the selected methods. However, since all regulatory limits and effects values are based on mass concentration, this is still default and needs to be reported.

2.2.2.1.2 Main recommendations for lung burden

- Data from existing studies performed with high doses of PSPs showing adverse effects cannot automatically be dismissed as irrelevant for humans.
- When planning/performing, new studies, the use of excessively high doses should be avoided (in order not to exceed the maximum tolerated dose). However, the doses in hazard tests should include an exposure concentration high enough to produce a toxic effect or an overload of particle clearance inducing lung inflammation, as well as concentrations not causing lung particle overload.
- Lung burden data provide useful information on the pulmonary (retained) dose as well as on clearance behaviour and may support the read-across and weight of evidence approaches. Lung burden and BALF must be measured for all concentrations [102].
- The most relevant metric should be used and mass metric should always be included. It is strongly recommended to use more than one metric.

2.2.2.2 Indirect genotoxicity

The term 'indirect genotoxicity' is mentioned in the REACH section on repeated dose studies (i.e. Annex VIII section 8.6.1, and Annex IX section 8.6.2): "*Further studies shall be proposed by the registrant or may be required by the Agency in accordance with Articles 40 or 41 in case of […] - indications of an effect for which the available evidence is inadequate for toxicological and/or risk characterisation. In such cases it may also be more appropriate to perform specific toxicological studies that are designed to investigate these effects (e.g. immunotoxicity, neurotoxicity, and in particular for nanoforms indirect genotoxicity)*,".

Formally, primary genotoxicity and secondary genotoxicity can be distinguished, as described by several authors [191], [192], [193]. Primary genotoxicity is a genotoxic effect caused by a substance that interacts with or is internalised in the target cell. Primary genotoxicity can be either 1) direct genotoxicity, where the substance enters the cell nucleus of the target cell, interacts directly with DNA (or disturbs the mitotic apparatus) to produce DNA lesions (or chromosomal modifications); or 2) indirect genotoxicity, where the substance does not directly interact with DNA of the target cell, but generates reactive oxygen/nitrogen species (ROS/RNS) inducing oxidative DNA damage, or induces damages to the mitosis apparatus or to enzymes involved in e.g. DNA replication or DNA repair, or antioxidant activity. Secondary genotoxicity involves mechanisms in which the substance does not interact directly with the target cell but produces a particle-induced inflammatory response in neighbouring cells/tissues that generates ROS/RNS and induces a (secondary) genotoxic effect in the target cell.

Both primary and secondary mechanisms (as defined above) were reported to play a role in the genotoxic effects induced *in vivo* by nanomaterials [194], [195], [196], [197]).

In the scope of REACH column 2 of Annex VIII, 8.6.1 and of Annex IX, 8.6.2, the term "indirect genotoxicity" refers to oxidative DNA damage (which results from ROS/RNS generated either in the target cell or by inflammatory response in neighbouring cells). The observed indirect genotoxicity may thus be due to both primary and secondary genotoxicity mechanisms (as defined above).

The most frequently described mechanism to explain the genotoxicity of nanomaterials is an indirect mechanism, i.e. via generation of oxidative species or indirect consequences of inflammation [198], [158], [199], [200], [201]. The specific toxicological studies to investigate the indirect genotoxicity (*i.e.* DNA damage due to high levels of reactive oxygen/nitrogen species) could be triggered by available robust data showing that the nanoform induces a significant inflammation and/or the generation of high levels of oxidative species by the tested nanomaterial after repeated dose administration (e.g. 28-day study at Annex VIII). The observed inflammation would be considered as relevant to trigger specific genotoxicity studies only in case it is persistent. In case an *in vivo* genotoxicity test required to address the data

requirement under section 8.4 of REACH show positive results that are relevant for classification and labelling for mutagenicity hazard, such 'specific toxicological studies' would not be needed. The comet assay appears to be a suitable test to assess such genotoxic effects, because 1) the standard comet assay can detect some DNA damages induced by oxidative stress and 2) its sensitivity can be increased by using endonuclease (e.g., OGG1, FPG) to detect oxidised DNA bases that are not detected in the standard comet assay [201]. It is noted that the modified comet assay to detect oxidative DNA damages is not yet validated and the current OECD TG 489 (2016) for the standard comet assay does not provide recommendations for the modified comet assay. Moreover, the influence of inflammation on the comet assay results needs to be further investigated.

2.2.3 Mutagenicity and Carcinogenicity

The parent guidance[12] Section R.7.7 provides the general testing strategy for mutagenicity and carcinogenicity. The advice provided in the parent guidance should be followed together with the recommendations given in this section. The text below provides advice only on the mutagenicity endpoint.
The assessment of mutagenicity/genotoxicity generally relies on the investigation of *in vitro* (and possibly *in vivo*) effects for three major endpoints: i.e. gene mutation, clastogenicity and aneugenicity. It is now widely accepted, based on international collaborative studies and the large databases available, that no single assay can detect all genotoxic substances and that a battery of tests should be implemented.
Different groups have published several reviews on the genotoxic assessment of nanomaterials [202], [203], [200], [204] and the most recent comprehensive review [201] extended previous works with critical analysis of published data. All these reviews agreed on a number of recommendations that are described in this section (and also mentioned in recent guidance documents from EFSA [104] and SCCS [89]).

2.2.3.1 Bacterial (Ames) mutagenicity assays are not recommended

The bacterial reverse mutation (Ames) test (OECD TG 471 [205]/EU B.12/13: Bacterial reverse mutation test (*in vitro*)) detects point mutations in *Salmonella typhimurium* and *Escherichia coli* ([206], [207]; [208]). However, the strains of bacteria used in the standard assays do not appear to have the ability to take up/internalise nanoparticles, as they lack mammalian mechanisms of endocytosis, pinocytosis, and phagocytosis [209], [210], [158], [211], [201]. The updated text of Annex VII of REACH quoted in section below reflects the agreement by the scientific community that the standard bacterial Ames assay test is usually not adequate to be part of the battery of mutagenicity tests for 'poorly soluble' particles ([158], [202], [209], [203], [200], [204], [201]). In 2014, OECD 43 [150] already stated that *'The use of the Ames test (TG 471) is not a recommended test method for the investigation of the genotoxicity of nanomaterials'*.

2.2.3.2 Recommended approach for gathering mutagenicity information on nanomaterials according to REACH

The revised Annex VII of REACH, for section '8.4.1 *In vitro* gene mutation study in bacteria', column 2 is now reflecting the agreement not to recommend the Ames test and, for the endpoint 8.4.1 *In vitro* gene mutation study in bacteria, column 2 reads "*The study does not need to be conducted for nanoforms where it is not appropriate. In this case other studies involving one or more in vitro mutagenicity study(ies) in mammalian cells (Annex VIII, sections 8.4.2. and 8.4.3 or other internationally recognised in vitro methods) shall be provided*".
In order to assess the *in vitro* mutagenicity of nanomaterials, the Ames test must not be used, unless there is evidence that the nanomaterial penetrates the cell wall of the bacteria or that

[12] Guidance on information requirements and chemical safety assessment Chapter R.7a: Endpoint specific guidance [3]

indirect genotoxic effects due to generation of reactive oxygen species outside the bacteria. In most cases, the Ames test is not appropiate and one or more *in vitro* test(s) on mammalian cells must be performed at Annex VII instead. The registrant must provide at least one of the following tests:

1. one test detecting gene mutation: the *in vitro* mammalian cell gene mutation test using the thymidine kinase gene (according to OECD TG 490, latest updated in 2016) or the *in vitro* mammalian cell gene mutation test using the Hprt and xprt genes (according to OECD TG 476, latest updated in 2016).
2. one test detecting chromosomal aberration: the *in vitro* mammalian cell micronucleus test (according to OECD TG 487, latest updated in 2016), or the *in vitro* mammalian chromosomal aberration test (according to OECD TG 473, latest updated in 2016).

Given that the standard REACH data requirement at Annex VII (Ames test) investigates gene mutation, in case of a nanoform registered at Annex VII, ECHA considers appropriate that the registrant provides data from an *in vitro* test detecting also gene mutation, i.e. performed according to OECD TG 490 or OECD TG 476. At Annexes VIII to X, the registrant should provide data from two *in vitro tests*, one detecting gene mutation (OECD TG 490 or OECD TG 476) and one detecting chromosomal aberration (OECD TG 487 or OECD TG 473).

2.2.3.3 Recommendations for the *in vitro* tests

During the OECD/WPMN[13] expert meeting on the Genotoxicity of Manufactured Nanomaterials in Ottawa, Canada in November 2013 [150], several consensus statements were agreed and recommended to investigate the genetic toxicity of nanomaterials. These recommendations are further supported by more recent scientific literature (e.g. see reviews by Magdolenova *et al*. [200], Pfuhler *et al*. [203], Doak *et al*. [209]; Dekkers *et al* [91]; Elespuru *et al*. [201]).

2.2.3.3.1 Nanomaterials characterization in the test medium

When investigating nanomaterials, one must have or generate detailed data on the intrinsic properties of the studied nanomaterial as described in section 2.1.1.3 (e.g. size, shape, crystallinity, surface treatment) as well as on its properties under the test condition. For *in vitro* genotoxicity test, the "characterization of the test material should be undertaken in the cell culture medium used during the test, both at the beginning of treatment and, where methodologies exist, after treatment" [201]. The recent JRC report describes relevant characterisation methods and apply them for gold, silica and silver nanoparticles [212]. Such characterisation helps to monitor the dispersion of the nanomaterial and to ensure that the cells are exposed to a sufficient effective dose under *in vitro* conditions.

2.2.3.3.2 Verification of uptake into target cells

The verification of cellular uptake by appropriate methods is highly advised for *in vitro* genotoxicity tests. The importance of cell uptake was pointed out by the reviews mentioned above, the Nanogenotox report [213], the OECD report [150], and recent guidance documents from EFSA [104] and SCCS [89]. Several parameters (e.g. agglomeration and protein coating) can influence cell uptake.

In vitro tests should be performed on cell lines that have demonstrated the ability to take up nanoparticles. A recent JRC report [214] showed that several cell lines (Caco-2, A549, CHO, V79 and TK6) demonstrated their ability to internalise the tested nanomaterials (gold, silica and silver nanoparticles). If a test is performed on a cell line for which the uptake capacity is unknown, the test data should demonstrate the uptake ability of the studied cell line. Such data can be generated using optical- and electron-based microscopy techniques (e.g. fluorescence microscopy, transmission electron microscopy) ([215], [216], [217], [218], [214]). It is noted that the fluorophore used for labelling may either influence the uptake or detach from the nanomaterial (which would complicate the interpretation of the analysis). The measurement of

[13] WPMN, Working Party on Manufactured Nanomaterials

intracellular nanomaterial can be more or less challenging depending on the nature of the studied nanomaterial.

2.2.3.3.3 Recommendations to avoid interference with uptake or endpoint analysis

An *in vitro* mutagenicity test usually includes an experiment in the absence of metabolic activation system (S9mix) and another experiment in the presence of S9mix. The use of S9 mix in *in vitro* studies can affect the outcome of the tests: like for any other tested chemical, S9 can induce the formation of mutagenic metabolites (in case the nanomaterial can be metabolised); also, the addition in the culture medium of proteins (contained in S9) can modify the cellular uptake of nanomaterials ([200], [209] and [203]). It it thus recommended to perform *in vitro* tests in the presence of S9 only for the NMs composed of organic materials.

Cytotoxicity measurements described in relevant test guidelines (e.g*. relative population doubling, cloning efficiency or relative total growth) are appropriate to determine the top concentration that should be used for nanomaterials in vitro tests.* Moreover, the cytotoxicity should be assessed concurrently with the genotoxicity (not in separate experiments) [150] [201]. Since a variety of proliferaton or cytotoxicity assays employ colourimetry or fluorometry, dye interference should be checked beforehand (section 2.1.2 above).
"The intent when applying nanomaterials to a cell culture medium is to create conditions that are comparable, to the extent possible, with the biological and physiological conditions within the *in vivo* system" [150].

According to Annex VIII 8.4.2 of REACH, a micronucleus test (OECD TG 487 [219]) or a chromosomal aberration test (OECD 473 [220]) is required. Nanogenotox concluded that the guideline for the testing of chemicals *in vitro* mammalian cell micronucleus test (OECD TG 487) is "*applicable for nanomaterials but may need some adaptation in order to provide predictive results in vivo*" [213].

A project on the adaptation of the *in vitro* mammalian cell micronucleus assay (TG 487 [219]) for nanomaterials testing is ongoing in the OECD WPMN rolling work plan (Project 4.95: Guidance Document on the Adaptation of *In Vitro* Mammalian Cell Based Genotoxicity TGs for Testing of Manufactured Nanomaterials). The project focuses on the physico-chemical characterisation of nanomaterials and protocol modifications (selection of cell type with respect to uptake mechanisms, use of cytochalasin B, timing of exposure to nanomaterials, specification of controls, dose ranges and dose metrics). Two intermediary reports studying gold, silica and silver nanoparticles were produced by this project and recently published: in 2018, on the physicochemical characterisation in water and in serum-containing cell culture media [212]; in 2020, on the *in vitro* cytotoxicity and cellular uptake in five different cell lines [214].
At this time, it is recommended to conduct genotoxicity tests for nanoforms according to the available OECD test guidelines, as for any other forms of a substance, with the exception of specific methods adaptations required for nanomaterials, as mentioned here and in the OECD guidance on Sample Preparation and Dosimetry [19]. For instance, if the *in vitro* micronucleus assay is performed using a mitosis blocking agent (cytochalasin B), it is recommended to add this agent post-treatment or to use a delayed co-treatment protocol, in order to ensure a period of exposure of the cell culture system to the nanomaterial in the absence of cytochalasin B ([150], [104], [89], [201]). Moreover, cell lines derived from the expected target tissue(s) should be utilised, if available and possible.

2.2.3.4 *In vivo* test and exposure of target tissue

Prior to conducting an *in vivo* genotoxicity study, there is a need to have relevant toxicokinetic data or to conduct toxicokinetic investigations to assess whether the nanomaterial reaches the target tissue, where the target tissue is not the site of contact. An *in vivo* test is not applicable

for detecting the genotoxicity of a nanomaterial if the nanomaterial does not reach the target tissue [150].
In the absence of toxicokinetic information demonstrating systemic availability and/or exposure of target tissue(s), it is recommended to investigate the genotoxic effects in the site of contact tissue(s). *The basis for selecting the route of administration for testing should be to consider the route most applicable to human exposure(s)* [150]. Currently inhalation is considered the most likely route for human exposure to nanoforms - at least for workers - (See parent Guidance Chapter R.7a: Section R.7.5.6 [3]). The selected route of administration should be justified and the issue of exposure of target tissues should be addressed.

Appendix 1 : Models for fate and exposure of nanomaterials

There is on-going research and development of modelling tools to assess the fate of nanomaterials. The list of methods given below is not exhaustive and includes methods based on attachment efficiency and dissolution rate of nanomaterials. More information on these methods that may be used to predict fate and transport of nanomaterials in the environment and organisms can be found at [221].
Further information on the models and their validation status can be found in the referenced publications for each model.

Table 4: Overview of some models for fate for nanomaterials

Model	Overview	Output	Link to the model tools	References
SimpleBox4nano (SB4N): Classical multimedia mass balance modelling system	The model expresses engineered nanoparticles (ENP) transport and concentrations in the environmental compartments (air, water, soil, etc.) accounting processes such as aggregation, attachment, and dissolution. The model solves simultaneous mass balance equations.	The output is mass concentrations of ENPs as free dispersive species, heteroaggregates with natural colloids, and larger natural particles in each compartment in time and at steady state.	http://www.rivm.nl/simplebox	[222]
NanoDUFLOW: Spatiotemporally explicit hydrological model	Feedbacks between local flow conditions and engineered nanoparticles (ENPs) fate processes, such as homo- and heteroaggregation, resuspension and sedimentation, are modelled.	The outputs are the concentrations of all ENP forms and aggregates in water and sediment in space and time, and retention.	DUFLOW Modelling Studio (v3.8.7) software package with a set of specific processes defined by the user via the NanoDUFLOW submodel.	[223]

Model	Overview	Output	Link to the model tools	References
Steady-state distribution model	Multimedia model was developed using nanospecific process descriptions such as homo- and heteroaggregation, dissolution and sedimentation to estimate the steady-state distribution	The output is nanoparticle/mass concentrations in water and sediment, and its distance from the source.	As a first case study in Praetorius *et al.*, [56] a river model was used.	[56]
NanoFATE	Considers a wider range of ENM processes, including emissions to air, water (freshwater and marine), and soils (urban, agricultural, undeveloped) from their manufacturing, use, and disposal; advection in and out of main environmental compartments; rate-limited transport across compartments; resuspension to air and attachment to aerosols; transformation into other ENMs or compounds; in natural waters aggregation, sedimentation, dissolution, filtration, and sorption to suspended particles and the subsequent deposition to sediment; considers long-term accumulation of NPs and dissolved metal ions; allows inclusion of key transformation processes (e.g. oxidation, sulfidation, adsorption of NOM, loss of primary coating)	Nanoparticle/mass concentrations in different environmental compartments; long term concentrations/releases	https://nanofate.eu/	Example of application: [224]
NanoFASE	Water-Soil-Organism model, a complex multimedia spatiotemporal model predicts the fate and bio-uptake, across space and in time, of nanomaterials entering the soil and aquatic environments. It works by coupling submodels for environmental compartments: soils, rivers, bed sediments, lakes, estuaries and the sea, and simulating the transport of nanomaterials between these compartments of nanomaterials in different forms and states; useful for identifying accumulation hotspots and studying the temporal dynamics of NM concentrations.	Spatiotemporal distribution of nanomaterials (NM) across multiple environmental compartments, making it distinct from lower-tier screening level models, such as SimpleBox4nano,	http://nanofase.eu/show/element_268	

Model	Overview	Output	Link to the model tools	References
LOTOS-Euros	A long-range (regional scale) spatiotemporal atmospheric substance transport and deposition model; open source	Wide range of applications such as air quality forecast, emissions, depositions etc.	https://lotos-euros.tno.nl/publications/model-documentation/	Open source, see link; [225]

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Appendix R7-1 for nanoforms applicable to Chapter R7a (Endpoint specific guidance)
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