ECHA
EUROPEAN CHEMICALS AGENCY

GUIDANCE

Guidance on information requirements and chemical safety assessment

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

Draft (Public) Version 4.0

2021

NOTE

Please note that the present document is a proposed amendment to specific extracts **only** of the *Appendix R7-1 to Chapter R.7a* of IR&CSA Guidance.

This document was prepared by the ECHA Secretariat for the purpose of this consultation and includes only the parts open for the current consultation, i.e. :

- Section 1.1.1 on sample preparation
- Section 1.2.1 Water solubility
- Section 1.2.2 Partition coefficient n-octanol/water
- Section 1.2.3. Granulometry
- Addition of the new Section 1.2.4. Dustiness
- Section 1.2.5 Adsorption/desorption

The full guidance document (version before proposed amendments) is available on the ECHA website at:
https://echa.europa.eu/documents/10162/17224/appendix_r7a_nanomaterials_en.pdf/1bef8a8a-6ffa-406a-88cd-fd800ab163ae?t=1633348005491

The numbering and headings of the sub-sections that are displayed in the document for consultation correspond to those used in the currently published guidance document; this will enable the comparison of the draft revised sub-sections with the current text if necessary.

After conclusion of the consultation and before final publication the updated sub-sections will be implemented in the full document.

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

Reference: ECHA-xxxx
Cat. Number: xxxx
ISBN: xxxxx
DOI: xxxx
Publ.date: XXXXXXX 2022
Language: EN

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1 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 1.2.1 on 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. changed to section 2.1.1. General advisory note on testing with nanoforms and sampling strategy and sample preparation for human health endpoints:** restructured and updated content as follows: ○ Updated Fig 1. and added 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 as well as sample preparation. ○ Section 2.1.1.4. Biological sampling. ○ Section 2.1.1.5. Use of Non-Animal Testing Approaches. • **Section 3.2.2. changed to section 2.2.2. Repeated dose toxicity:** update content: ○ 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.	October 2021

	○ Section 2.2.2.1. The concept of lung overload: updated references. ○ Section 2.2.2.2. Indirect genotoxicity • **Section 3.2.3. changed to section 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. Nanomaterial 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 the advisory note on testing and sampling strategy and sample preparation for ecotoxicological endpoints. • Update of the section 1 for water solubility, granulometry, K_{ow}, adsorption/desorption. • Addition of Section 1.2.4 Dustiness.	2022

1

PREFACE

Three appendices (appendices to IR&CSA Guidance Chapters R7a, R7b and R7c) specifying information requirements have been developed to provide advice to registrants to use 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 the guidance. As this appendix is specifically addressing REACH information requirements, the term nanoform is the preferred one 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, when such advice has been included, it is because it is considered especially relevant for nanoforms and should be part of the nanoform specific guidance. If such nano specific 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 guidance is considered equally applicable to nanoforms or c) more research is needed to develop nano specific advice. This appendix is providing advice specific to nanoforms and does not preclude the applicability of the general principles given in Chapter R.7a [3], i.e. the parent guidance and the parent guidance is applicable in case of no specific advice given for any given endpoint in this appendix. Please note that this document and its parent guidance provides specific guidance on 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 Chapter 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 to address nanoforms of substances.

[2] In this document often the term "set of nanoforms" is used instead of "set of similar nanoforms", but it should be always interpreted as "set of similar nanoforms", as defined in Annex VI.

Table of Contents

Table of Tables

Table of Figures

GLOSSARY[3]

Accumulation: The gradual build-up over time of particles in a tissue or organ.

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: Agglomeration 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 [8].

Aggregate: A particle comprising of strongly bound or fused particles [2].

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 anesthetized.

BALF: Bronchoalveolar lavage fluid.

Bioavailability (toxicological application): The proportion of a substance in the systemic circulation compared with the total amount of substance that has been ingested or inhaled (modified from [9]).

Bioavailability (ecotoxicological and fate application): The amount of a substance accessible to an organism for uptake or adsorption across its cellular membrane [10].

Biodegradation: Degradation of a substance resulting from interaction with the biological environment [9].

Biodurability: The tendency to resist chemical and biochemical alteration through dissolution and enzymatic biodegradation or chemical disintegration within biological media (modified from [9]). 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 [9]. It is determined using *in vivo* methods.

Biotransformation: Alteration of a substance resulting from interaction with biological systems [9].

Clearance: (1) In general (eco)toxicology, volume of blood or plasma or mass of an organ effectively cleared of a substance by *elimination* (*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 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 [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 the "nanoform". Nanoform is a recently defined REACH regulatory term which was therefore not used previously in the scientific literature or authority reports referred to in this guidance.

Dissolution half-life/half-time: A time interval that corresponds to a concentration decrease by a factor of 2 [9].

Dissolution: Dissolution as used in this guidance is the process by which a soluble nanomaterial in an aqueous medium or biological environment is converting/transforming into its constituent ions or molecules [7].

Dissolution rate: The rate at which ions or molecules are released from the surface of a solid into the surrounding liquid medium [9].

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 [9]. Dispersion may also refer to the "act of" dispersion.

Heteroagglomeration: Agglomeration of nanomaterials with different particles such as SPM

αhetero: Heteroagglomeration attachment efficiency

Homoagglomeration: A special form of hetero agglomeration as this describes the agglomeration of the same type of particles, e.g. the nanoparticles with each other, or naturally occurring suspended particulate matter (SPM) [12] and [13].

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 [11]). 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 [11]).

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) [14].

Nanofibre: Fibre with a length-to-diameter ratio of > 3:1 (by partial analogy to the WHO fibre concept [15]) and with one or more external dimensions below 100 nm.

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 [5], [2].

Nanomaterial: A natural, incidental or manufactured material containing particles, in an unbound state or as an aggregate or as an agglomerate and where, for 50 % or more of the particles in the number size distribution, one or more external dimensions is in the size range 1 nm-100 nm. In specific cases and where warranted by concerns for the environment, health, safety or competitiveness the number size distribution threshold of 50 % may be replaced by a threshold between 1 and 50 %. By derogation from the above, fullerenes, graphene flakes and single wall carbon nanotubes with one or more external dimensions below 1 nm should be considered as nanomaterials [5].

Nanoparticle: A nano-object with all three external dimensions in the nanoscale where nanoscale is defined as the size range from approximately 1–100 nm [16]. 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).

NOAA: Nano-objects and their aggregates or agglomerates.

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 [11]. 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 [11]. Examples of dissolvent 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 [8].

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"* [11].

(Q)SAR: Quantitative structure–activity relationship.

Set of similar nanoforms: Under 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 in a solvent under equilibrium conditions, i.e. in a saturated state [17].

SPM: Suspended particulate matter.

[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

1.1.1 Sample preparation

The following section focuses on preparation of the sample for testing implying that a choice of nanoform being representative testing material(s) has already been made. Sample preparation is widely recognised as one of the most critical steps towards successful testing of nanoforms requiring a successful monitoring of the exposure situation. There are many variables to consider when designing 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 chemical composition of the test media, characterisation of stock dispersions and characterisation of samples (prepared from stock dispersions) prior to administration/testing [19]. The hazards posed by all possible forms of the substance, including nanoforms, covered in a registration, must be addressed within the provided toxicological and ecotoxicological information in the registration dossier. Equally important is that the fate information for all possible forms of the substance, including nanoforms, covered by a registration, must be addressed 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).

In effect, recital 12 of the REACH amended Regulation (EU 2018/1881) [20] 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:

- Names 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)

In addition, detailed information regarding the test conditions, 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 other than the experimental test data has to be provided.

Moreover, appendix R6-1 for nanomaterials 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 consider characterising the test material according to these parameters, in order to be able to follow the above-mentioned guidance. For example, baseline information for the grouping of nanoforms [4] dissolution rate, surface chemistry and dispersibility have to be reported.

1.1.1.1 Test material and sample preparation for ecotoxicological and fate tests

OECD GD 318 [21] provides guidance on the assessment of dispersion and dissolution of nanomaterials as well as indication of the applicability of the (non-nano specific) available methods to test nanomaterials in aqueous media, i.e. environmental media.
When considering aqueous media and environmental organisms and compartments, 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) such that a single, homogeneous, stable phase results; and it is relevant to 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 part of a liquid sample that had passed through a 0.45µm (or similar) filter. However, as dispersions of nanoparticles will pass through such filters, "dispersed" is the term to use when both liquid and particulates are present and to restrict the term "dissolved" for the formation of solutions in the strict sense of the definition ([19], [22]).

Because dispersions may falsely visibly present as a solution, dispersion stability is an important parameter to assess in the context of sample preparation. The dispersion of particles 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. As a result, the state of dispersion is dynamic and changes with time to potential dissolution and/or higher agglomeration. Dispersion stability is determined by interactions between the properties of the particles of the nanoform and properties of the surrounding matrix. In liquid media, slight modifications in pH, ionic strength and concentrations of molecular constituents can significantly alter the particle dispersion.
The stability of a dispersion is typically assessed using comparative particle size measurements and requires a reliable method of measuring the baseline particle size distribution of the material. By comparing changes in particle size distribution, a qualitative assessment or proxy measure of the state of dispersion can be made. Examples of measurement methods applicable for spherical particles are Zeta potential measurement, combined with Dynamic Light Scattering (DLS) or UV-VIS spectroscopy to monitor the stability of nanoparticle dispersions and to gain a qualitative understanding of the agglomeration process. Other methods, when suitable, e.g. particle tracking analysis, can also be used for the test particles [23]. Further details and specific analytical methods on the sample preparation (whether for stock or test dispersion) are provided in the OECD GD 317 [24] under Section 4 and should be followed.

Draft test guidelines on agglomeration and dissolution rate of nanomaterials in aquatic environments are under development within the OECD (through the NanoHarmony project WNT 1.5 and 3.10 [25] [26]) and will allow eventually characterisation and quantification of dissolution, dispersion and agglomeration behaviour in more complex media (see section 1.2.2). OECD GD 318 [21] provides guidance on the applicability and adaptation possibilities of OECD TG 318 [25] and other relevant test guidelines available, to identify and quantify the dispersed and truly soluble fractions of nanoforms. This recently published guidance document consolidates the current knowledge on dispersion stability, dissolution, homo- and hetero-

agglomeration, but also highlights limitations of the currently available test methods.

1.1.1.2 Test material and sample preparation for toxicological tests

Besides all these parameters, ISO 14887:2007 [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., in [28] and [29].

However, such procedures should be carefully examined to determine if they are adequate for the test material under consideration and modifications may be required for different materials in the context of toxicological testing preparation. 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].
An important component of sample preparation is "reliable" sampling. In reliable sampling the test aliquot used for measurement 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 to be followed, as well as careful storage if aliquot is not characterised straight after sampling. ISO 14488:2007 [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.

In order to prevent errors in the interpretation of results due to particle contaminants/impurities, data from the characterisation of the test material including its purity and, if technically feasible, quantities of identified contaminants and impurities have to be characterised and analysed prior to the start of a study, consistently with the substance identification requirements.

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 a number of 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 state of agglomeration or aggregation is recognised as an important parameter influencing the interpretation of the characterisation results and the actual testing of nanoforms ("as received", "as used", "as dosed / as exposed") and has therefore to be considered during sample preparation. A number of measurands have been proposed for assessing agglomeration and/or aggregation state, including the effective cross-section, determined by measuring aerodynamic/light scattering properties or by electron microscopy ([30], [31]).

1.1.2 General considerations for (Eco)-Toxicological testing

1.1.2.1 Considerations for ecotoxicological testing

In order to start with relevant sample preparation, the Guidance on Sample Preparation and Dosimetry for the Safety Testing of Manufactured Nanomaterials OECD GD 36 [19] should be considered. Further guidance may be found in Ecotoxicology and Environmental Fate of Manufactured Nanomaterials: Test Guidelines [32] reflecting the outcome of the discussion of the OECD's work on nano safety during the Testing Programme of Manufactured Nanomaterials

[23], while the latest developments were compiled in the guidance document on aquatic and sediment toxicological testing of nanomaterials OECD GD 317 [24]. This guidance, besides providing specifications on sample preparation, e.g. starting materials, stock and test suspension preparation, also includes analytical techniques to characterize the as-produced or as-received test material, as well as the test material in stock and test suspensions.

The following aspects are important and need to be applied for sample preparation:

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

- Nanoform test item preparation and dispersion (including stability) have to consider key characteristics and composition of the test medium (such as pH, OM, salts etc) as interactions between the particles of a nanoform and the test medium define their physico-chemical properties and determining as a consequence then fate and potential adverse ecotoxicological effects. Thus, testing has to be accompanied with suitable analytics to monitor the exposure situation, such as exposure concentration but also exposure form of the tested nanoform (dissolved or in particulate form). For nanoforms, the use of only chemical analysis to determine mass based concentrations/metric is not sufficient, as further explained in the next bullet point, regarding dose metrics (see OECD GD 317 [24], Section 4).

- 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 increase the ability to interconvert doses 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 and consequently reduces the amount of required testing.

- Sample preparation needs to be controlled, consistent, relevant, reliable and robust, as the testing stages may include e.g. the use of powder and/or dispersion depending on the endpoint. This carries the risk that the test item may have undergone physico-chemical changes already during the different preparation stages so that monitoring of this progress is needed to be able to precisely describe the test item at the start of the exposure.

- The applied sample preparation protocol and control procedures need to be justified and sufficiently reported in the study summary.

If a nanoform is soluble and has a high dissolution rate in relevant organisms and biological (see Section 2.1.1) or environmental (see section 1.2.1) media , then it is likely to present itself to the test system in its molecular or ionic form and can therefore be expected to elicit the same response as the non-nanoform of the substance e.g. the metal salts ('ionic form') used as a positive control vs the metal ions released from the nanoform. If, however, the nanoform under investigation is insoluble or sparingly soluble in biological and/or environmental media, then it will probably present itself to the test system in a particulate form. In this case, the advice provided in *Appendices for nanomaterials applicable to Chapters R.7a (this document), R.7b and R.7c* would apply.

In addition, nanoparticles likely interact with the components of the environmental or biological media, partially or totally yielding soluble or dispersed transformation products (as well as some dissolved nanomaterial itself) that will influence the overall toxicity and fate processes. This possibility needs to be taken into account when selecting the media and

procedures as well as in the assessment of the results of any test ([19], [33]).
Due to differences in fate and behaviour between nanoforms and non-nanoforms in different test environments, a testing strategy in form of a decision tree on dispersion, dissolution, dispersion stability and agglomeration/aggregation was initially recommended in OECD GD 40 [22]. However, following latest developments and publications from OECD flowcharts on specific preparation and dispersion the choice of testing strategy is specifically described in OECD GD 317 [24], Section 5 and OECD GD 318 [21] Sections 2 and 3. Considerations and measurements of dissolution rate and dispersion stability in test media do not only help to find the appropriate testing strategy and test conditions, but also to interpret the results. This information is also considered to be useful for nanoform grouping and read across [34].

1.1.2.2 Further considerations for (eco)toxicological testing

Notwithstanding the ecotoxicological considerations for sample preparation and testing preparation, other important considerations for sample preparation include the influence of contaminants (including biological contaminants) and impurities on (eco)toxicological test results. For example, metallic impurities such as Co and Ni catalysts used in the production process of the nanoparticles were shown to inhibit hatching in zebrafish embryos (e.g. [35]).

Also of particular concern for nanomaterials is the influence of endotoxin on certain test results. 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 a confounding of results (including a potential false positive) in biological assays. Therefore, establishing the presence or level of endotoxin in a test sample is an important step during the preparation of a sample for toxicological testing. Endotoxin can be measured using *in vitro* methods such as the macrophage activation test, which has been validated by European Committee on Validation of Alternative Test Methods and proposed as a reliable method for determining the pyrogenicity of engineered, research-grade nanomaterials [36]. International standards are available for the testing of nanoforms [37]. Although issues regarding contamination are not nano-specific, the increased relative surface area of nanophase systems compared to other particles means that the possible amounts of adsorbed endotoxin (e.g. grams adsorbed endotoxin per gram of material) are significant [38]. The existence of false negatives has also to be accounted for, for instance in cases where exposure of the organism is underestimated (e.g. Ames test, insoluble particles etc.).

Further specifications on the toxicological testing and preparation for exposure are detailed under Section 2.1.1 General advisory note on testing with nanoforms and sampling strategy and sample preparation for human health endpoints of this Appendix.

1.2 Specific advice for endpoints

1.2.1 Water solubility

Water solubility is covered in Section R.7.1.7 of the parent guidance. In the case of nanoforms, it is necessary to take into account that water solubility has the potential to increase for materials in the nano-size range due to their generally smaller particle size, decreasing size depending on their interactions with the surrounding media or the impact of their shape and surface coating. The transport of nanoforms is affected by their dissolution rate and their degree of dispersion. However, it can be difficult to distinguish between a dispersed nanoform and a dissolved nanoform, due to its small particle size. It is important to distinguish between the different phenomena solubility and dispersibility as this has implications on respective testing and characterisation strategies. 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. Further information on these issues is provided in the section on Sample Preparation. Furthermore, it is important to ensure that no undissolved material contributes to what is being measured as dissolved material.

The OECD has examined the applicability of its test guidelines for nanoforms and OECD publications have stated that OECD TG 105 [39] (Water solubility) is mostly not appropriate for testing of nanoforms [32]. OECD GD 318 [21] refers to OECD TG 105 in a section dedicated to other relevant method, comparing OECD TG 105 to the methods proposed for dissolution rate assessment (referred in 1.2.1.1) and establishes the test limitations. The flask method presented in this test guideline recommends determination of (total) dissolution after 24-hour equilibration. The measurements are to be performed after a separation step. However, the method recommended in OECD TG 105 is not considered adequate for nanoform testing. This method resembles the static batch method described in section 1.2.1.1 with the limitation that it does not allow determination of dissolution rate, due to limited measured data (single time point).
Measurement of water solubility using the OECD TG 105 guideline may still be of value for nanoforms that are water soluble and have a high dissolution rate.

OECD GD 29 [40] transformation /dissolution protocol allows to test dissolution and transformation of metals with test duration varying between 1 up to 28 days and a common duration of 7 days. OECD GD 29 protocol provides advice on how to determine the transformation or dissolution and provides knowledge to which extent metals and sparingly soluble metal compounds can produce soluble ionic forms and other metal bearing species in aqueous media. When choosing the testing material for this endpoint, it should be noted that testing the smallest particle size (as recommended by the guideline) may not be adequate in the case of nanoforms. Furthermore, a 0.20 um filtration, as recommended in OECD GD 29, is not appropriate for nanomaterial testing either.

In the OECD GD 318: OECD 29 and other TGs have been reviewed to establish their applicability for measuring water solubility and dissolution rate in biological and environmental media. Guidance on the applicability of static batch mode (screening test, adapted from OECD GD 29) and dynamic mode (based on ISO TR 19057 [41]) is further provided in the OECD GD 318 and under this section.
For instance, the static batch method allows quick determination of solubility of nanomaterials as "poorly" or "highly" soluble, based on a 24-hour test. When nanoforms dissolve fast, the time resolution of static batch mode does not allow a clear determination of dissolution rates and the time resolution has to be increased in a new batch mode test to be able to do so as explained further in OECD GD 318 (**Figure 1**).
When a nanoform is fully dissolved in a solubility screening test, as referred to in OECD GD 318, it can be considered highly soluble i.e. no particles are present) and under this condition

the parent guidance could be followed for environmental and human health endpoints, providing satisfactory justification and documentation. Even in case of highly soluble nanoforms, the assessment of the solubility is related to dissolution in water and cannot be neglected as explained as per REACH column 1 information requirement and in the following Section 1.2.1.1.
For specific considerations to human health endpoints and dissolution in biological media Section 2.1.1 of this Appendix has to be consulted.

1.2.1.1 Dissolution rate

Annex VII 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 dissolution rate has to be provided as part of the solubility endpoint and in, addition, should be investigated in relevant media, i.e. as used in the applied (eco)toxicological tests).

Dissolution rates in the different biological and environmental media used for (eco)toxicological and fate tests are variable and affect the bioavailability of substances in the (biological) environment and will help to predict toxicokinetic behaviours of the particles.

Currently, no test guideline is available for determining the dissolution rate of nanomaterials although some projects are working on developing specific TG (OECD WNT projects 3.10 "Dissolution Rate of Nanomaterials in Water and in environmental media" and 1.5 on "Determination of Solubility and Dissolution Rate of Nanomaterials in Water and Relevant Synthetic Biological Media) for dissolution rate of nanomaterials in water and biological or environmental media. The recently published OECD GD 318 [21] provides guidance on how to adapt available test guidelines for determination of dissolution, dispersion and agglomeration of certain nanoforms. 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 tested less and analytical methods are lacking 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.

The testing strategy proposed in OECD GD 318 aids at determining when nano specific testing is required (see **Figure 1** below).

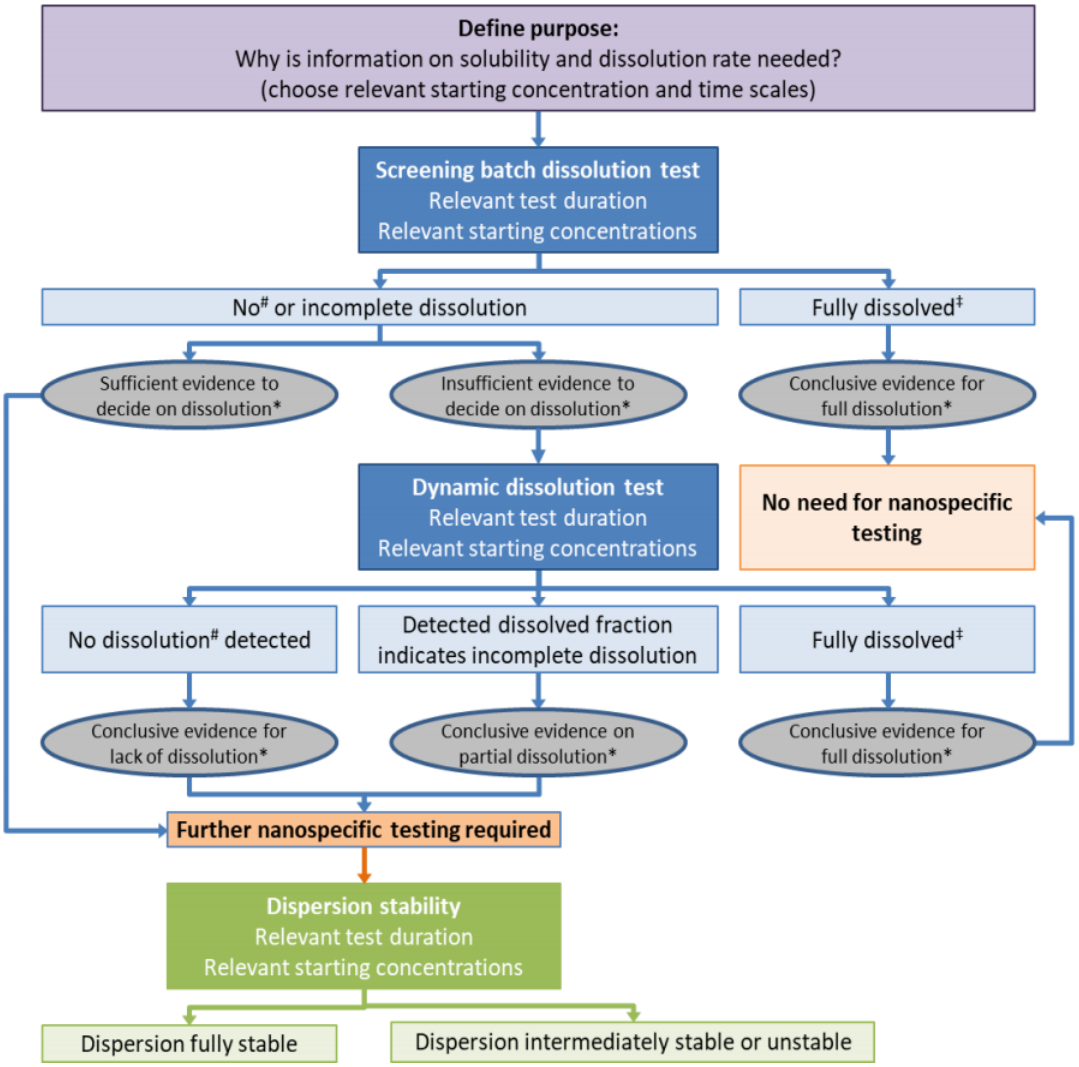

Figure 1: Dissolution rate tier testing strategy for nanoforms (based on OECD GD 318)

Regarding dissolution, static batch mode (screening test, adapted from OECD GD 29) and dynamic mode (based on ISO TR 19057) are used as reference methods.

The determination of dissolution rates depends on an appropriate separation of particulates from the dissolved fraction and appropriate time resolution which can be a drawback for static batch mode as a screening test, especially in cases of rapid and full dissolution of the nanoform. For slowly dissolving nanoforms, batch method applicability 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.

ISO TR 19057 reviews separation techniques applicable for nanomaterials. As it is explained under OECD GD 318 centrifugal ultrafiltration promotes fast enough separation to allow calculation of dissolution rates and is therefore the recommended method. 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, there is some danger that ions pass through the filter adsorbed to solids and further consideration is needed when using NOM as it can clog filters with smaller cut-off.

The dynamic method is performed in a flow-through system. This method precludes the use of

a test medium delivered at constant flow rate through a compartment which entraps the nanoparticles, i.e. using ultrafiltration membranes. Nevertheless, sensitivity can be partially compensated by adapting the low flow rate and nanoform concentration in the dynamic method.

For both methods, static batch and dynamic, their applicability in testing non-metallic nanoforms depends on the specificities of the analytical method used. Further specific considerations to the adaptation of analytical methods to nanoform testing is not yet fully developed. Nevertheless, experience showed that for both screening and dynamic dissolution tests may be applicable to nanoforms within a solubility range 0.1 to 10mg/L.

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

Solubility should be plotted as ionic concentration (in mg/L) versus 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.

In the OECD GD 318, advice is also provided on how both static batch and dynamic methods can be adapted to measure dissolution in natural or artificial environmental media. For the dynamic method, applicability has already been successfully demonstrated for toxicological test media, i.e. in lung and gastro fluids) and considerations to its applicability are further described in OECD GD 318. In addition, the OECD WNT 1.5. project will provide a specific GD to determine dissolution in biological media and water. 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.

For environmental media, the impact of key parameters such as pH, ionic fraction or suspended particulate matter should be evaluated carefully when testing dissolution, dispersion and agglomeration of nanoforms.

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 have to be warranted.

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.

Taking into account the nano-specific properties and constraints in assessing the solubility of nanoforms, waiving the information requirement based on high insolubility 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. The fate of nanoparticles may not be predicted by equilibrium partitioning ([42], [43]) as nanoparticles cannot reach thermodynamic equilibrium by distributing between two phases, water and n-octanol, due to their particulate nature. Therefore, OECD TGs recommended in the parent ECHA Guidance for partition coefficient n-octanol/water, i.e. OECD TG 107 [44], OECD TG 117 [45] and OECD TG 123 [46], are in most cases not applicable to nanoforms ([30], [22], [47]). Results will be impacted upon by the presence of a colloidal suspension, which could be present if the manufactured nanomaterial does not completely dissolve ([19], [22], [34]).

If it is shown that the nanoform is highly dissolved, as explained under Section 1.2.1, and the presence of particles can be excluded the parent guidance will apply. Taking into account the above, measurement of n-octanol/water partition coefficient may still be of value for organic nanoforms and organic coated nanoforms that are water soluble and have a high dissolution rate.

If on the other hand, it is shown that the nanoform tested is dissolving partially, K_{ow} can be used for the dissolved fraction but cannot replace the need for data on dispersion stability and is not sufficient to waive the generation of further data on its own (see Section 1.2.5 and R7c).

Section R.7.1.8.3 of the parent guidance, includes information regarding experimental data on n-octanol/water partition coefficient including testing methods.

1.2.2.2 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".*

Regarding the use of K_{ow} as a waiver, it might lead to erroneous interpretation of the environmental fate or bioconcentration [42]. Taking into account the nano-specific properties and constraints in assessing the n-octanol/water partition coefficient (K_{ow}) of the nanoforms by currently available standard methods, waiving the information requirement based on n-octanol/water partition coefficient should always be accompanied by a robust technical and scientific justification on the applicability of the used test method (e.g. nanoform being water soluble or having a high dissolution rate).

When the K_{ow} is not applicable, the dispersion stability test according to OECD TG 318 [25] has to be performed.

Thus, the use of the dispersion stability alone does not fulfil the K_{ow} information requirement.

And the use of dispersion stability cannot be applied (in isolation) to waive other tests such as bioaccumulation.
Other 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 312 [48]). A list of the models and specific parameters under development as alternative methods to K_{ow} and K_{oc} is available in Appendix 1.

1.2.2.3 Dispersion stability

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 ([42], [43]).

Annex VII proposes to use dispersion stability for nanoforms, however other properties may be used to predict fate and transport of the nanoforms in the environment and organisms. Agglomeration, aggregation, deposition and attachment are considered informative properties to predict the environmental behaviour of the nanoparticles ([42], [49], [50], [51]). In this line, OECD GD 318 includes recent developments on agglomeration measurements, considering both homoagglomeration, as per OECD TG 318, and heteroagglomeration. Furthermore, dispersion stability (as per OECD GD 318) is a relevant parameter to consider for aquatic toxicity testing while adsorption/desorption is more suitable when testing soil and sediment toxicity (as per OECD TG 312) and will complement the information on the environmental behaviour of nanoparticles.

As stated in section 1.2.1.2 of this guidance, a dissolution screening test evaluates the nanoforms solubility by determining the ionic content of the sample.

To avoid misinterpretation on the dissolution results or confounding effects between dispersed and truly dissolved fractions of the nanoform, assessment of dispersion stability of all used stock dispersions is required, i.e. the original dispersion (either as delivered to the lab or prepared from a powder prior to testing), the nanoform stock dispersion as prepared in the respective test medium and all intermediate nanoform stocks which might be required for a dilution series down to the desired test concentration. The dispersion stability is performed following the method described in OECD TG 318 [25].

OECD TG 318 proposes testing for dispersion stability in aquatic media stabilised with NOM where, for comparison purposes, the tests are performed on particle number concentration basis. The dispersion stability provides information on the quantity or relative percentage of nanoparticles that remains dispersed in the aquatic medium tested.

Hence, these allow to establish the tendency of the nanoform to attach, sediment, agglomerate and potentially dissolve. These results can then be used to define the testing strategy in ecotoxicological tests and environmental compartments (see Appendix R7b).

Homoagglomeration

Agglomeration is the process by which two particles interact. When the two particles are of the same kind, this process is called homoagglomeration. The test guideline OECD 318 is designed to determine homoagglomeration; i.e. particle-particle attachment of nanomaterials in ecotoxicological test media; although the use of other test media, such as natural waters, should in principle, be also feasible.

As for the dissolution test, the dispersion stability test follows a 2-tier approach (described in TG 318 and under Section 3 of GD 318):

- Tier 1 (screening test): where measurements are done at two time points (0 and 6h) at different pHs (pH 4, 7 and 9 are recommended), 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): where, in addition to the conditions 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 buffering agent. The stability of the dispersion is measured hourly, from sub-samples, during the test i.e. over 6 hours).

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

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

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

Where the measured concentration is within 10 and 90% of the initial test concentration, i.e. intermediate stability, the extended test (Tier 2) needs to be performed. The extended test would also provide information on the type of nanoparticles, with regards to density, as explained in **Figure 2** below. Specifically, the red line represents a NP which agglomerate however, due to low density, do not settle. On a more complex example, the green line may represent either high density NPs that agglomerate and settle quickly or a mixed sample composed of an unstable, high density, fraction and a stable one.

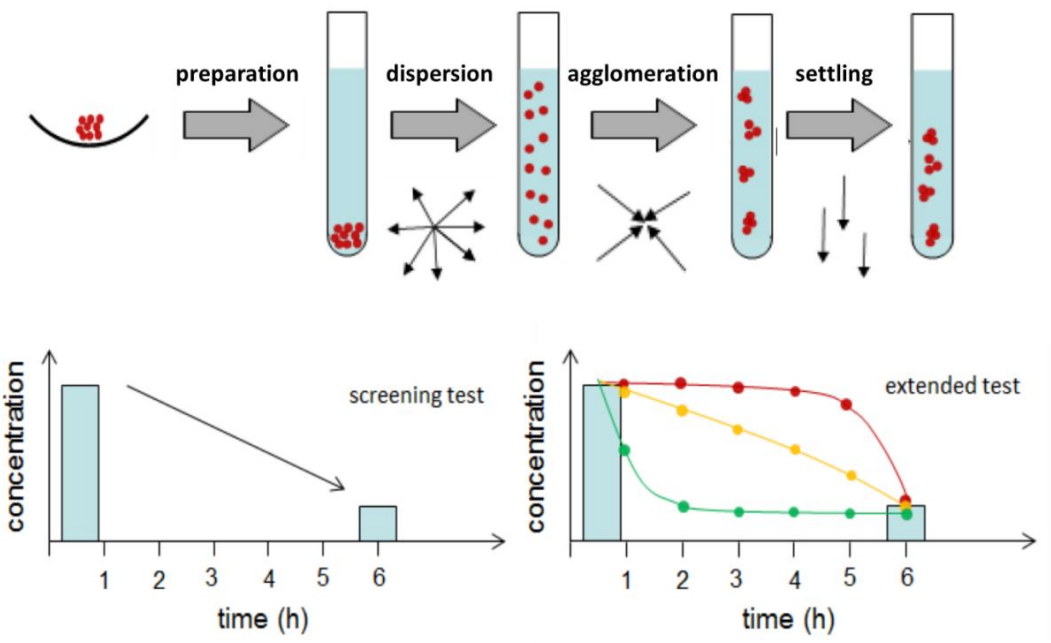

Figure 2: Dispersion stability tier testing (from OECD TG 318)

Overall, OECD TG 318 applicability to nanoforms should be considered based on:

- material density i.e. >1g/cm3);

- analytical quantification sensitivity should allow quantification of the nanoforms from 100 to 10% of the particles present in the water column, i.e. LOQ equal to 10% of initial mass concentration;

- analytical sensitivity.

When applying the test in different settings, namely when testing 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. As a remark, this sample is 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 hetero-agglomeration considerations below).

Data comparability must be targeted therefore, media constituents interfering with agglomeration need 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 according to OECD TG 318, namely:

- Although NOM-nanoparticles interaction varies with particle properties (e.g. surface charge), NOM is known to form nanoparticles "corona", i.e. surface interaction/coverage, which highly influences the dispersion behaviour of the test material, by promoting stabilisation. Use of synthetic commercial NOM (such as 2R101N Suwannee River NOM (SRNOM)) is recommended in view of reproducibility and comparability of results.

- Use of stabiliser aids dispersing extremely hydrophobic materials; however, it will impair the evaluation of pristine material stability.

- A 6-hour long test performed with fixed particle number concentration (e.g. 1012 particles/L) 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 method 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 the 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 analytical methods applicable to nanoforms are limited only by their sensitivity. ICP-MS and ICP-OES are recommended in the TG 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 quantitative method. However, careful considerations of its applicability based on material properties is needed. This method provides indication of the maintenance of particles size and number in suspension therefore, nanoform stability can, at some extent, be

monitored. Additionally, non-quantitative measurements such as electrophoretic mobility i.e. Zeta potential can also be used as an indicator of particles (in)stability. Further information on methods applicability can be found in "NanoDefine Technical Report D7.6" [52].

Heteroagglomeration

Heteroagglomeration, i.e. the interaction between two particles of different nature, is considered the most relevant type of agglomeration process to be investigated under hazard evaluation. The heteroagglomeration kinetics depend on number ratios of nanoparticles and suspended particle 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 the most suitable measurement for fate and hazard evaluation, as it measures the fraction of collisions resulting in attachment.

A test guideline to determine attachment efficiency is not yet available. A test performed, as described in OECD TG 318 would theoretically allow categorisation as "low", "medium" or "high" heteroagglomeration, based on attachment affinity (αhetero), t1/2 and the fraction of free nanomaterials left after a certain time. These thresholds are under discussion and should be reconsidered depending on the nature of nanoform tested, the SPM used and the test media composition.

According to the recommendation of OECD GD 318, categorisation would be done by setting the remaining fraction of free nanomaterials (e.g. to 10%) and the test span to e.g. 3 h (considered conservative). Illustratively it would provide:

- fast heteroagglomeration = αhetero > 0.1 <1, if less than 10% of the nanomaterials is left after 3 h
- slower heteroagglomeration = αhetero ≤0.1, if more than 10% are left after 3h
    - intermediate heteroagglomeration = >0.01 ≤0.1
    - low heteroagglomertion = αhetero ≤0.01

Where heteroagglomeration is shown slower, i.e. ≤0.1, a kinetic study to determine αhetero should be conducted.

As set in OECD GD 318, the total collision frequency can be calculated according to the following equation (Section 3.4 OECD GD 318):

$$k_{\mathrm{coll}} = \frac{2kT}{3\mu}\,\frac{(r_{\mathrm{SPM}} + r_{\mathrm{NM}})^2}{r_{\mathrm{SPM}}\, r_{\mathrm{NM}}} + \frac{4}{3}G(r_{\mathrm{SPM}} + r_{\mathrm{NM}})^3 + \pi(r_{\mathrm{SPM}} + r_{\mathrm{NM}})^2 |v_{\mathrm{SPM}} - v_{\mathrm{NM}}|$$

k: Boltzmann's constant [m²kg/s²K]
T: absolute temperature [K]
μ: dynamic viscosity of dispersion medium [Ns/m²]
r: particle/aggregate radius [m]
G: shear rate (velocity gradient) [s^{-1}]
g: gravity acceleration [m/s²]
ρ_S: density of particles [kg/m³]
ρ_L: density of liquid [kg/m³]
$v_{SPM;\,NM}$: settling velocity of SPM or nanomaterials [m/s]
ϵ: power input per unit mass [Nm/skg]
v: kinematic viscosity (μ/ρ_L) [m²/s]
P: power dissipated by liquid turbulence [W]
V: volume of liquid [m³]

Considering heteroagglomeration as a pseudo first-order reaction with linear relation between natural logarithm of the particle concentration plotted and time, the heteroagglomeration attachment efficiency, i.e. αhetero, can then be calculated from the slope ($khet* = \alpha het\ nSPM\ kcoll$).

These kinetics are strongly dependent on nanomaterial/SPM number concentrations and sizes. The time span of the test needs to take into account the respective nanomaterial/SPM number-ratio applied.

Media components such as NOM or impurities of the nanoforms, able to modulate its surface properties, can promote stabilisation of a sub-fraction of the nanoforms. In this respect, careful consideration is required when setting the remaining free nanomaterial fraction threshold. Ideally, a longer test should be performed (e.g. 24 h) to determine if a plateau is reached, in order to set a relevant threshold value.

Additional uncertainties on the measurement and method applied, as set in OECD GD 318, include:

- The impact of homoagglomeration as well as the impact of shear forces, in case SPM are in the µm range, needs to be considered.

- Similarly to homoagglomeration, the presence of divalent ions plays an important role. Therefore, the use of counter ions in media, compensating for the SPM surface charge is required.

- The settlement of particles and consequential need for agitation must be carefully judged, as it would impact collision and, consequently, heteroagglomeration rate. In this line, the separation method for analysis of free particles needs to be fast and non-intrusive, therefore centrifugation is still considered appropriate.

- The type of SPM to be used is yet uncertain. "Model SPM" are currently under development considering factor such complexity/representativeness, stability or density. The impact of the type of SPM on the attachment evaluation must be carefully evaluated as it could ultimately be indicative of bioaccumulation potential i.e. based on free nanoparticles availability.

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 practise 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 approriate 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 fullfil other Annex VII-X

data requirements.

The different characterisation parameters of nanoforms, such as the constituent particle size, the shape of the particles and the surface treatement, 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. 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 toxicity studies via the inahalation route, the most relevant parameter to measure and report is the aerodynamic diameter of the particles. The aerodynamic diameter is defined as diameter of a spherical particle with a density of 1 000 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 atmospheres for the purpose of inahalation toxicity studies for nanomaterials. Some guidance on this subject can be found in the ISO standard ISO/TR 19601:2017(en) [53]: The generation of aerosols for the purpose of inhalation toxicity studies.

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 [54] 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 1.1.1 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 draft Test Guideline for particle size and particle size distribution of nanomaterials **[55]** and the method listed **Table 1** can be used to measure the particle size size distribution of nanoforms to fulfil the Granulometry endpoint. Under Section 1.2.4. **Table 2** is also collecting methods to measure particles and can be consulted. The measurement techniques have limitations in respect to the size range of which can be measured with each of them and this must be taken into account when a method is selected for a certain nanoform. 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.

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 a safety hazard and may 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.

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 15900:2009 [56]**; ISO 10808:2010** [29]**; ISO 28439:2011** [57]**)** 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 nanoparticles, 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 nanoparticle 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 nanoparticle aerosols exposed to the test system during the toxicity test; iii) particle size and concentration measurements are sufficiently accurate for nanoparticle 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. HARN), 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	Particles in an aerosol Size range: ~3 – 800 nm -115 microns	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.

not appropriate, due to the different principles employed by the two methods [58]. 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-micrometer aerosol particles, up to 10^7 particles / cm^3 (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 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	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.

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 [59] 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 [58]. ISO/TR 27628:2007 [59] 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 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 1:3 for non-spherical particles. This method has limited	Particles of all kind Size range: 0.06-100 µm	Particle size/size distribution, from which mass median diameter can be calculated, with knowledge of the density of the particles.

applicability really suitable in the sub-100 nm range. In the range below several microns, results strongly depend on optical constants of 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

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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 particle size instrumentation have ranges of particle size limited by 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. Depending on the number based particle size distribution the particle number concentration will be determined too low and particle counters with different valid lower size limit will give different particle number concentrations. 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**.

Published data on granulometry

Particle size measurements have been published in the peer-reviewed literature. Some electronic databases exist collecting published information on properties of specific nanomaterials, including information on particle size distribution. 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.

Regarding the evaluation of available information on granulometry (Section R.7.1.14.3), it is advised to perform particle size characterisation not only of the material under investigation but also of the airborne dust where appropriate. It as also important to remember that the original particle size distribution is highly dependant of the industrial processing methods used and care should be taken to ensure that the measurement and assessment activity considers any changes to the particle size distribution by subsequent environmental or human transformations.

When considering the uncertainty on granulometry it has to be noted that 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.

For reaching conclusion on granulometry (See Section R.7.1.14.4) 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 a safety 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 or fibres, 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.

In addition, the particle size distribution is needed to inform the decision regarding which route of administration is most appropriate for the acute toxicity and repeat dose toxicity animal studies. A number of methods are provided for determining the particle size fractions 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.

Finally, although granulometry and dustiness are separate information requirements for nanoforms of a substance, some of the test methods used to determine dustiness can provide information regarding the MMAD of a particle (see **Table 2**, under the section on dustiness). However, it is necessary to respect the limitations of each method outlined, to ensure that the method is applicable to the nanoform(s) in question.

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 is not an intrinsic physical or chemical substance property, as 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) or 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 by 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 CEN has published 5 standards (EN 17199: 1-5) for the testing of dustiness of materials that release or contain nanomaterials. The EN 17199-1 [60] gives advice on the methodology and provide guidance to choose the more adequate tests method. The other 4 standards, EN 17199-2 to 5 [61], [62], [63], [64], provide 4 different test methods.

- Rotating drum (EN 17199-2) [61]
- Small rotating drum (EN 17199-4) [63]
- Continuous drop (EN 17199-3) [62]
- Vortex shaker (EN 17199-5) [64]

Table 2 summarises methods to generate/sample airborne dispersed or nebulised particles.

Table 2: Methods to generate/sample airborne dispersed or nebulised particles and 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
Cascade impaction Cascade impactors can be used to obtain the size distribution of an aerosol i.e.. in this context a dust cloud). Air samples are drawn through a device which consists of several stages on which particles are deposited on glass or glass fibre. Particles will impact on a certain stage depending on their size. The cut off size can be calculated from the jet velocities at each stage by weighing each stage before and after sampling and the MMAD derived from these calculations. A well established technique to measure the distribution of particles of respirable or inhalable size. However, cascade impaction may fail to describe the dimension of high aspect ratio nanoparticles when they no longer follow aerodynamic rules [58]. Conventional cascade impactors will have size selective stages limited to the capture of particles greater than ~250 nm. This is a sampling method and also requires aerosolisation. ISO/TR 27628:2007 [59] provides an informative description.	Particles in an aerosol Size range: 0.1-20 µm and 0.5-80 µm	MMAD can be determined via an appropriate coupled analytical technique.
Low Pressure Impactor (ELPI) ELPI is a type of cascade impactor that combines inertial collection with electrical particle detection to provide near-real-time aerosol size distributions for particles larger than 7 nm in diameter. Aerosol particles are charged in a unipolar ion charger before being sampled by a cascade impactor. The upper size limit of the instrument is 10 µm, but in practice reliable data can be obtained only up to about 2.5 µm due to significant losses at larger particle sizes. Collected aerosol particles are available for offline analysis, but this is also a limitation as it does not provide a direct measurement. It does however enable a range of off-line analytical methods to be used with samples, including electron microscopy and chemical speciation. ELPI has useful application in relation to exposure estimation. Data from the lowest stage have relatively large uncertainty due to losses and uncertainties of the true size channel width. ISO/TR 27628:2007 [59] provides an informative description.	Particles in an aerosol Size range: 7 nm – 10 µm	MMAD can be determined via an appropriate coupled analytical technique or by calculation.

Rotating drum method (EN 15051-2) [65] **and small rotating drum method** 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, airbone nano-objects and strutures can be collected for off-line (analytical)electron microscopy analysis. For the small rotating drum, a respirable 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 [66] and EN 15051-2 [65]. The small rotating drum require smaller amounts of bulk material for testing (2 to 6 g) compared to the rotaling drum method.	Dry powders/granulates/friable products Size range: 0.5-10,000 µm	MMAD can be determined via an appropriate coupled analytical technique.
Continuous drop method (EN 15051-3) [67] 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 [68].	Dry powders/granulates/friable products Size range: 0.5-10,000 µm	MMAD can be determined via an appropriate coupled analytical technique.

Vortex shaker The vortex shaker method consists of especially designed cylindrical container that is continuously shook 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 more 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 with one of the methods. This includes the sample preparation, determination of moisture content etc. The standards propose a number of measurands of dustiness to be determined. In addition, it establishes 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 as proposed by the standard:

- Respirable dustiness mass fraction (mg of airborne resp. 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)

Further information on the methodology described above can be found in the following reference standards:

- EN 17199-1:2019-1 [60] - Part 1: Requirements and choice of test methods
- EN 17199-2:2019-2 [61] - Part 2: Rotating drum method
- EN 17199-3:2019-3 [62] - Part 3: Continuous drop method
- EN 17199-4:2019-4 [63] - Part 4: Small rotating drum method
- EN 17199-5:2019-5 [64] - Part 5: Vortex shaker method

[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.

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 in the environment. 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[6] 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, as set in Section 1.2.1). K_d measurement is based on the assumption of thermodynamic equilibrium between liquid and solid phase. Equilibrium partitioning does not apply to undissolved nanoparticles ([47], [42], [43], [30]) as described in section 1.2.2. Hence, nanoparticles mostly do not form solutions, but instead form dispersions, which are multiphase systems and thermodynamically unstable. Thus, nanoparticle 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 [43], [69] [70].

Therefore, nanoparticles 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 with e.g. organic matter, or
- to the interface between phases (deposition or attachment).

Because of our 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 (hetero-agglomeration) or deposition experiments [42], [70].

[6]Please note that distribution/partitioning does not equal adsorption, and neither does sorption, which consists of aDsorption and aBsorption phenomena.

OECD TG 106 Adsorption – Desorption Using Batch Equilibrium Method [71] 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 nanoparticles settled during the centrifugation step, and a new TG needs to be developed ([22], [72]). 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.

1.2.5.1 Relevant method to measure adsorption/desorption of nanoforms

The advice provided here are not applicable to nanomaterials with high dissolution rate or poor dispersibility in aqueous media (with definitions given in OECD TG 318 (2017) [25]). Guidance on how to determine dispersion stability/dissolution rate of the nanoforms can be gained from OECD GD 318 for simulated environmental media as well as the use of the data for further environmental testing and assessment strategies [21]. Testing of these parameters have to be performed before applying OECD GD 312.

OECD TG 312 Leaching in Soil Columns [73] allows study of 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 312 [48].

This guidance for nanoform testing using OECD TG 312 [73], 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 quantitatively the adsorption potential and mobility of nanomaterials between soil and water.

The parent guideline, OECD TG 312, uses thermodynamic processes where non-nanoforms of substances often will reach an equilibrium ([73]). The adaptation of OECD TG 312 is mainly needed because thermodynamic processes are not applying for undissolved nanoforms. Instead, they form colloidal dispersions and are thermodynamically unstable with dominating processes such as (hetero)agglomeration and sedimentation ([42]). Hence, estimations of parameters such as K_{oc} and K_{ow} as presented in the parent OECD TG 312 are not applicable for nanomaterials. For the nanoforms, particle attachment efficiency (αhetero) can be calculated instead.

Alpha (α) 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 ([74]). A quantitative estimation of α can be obtained where a continuous nanomaterial input is applied into the column transport test and the NM concentration is monitored over time in the eluate. However, it needs to be noted that the determination of α 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 present. Outside these settings more complex mechanisms can influence the particle transport (e.g. blocking, ripening) and α is not able to accurately describe the system anymore, leading to misinterpretation and misuse of the data. Here, more complex and comprehensive models are necessary, requiring moderate to high modelling skills, to perform a reliable quantitative analysis of the results. Examples of such models are:

- STANMOD – (Studio of Analytical MODels ,https://www.pc-progress.com/en/Default.aspx?stanmod), or
- NMMs 2021 (Micro – and Nanoparticle transport, filtration and clogging Model – Suite (https://areeweb.polito.it/ricerca/groundwater/software/mnms/), or

- Hydrus-1D (https://www.pc-progress.com/en/Default.aspx?hydrus-1d), or
- ColloidFit (https://tuceel.tuc.gr/colloidfit).

Besides, the overall recovery (mass balance) of nanomaterials should be reported. In accordance with the GD 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. 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.

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 ([75]) 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 (they strongly attach to the clay minerals preventing break through ([76], [77], [78]) and sandy soils with high carbon content (soil 5 in OECD TG 312) are only limited availability.

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), 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 senario 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.

1.2.5.2 Alternative methods adsorption/desorption of nanoforms

A list of available models to predict alternative fate descriptors for nanomaterials is available in **Appendix 1**.

These models are still under development and further validation is needed in particular with regard to the uncertainties and applications mainly when these are used more for exposure of the environmental compartment and organisms than for estimation of adsorption potential to soil as information. When they are available, they will be recommended as a mean to provide suitable alternative information on the sorption and agglomeration/aggregation of nanomaterials. In addition, pre-assessment of dissolution rate and agglomeration behaviour of nanomaterials is needed before proceeding with any alternative measurement of their attachment or deposition ([22], [47], [69]).

An OECD Study Report on a test for removal in wastewater treatment plants of gold manufactured nanomaterial: activated sludge sorption isotherm [79] was published recently and could be used to estimate sludge sorption isotherm of nanomaterials. This work constitutes an alternative that would be relevant for exposure purposes of the sludge and therefore is not applicable as a surrogate to K_{oc} or attachment efficiency nor to hetero/homo

agglomeration to particles.

1.2.5.3 Waiving of adsorption/desorption for 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 of nanoparticles 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 waiving should always be accompanied by a robust technical and scientific justification on the applicability of the used test method (e.g. nanoform being water soluble or having a high dissolution rate, not being dispersed or having no data proving it agglomerates or aggregates as detailed under Section 1.2.2) with further justifications on nanoforms behaviour in soil and sediment.

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 in below is not exhaustive and includes methods based on attachment affinity and dissolution rate of nanomaterials. Further Information on these methods that may be used to predict fate and transport of nanomaterials in the environment and organisms can be found at for instance at [80]. Further information on the models and their validation status can be found in the referenced publications for each model.

Table 3: 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	[81]
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.	[82]

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*., [51] a river model was used.	[51]
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: [83]
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	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
	identifying accumulation hotspots and studying the temporal dynamics of NM concentrations.			
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; [84]

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