

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

2022



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 Information Requirements and Chemical Safety Assessment (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.

LEGAL NOTICE

This document aims to assist users in complying with their obligations under the REACH Regulation. However, users are reminded that the text of the REACH Regulation is the only authentic legal reference and that the information in this document does not constitute legal advice. Usage of the information remains under the sole responsibility of the user. The European Chemicals Agency does not accept any liability with regard to the use that may be made of the information contained in this document.

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: XXXXXXXX 2022

Language: EN

© European Chemicals Agency, 2022

Permission to reproduce extracts from BS ISO 9276-6:2008 is granted by BSI. No other use of these extracts is permitted. British Standards can be obtained in PDF or hard copy formats from the BSI online shop: www.bsigroup.com/Shop or by contacting BSI Customer Services for hardcopies only: Tel: +44 (0)20 8996 9001, Email: cservices@bsigroup.com.

If you have questions or comments in relation to this document please send them (indicating the document reference, issue date, chapter and/or page of the document to which your comment refers) using the information request form. The information request form can be accessed via the Contact ECHA page at: <http://echa.europa.eu/contact>.

European Chemicals Agency

Mailing address: P.O. Box 400, FI-00121 Helsinki, Finland

Visiting address: Telakkakatu 6, Helsinki, Finland

1 DOCUMENT HISTORY

Version	Changes	Date
Version 1	First edition	April 2012
Version 2	<ul style="list-style-type: none"> 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 <p>Please note that the numbering of the sections has changed, the section numbers above refer to the updated numbering of the guidance sections.</p>	May 2017
Version 3	<ul style="list-style-type: none"> 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: <ul style="list-style-type: none"> 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: <ul style="list-style-type: none"> The mandatory status of some parameters (e.g. dissolution rate in water and relevant biological media). The conditions under which an older study can be used. The doses to be used in repeated dose inhalation studies. The consequences of the dose on agglomeration for designing and interpreting repeated dose toxicity studies (RDT). The mechanism of toxicity for fibres. The type of toxicokinetic investigations that can be done under RDT studies. Section 2.2.2.1. The concept of lung overload: updated references. 	October 2021

	<ul style="list-style-type: none"> ○ Section 2.2.2.2. Indirect genotoxicity • Section 3.2.3. changed to section 2.2.3. Mutagenicity and Carcinogenicity updated and restructured: <ul style="list-style-type: none"> ○ 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 <ul style="list-style-type: none"> ▪ 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	<ul style="list-style-type: none"> • Update of the advisory note on testing and sampling strategy and sample preparation for ecotoxicological endpoints: <ul style="list-style-type: none"> ○ Update and changing of Section 1.1 and sub sections with 1.1.1. Characterisation of test materials, 1.1.2 Sample preparation of test materials and 1.1.3 General considerations for Fate and (Eco)-Toxicological testing • Update of the section 1 for water solubility, granulometry, K_{ow}, adsorption/desorption: <ul style="list-style-type: none"> ○ Addition of sub-section for Section 1.2.1 Water solubility with waivers and dissolution rate, ○ Addition of sub-section for section 1.2.2 K_{ow}, with waivers and request for dispersion stability, ○ Addition of Section 1.2.4 Dustiness, ○ Addition of sub-section for alternative methods to Koc under section 1.2.5. 	2022

PREFACE

Three appendices (appendices to Information Requirements and Chemical Safety Assessment Guidance Chapters R7a, R7b and R7c) specifying information requirements have been developed to provide advice to registrants when preparing REACH registration dossiers that cover “nanoforms” [1]. The advice provided in this document focuses on specific recommendations for testing materials that are nanoforms of substances¹. As most of the guidelines and publications are referring to nanomaterials or nanoparticles, also the terms “nanomaterial” and “nanoparticle” are used. Annex VI of REACH defines the terms “nanoform” and “set of similar nanoforms”² and establishes the requirements for characterisation of the identified nanoforms of the substance. Advice on substance identification and registration of nanoforms can be found in the guidance “Appendix for nanoforms applicable to the Guidance on Registration and Substance Identification” and shall be applied when registering nanoforms of a substance under REACH [2]. A glossary is available to define the terms used in this guidance. As this appendix is specifically addressing REACH information requirements, nanoform is the preferred term and used whenever possible.

Part of the provided advice is not strictly nanoform specific and may, for instance, also be applicable to other particulate forms of substances (e.g. where dissolution rate is relevant). However, advice has been included when it is considered especially relevant for nanoforms and should therefore be part of the nanoform specific guidance. If such 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 parent 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 supersede the applicability of the general principles given in Chapter R.7a [3], i.e. the parent guidance which is applicable in case of the absence of nano specific advice in this appendix. Please note that this document and its parent guidance provides specific guidance for meeting the information requirements set out in Annexes VII to XI to the REACH Regulation. General information for meeting the information requirements such as collection and evaluation of available information, and adaptation of information requirements, is available in Chapters R.2 to R.5 of Guidance on IR&CSA. Moreover, when considering the use of data already available, “Guidance on information requirements and chemical safety assessment –Appendix R.6-1 for nanoforms applicable to the Guidance on QSARs and Grouping of Chemicals” [4] may be useful as it provides how to approach read-across for hazard data between nanoforms, as well as nanoforms and the non-nanoform of the same substance.

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

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

1	Table of Contents	
2	NOTE	2
3	LEGAL NOTICE	3
4	DOCUMENT HISTORY	4
5	PREFACE	6
6	GLOSSARY	8
7	1 RECOMMENDATIONS FOR PHYSICO-CHEMICAL PROPERTIES	11
8	1.1 GENERAL REMARKS.....	11
9	1.1.1 <i>Characterisation of test materials</i>	11
10	1.1.2 <i>Sample preparation of test materials</i>	12
11	1.1.3 <i>General considerations for Fate and (Eco)-Toxicological testing</i>	14
12	1.2 SPECIFIC ADVICE FOR ENDPOINTS.....	17
13	1.2.1 <i>Water solubility</i>	17
14	1.2.2 <i>Partition coefficient n-octanol/water</i>	22
15	1.2.3 <i>Granulometry</i>	27
16	1.2.4 <i>Dustiness</i>	33
17	1.2.5 <i>Adsorption/desorption</i>	37
18	APPENDIX 1 MODELS FOR FATE AND EXPOSURE OF NANOMATERIALS	40
19	REFERENCES	43
20		
21	Table of Tables	
22	TABLE 1: METHODS OF MEASURING AIRBORNE DISPERSED OR NEBULISED PARTICLES	29
23	TABLE 2: TEST METHODS TO MEASURE DUSTINESS OF BULK MATERIALS THAT CONTAIN OR RELEASE NANO-	
24	OBJECTS OR SUBMICROMETER PARTICLES	35
25	TABLE 3: OVERVIEW OF SOME MODELS FOR FATE FOR NANOMATERIALS	40
26		
27		
28	Table of Figures	
29	FIGURE 1: SCHEME OF THE TESTING REQUIRED TO FULFILL ANNEX VII, SECTION 7.7 INFORMATION	
30	REQUIREMENT OF NANOFORMS OF A SUBSTANCE AND THEIR IMPACT ON TESTING STRATEGIES	19
31	FIGURE 2: SCHEME OF THE TESTING REQUIRED TO FULFILL ANNEX VII, SECTION 7.8 INFORMATION	
32	REQUIREMENT OF NANOFORMS OF A SUBSTANCE AND THEIR IMPACT ON TESTING STRATEGY	23
33	FIGURE 3: SCHEMATIC REPRESENTATION OF EXEMPLIFIED DISPERSION STABILITY RESULTS FROM AN EXTENDED	
34	TEST (EXTRACTED FROM OECD TG 318)	25

GLOSSARY

Accumulation:

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

ADME: Absorption, distribution, metabolism, excretion.

Agglomerate: A collection of weakly bound particles or aggregates where the resulting external surface area is similar to the sum of the surface areas of the individual components [2], [5], [6] and [7].

Agglomeration: Process of contact and adhesion whereby dispersed particles are held together by weak physical interactions ultimately leading to enhanced sedimentation by the formation of particles (agglomerates) of larger than colloidal size. The process occurs when two particles, i and j , collide and attach to one another to form an agglomerate. The agglomeration rate constant, k_{agg}^{ij} , provides a quantitative description of the speed of the agglomeration process and depends on the collision rate constant, k_{coll}^{ij} 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]. In contrast to agglomeration, aggregation occurs where particles are held by strong bonds like sinter bridges.

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

BALF: Bronchoalveolar lavage fluid.

Bioavailability : The amount of a substance accessible to an organism for uptake or absorption across its cellular membrane. In toxicology this is measured as the proportion of a substance in the systemic circulation compared with the total amount of substance that has been ingested or inhaled (modified from [9]). In ecotoxicology it is also measured as the amount taken up via cell surfaces (e.g. gills for fish) from the aqueous, sediment and/or soil compartments.

Biodegradation: Degradation of a substance resulting from interaction with the biological environment [10].

Biodurability: The ability to resist chemical and biochemical alteration through dissolution and enzymatic biodegradation or chemical disintegration within biological media (modified from [10]). 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 [10]. It is determined using *in vivo* methods.

Biotransformation: Alteration of a substance resulting from interaction with biological systems [10].

Clearance: (1) In (eco)toxicology, the volume of blood or plasma or mass of an organ effectively cleared of a substance by *elimination* (including *metabolism* and *excretion*) divided by the time of elimination. Total clearance is the sum of the clearances of each *eliminating* organ or tissue for a given substance. (2) In pulmonary toxicology, the volume or mass of the lung cleared divided by the time of *elimination* is used qualitatively to describe removal of any inhaled substance which deposits on the lining surface of the lung [11].

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

Dissolution half-life/half-time: A time interval that corresponds to a concentration decrease by a factor of 2 for the nanomaterials and a corresponding increase of its ions or molecular forms [10].

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

Heteroagglomeration: Agglomeration of particles (here nanomaterials) with other particles, including other nanomaterials and non-nanomaterials (synthetic or natural), that differ in composition or size, for example Suspended Particulate Matter (SPM) [10] and [12].

α_{hetero} : Heteroagglomeration attachment efficiency

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

Nanoform: A form of a natural or manufactured substance containing particles, in an unbound state or as an aggregate or as an agglomerate and where, for 50 % or more of the particles in the number size distribution, one or more external dimensions is in the size range 1 nm-100 nm, including also by derogation fullerenes, graphene flakes and single wall carbon nanotubes with one or more external dimensions below 1 nm [2].

Nanomaterial: a natural, incidental or manufactured material consisting of solid particles that are present, either on their own or as identifiable constituent particles in aggregates or agglomerates, and where 50 % or more of these particles in the number-based size distribution

fulfil at least one of the following conditions:

- (a) one or more external dimensions of the particle are in the size range 1 nm to 100 nm;
- (b) the particle has an elongated shape, such as a rod, fibre or tube, where two external dimensions are smaller than 1 nm and the other dimension is larger than 100 nm;
- (c) the particle has a plate-like shape, where one external dimension is smaller than 1 nm and the other dimensions are larger than 100 nm.

In the determination of the particle number-based size distribution, particles with at least two orthogonal external dimensions larger than 100 µm need not be considered. A material with a specific surface area by volume of < 6 m²/cm³ shall not be considered a nanomaterial [5].

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

NOM: Natural Organic Matter.

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

PEO: Post-exposure observation.

Poorly soluble particle (PSP): Solid aerosol particles deposited in the lung that do not undergo rapid dissolution and clearance [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 solvent 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].

QSAR: 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 jointly³ [2].

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

Solubility: The proportion of a solute that dissolves in a given quantity of solvent, at a given temperature under equilibrium conditions, i.e. in a saturated state [15].

³ A justification shall be provided to demonstrate that a variation within these boundaries does not affect the hazard assessment, exposure assessment and risk assessment of the similar nanoforms in the set. A nanoform can only belong to one set of similar nanoforms.

1 RECOMMENDATIONS FOR PHYSICO-CHEMICAL PROPERTIES

1.1 General remarks

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

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

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

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

1.1.1 Characterisation of test materials

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

Recital 12 of the REACH amended Regulation (EU 2018/1881) [16] for nanoforms stipulates: *"to allow for adequate assessment of the relevance of any physicochemical, toxicological and ecotoxicological information for the different nanoforms, the test material should be appropriately characterised. For the same reasons, test conditions documented and a scientific justification for the relevance and adequacy of the utilised test material as well as documentation for the relevance and adequacy of the information obtained from means other than testing for the different nanoforms should be provided."*

Consequently, the following parameters have to be provided in line with Annex VI Section 2.4 requirements for the tested nanoform:

- Name(s) or other identifiers of the nanoform of the substance
- Number based particle size distribution with indication of the number fraction of

constituent particles in the size range within 1 nm – 100 nm.

- Description of surface functionalisation or treatment and identification of each agent including IUPAC name and CAS or EC number.
- Shape, aspect ratio and other morphological characterisation: crystallinity, information on assembly structure including, e.g. shell like structures or hollow structures, if appropriate
- Surface area (specific surface area by volume, specific surface area by mass or both)

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

1.1.2 Sample preparation of test materials

The following section focuses on the preparation of the sample for testing. Sample preparation is widely recognised as one of the most critical steps towards reliable testing of nanoforms and requires monitoring of the nanoforms stability. There are many variables to consider when establishing a method for sample preparation. Common issues to be considered include storage (see for instance ACEnano [17]), colloidal and chemical stability of the tested nanoform, the media composition, characterisation of stock dispersions and characterisation of samples (prepared from stock dispersions) prior to administration/testing [18].

When considering aqueous media, it can be difficult to distinguish between a *dispersed* and a *dissolved* nanoform due to its small particle size in aqueous media. It is important to recognise that solubility and dispersibility are two distinct phenomena. Solubility is the degree to which a material (the solute) can be dissolved in another material (the solvent) resulting in a single, homogeneous, stable phase and it is relevant for solids, liquids and gases. Dispersibility is the degree to which a particulate material can be uniformly distributed in another material (the dispersing medium or continuous phase). Historically, the term "*dissolved*" was defined as the part of a liquid sample that had passed through a 0.45µm filter. However, dispersions of nanoparticles will pass through such filters. Therefore, "*dispersed*" is the term to use when particles are present in the aqueous media, while the use of the term "*dissolved*" is restricted to solutions in the strict sense of the definition ([18], [19]). In the context of sample preparation next to information on dissolution, knowledge about the dispersion of a nanoform and how stable it is under given conditions is important.

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

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

The stability of a dispersion is determined by intermolecular forces involving particle-particle interactions as well as those between the particles and their surrounding matrix. Due to

attractive forces (e.g. van der Waals interactions) particles tend to agglomerate unless stabilised by surface charge or steric effects. In liquid media, modifications in pH, ionic strength and concentrations of molecular constituents can significantly alter the particle dispersion.

The stability of a dispersion is also typically assessed using comparative particle size measurements and requires a reliable method of measuring the baseline particle size distribution of the material. To assess the initial agglomeration and/or aggregation state a number of parameters are recommended including the effective cross section and can be measured via aerodynamic/light scattering or by electron microscopy. ([20], [21]).

For example, by comparing changes in particle size distribution, a qualitative assessment or proxy measure of the state of dispersion can be made. Examples of methods applicable for spherical particles are Zeta potential measurement, combined with Dynamic Light Scattering (DLS) or UV-VIS spectroscopy to monitor the stability of nanoparticle dispersions in stock or test media and to gain a qualitative understanding of the agglomeration process. Other methods [22], when suitable, e.g. particle tracking analysis, can also be used [23]. For aquatic and sediment toxicity testing, OECD GD 317 also includes analytical techniques to characterize the *as-produced* or *as-received* test material, as well as the test material in stock and test dispersions.

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

- Sample Preparation and Dosimetry for the Safety Testing of Manufactured Nanomaterials OECD GD 36 [18]
- Ecotoxicology and Environmental Fate of Manufactured Nanomaterials: Test Guidelines [24]

However, most recently the advice provided in guidance document OECD GD 317 [25] has to be followed to the extent possible.

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

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

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

- The preparation of the nanoform dispersion (including stability) and composition of the test medium (such as pH, organic matter, salts etc) are key characteristics and interactions between the nanoform and the test medium influence their physico-chemical properties ultimately determining their fate and behaviour and consequently potential adverse (eco)toxicological effects.
- The applied sample preparation protocol detailing procedures for controlled, reliable, and robust processes need to be scientifically justified and reported in the study summary.

ISO 14887:2000 [26] 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., [27] [28].

However, such procedures should be carefully assessed to determine if they are adequate for the respective test material. Modifications may be required for different materials in the context of (eco)toxicological testing. For example, for testing inhalation toxicity, standards are available that outline procedures for the generation of metal nanoparticles using the evaporation/condensation method (ISO 10801:2010 [28] and support the characterisation of nanoforms in inhalation exposure chambers [28].

Another important component of sample preparation is the reliability of the sampling itself which means that the test aliquot represents the physical and chemical characteristics of the entire sample. The characterisation of particle properties like size, form and specific surface area requires very careful sampling and sample aliquoting, as well as considerate storage if the sample is not processed immediately to prevent transformation of the nanoform in question. ISO 14488:2000 [26] 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 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.

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

The interactions between nanoforms and their surroundings need to be accounted for, when testing fate and (eco-)toxicity, as this will influence the overall (eco)toxicity and fate processes. In particular, the interaction of nanomaterials with the the environmental or biological media have to be considered in case dissolution occurs. The test design and more specifically the test conditions (e.g. composition of test media, agitation, light, exposure method, temperature) influence the dissolution of nanomaterials affecting then the test results [18] [29].

If a nanoform is well soluble in water (see Sections 1.2.1 and 2.1.1 for thresholds) and has a high dissolution rate in relevant biological (see Section 2.1.1) or environmental (see Section 1.2.1) media, then it is likely that the nanoform is present in its molecular or ionic form. Therefore, it can be expected to behave similarly and elicit the same response as the non-nanoform of the substance. This could be the case for the metal ions released from the nanoform, where e.g. the metal salts ('ionic form') are used as a positive control, compared to the nanoform. If, however, the nanoform under investigation is poorly or only partially soluble with a low dissolution rate in biologically or environmentally relevant test media, then it will likely be present in the test system in a particulate form. In this case, the advice provided in *Appendices for nanoforms applicable to Chapters R.7a (this document), R.7b and R.7c* would apply including the considerations for partially soluble nanoforms where both the dissolved and particulate forms need to be accounted for.

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

- Any (eco)toxicological and fate testing has to be accompanied with suitable characterisation methods to monitor the exposure situation, such as exposure concentration and form of the tested nanoform (dissolved and/or particulate form). Concerning dose metrics for nanoforms using exclusively chemical analysis to determine

mass based concentrations/metrics is not sufficient as further explained in the last bullet point, (for aquatic and sediment toxicity testing see OECD GD 317 [25], Section 4).

- Monitoring of dissolution and dispersion stability has to be conducted at least at the beginning and at the end of the test, but depending on the results additional intermediate monitoring may be required if a high dissolution rate or reduced stability of the test dispersion is observed.
- Since the most appropriate dose metrics may not be known, the use of other dose metrics than mass-based, such as surface area and particle counts, are a favourable addition to the mass metrics. These measurements will allow for a conversion from mass to particle counts and/or to surface area and are considered essential. While diminishing the uncertainty related to the conversion when the metrics are used independently it will also consequently reduces the amount of required testing.

As fate and behaviour of a nanoform can differ from that of another nanoform and/or their non-nanoform counterparts, appropriate analytical methods are needed for fate and (eco-) toxicity testing of nanomaterials. These also need to consider the how the dissolution and the stability of the dispersion of the investigated nanomaterial influence each other. With respect to aquatic and sediment toxicity testing OECD flowcharts outlining strategies for preparing, controlling and maintaining test dispersions are given in OECD GD 317 [25], Section 5. OECD GD 318 [30] Sections 2 to 4 are advising how solubility, dissolution rate and dispersion stability testing informs further fate and ecotoxicological testing. Characterising the dissolution rate and dispersion stability in the respective test medium does not only help to find the appropriate testing strategy and test conditions, but also amplifies the correct interpretation of the results. General information on dissolution and dispersion stability for nanoforms is considered to be beneficial when building grouping and read across hypothesis [31].

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

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

Also of particular concern is the adverse impact of endotoxin contamination in nanomaterial samples on 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 confounding results (including a potential false positive) in biological assays. Therefore, establishing the presence and levels of endotoxin in a test sample is an important step during the sample preparation for (eco)toxicological testing. Endotoxin can be measured using *in vitro* methods such as the macrophage activation test (MAT, validated by European Committee on Validation of Alternative Test Methods) [33] or the Recombinant Factor C (rFC) test. [34]. Both rFC assay and MAT assay have been adopted by the European Pharmacopoeia [35], [36] and [37]. With ISO 29701 an international standard, the limulus amoebocyte lysate (LAL) test, for analysing endotoxin contamination in nanomaterial samples is available. [38]. Although issues regarding contamination are not nano-specific, the increased relative surface area of nanosized systems compared to other particles means that the possible amounts of adsorbed endotoxin (e.g. grams

1 adsorbed endotoxin per gram of material) are significant and deserve therefore special attention
2 [39].

1.2 Specific advice for endpoints

1.2.1 Water solubility

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

Water solubility is covered in Section R.7.1.7 of the parent guidance. There is no specific test guideline to test water solubility of nanomaterials. However, OECD GD 318 [30] describes applicability and limitations of OECD TG 105 [40] and OECD GD 29 [41] for testing solubility and dissolution of nanomaterials in simulated environmental media.

For water solubility, both OECD TG 105 and GD 29 adapted for nanomaterial testing [30] are applicable.

The flask method presented in OECD TG 105 states to measure solubility after a 24-hour equilibration period. Analysis is to be done after a separation step. However, the recommended method in OECD TG 105 is only considered to be adequate when using ultra-filtration.

OECD GD 29 [41] allows to test dissolution and transformation of metals with test durations varying between 1 up to 28 days (common duration of 7 days). The OECD GD 29 protocol provides advice on how to determine the transformation or dissolution and provides knowledge to which extent metals and poorly soluble metal compounds can produce soluble ionic forms and other metal bearing species in aqueous media, at different pHs. It should be noted that if the recommendations provided under OECD GD 318 are not considered, the method as described in OECD GD 29 is not applicable to nanoform testing (e.g. 0.20 µm filtration is not adequate). Nevertheless, with appropriate adaptations (in particular for the filtration step) the method can be applied for nanoform testing. Ultra-filtration using a 3-10 kDa cut-off membrane is considered an applicable separation method for nanomaterials [15].

OECD GD 318 provides further guidance on the application of OECD GD 29 and other TGs for measuring water solubility and dissolution rate of nanomaterials in environmental relevant conditions, including the applicability of the static batch mode (screening test, adapted from OECD GD 29) and the dynamic mode (based on ISO TR 19057 [42]).

For instance, the static batch method allows quick determination of solubility of nanomaterials as “poorly” or “highly” soluble, based on test duration with a minimum of 24-hours.

Overall, solubility measurements should be performed after at least 24-hour equilibration and must consider different pHs, as per OECD GD 29 recommendations, and appropriate separation method *i.e.* ultrafiltration with 3-10 kDa cut-off membrane.

Under these conditions, a nanoform is considered highly soluble *i.e.* no detectable particles are present, if its water solubility is above 100 mg/L (see **Figure 1**). The threshold of 100 mg/L for solubility in water is set based on the work done in support of OECD GD 29 [41] and considered as a conservative approach prioritising with environmental safety. The duration of solubility studies should be set to 24h, longer tests will not improve significance and value of ecotoxicological testing design, the results or their interpretation. The analytical method should be sufficient to measure reliably the water solubility hence, the limit of quantification (LOQ) of the analytical method must be justified.

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

For nanoforms, it is necessary to take into account that the dissolution rate may be affected by the specific properties of the nano-sized materials. Properties such as particle size and shape, impact the materials’ surface area and the specific surface area and surface coating, will influence the nanoform-solute interactions. Therefore the impact of such properties on the determination of the dissolution rate has to be considered. Further information regarding the

1 dissolution rate can be found in Section 1.2.1.1 below.

2
3 Providing information on dissolution is needed to determine the potential release to the aquatic
4 environment and therefore the aquatic exposure.

5 The fate and behaviour of nanoforms is affected by their dissolution rate and their degree of
6 dispersion. Distinction between a dispersed nanoform (where both solid and liquid fractions are
7 present) and a dissolved nanoform (where no detectable solid fraction is present) is important
8 as it has implications on the testing and characterisation strategies, as illustrated in **Figure 1**.

9
10 This situation is not unique to nanoforms, and indeed the parent guidance already highlights
11 that "*measurement of the solubility of sparingly soluble compounds requires extreme care to*
12 *generate saturated solutions of the material without the introduction of dispersed material*".
13 However, this problem is further amplified in the case of sparingly soluble nanoforms. It is
14 important to ensure that no undissolved material contributes to what is being measured as
15 dissolved material, meaning that an appropriate separation method is used.

16
17 For environmental endpoints covering fate and ecotoxicity the choice of nanospecific testing
18 and/or following the parent guidance must be duly justified and documented based on the
19 presence of soluble and/or solid fraction. Where partial dissolution is observed, the impact of
20 each fraction must be considered (i.e. data on non-nanoforms of the substance may inform on
21 the hazards of the solubilised fraction).

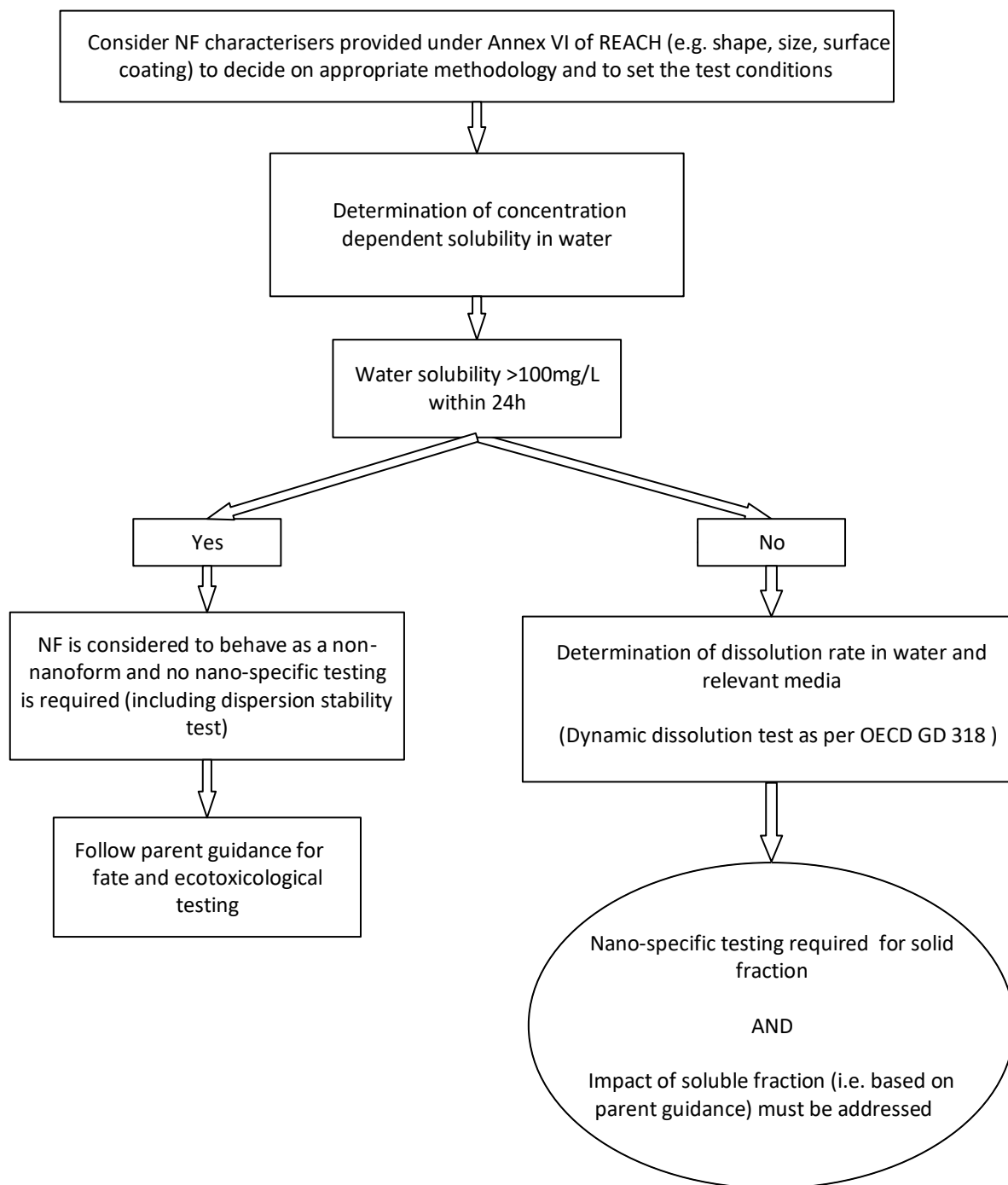


Figure 1: Scheme of the testing required to fulfill Annex VII, Section 7.7 information requirement of nanoforms of a Substance and their impact on testing strategies. Solubility in water defines whether determination of dissolution rate is required or the nanoform is considered to behave as a soluble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

1.2.1.1 Dissolution rate

Annex VII, section 7.7 of REACH states that: "For nanoforms, in addition the testing of dissolution rate in water as well as in relevant biological and environmental media shall be

1 *considered.*". Thus, information on the dissolution rate has to be provided as part of the solubility
2 endpoint and should be investigated in relevant media, such as the ones used in the
3 (eco)toxicological tests. For nanoforms with a water solubility above 100 mg/L this information
4 requirement is considered of low value.

5
6 Dissolution rates in the different biological and environmental media used for (eco)toxicological
7 and fate tests are variable and may affect the bioavailability of substances. Knowledge on
8 dissolution rates will also help to predict the toxicokinetic behaviour of particles.

9
10 Currently, no test guideline is available for determining the dissolution rate of nanomaterials
11 although some projects are working on developing a specific TG for dissolution rate of
12 nanomaterials in water and biological media (OECD WNT 1.5 project on "Determination of
13 Solubility and Dissolution Rate of Nanomaterials in Water and Relevant Synthetic Biological
14 Media) and in environmental media (OECD WNT projects 3.10 "Dissolution rate of nanomaterials
15 in the aquatic environment"). Nevertheless, OECD GD 318 [30] provides guidance on how to
16 determine dissolution, dispersion and agglomeration of certain nanoforms in environmental
17 media, based on available TGs. Such methods are also considered relevant to determine
18 nanoform dissolution rates in water as required under Section 7.7 of REACH. Where the
19 nanoform is outside the applicability domain of the available TGs and GDs, the registrant is
20 required to adapt such an information requirement with a robust technical and scientific
21 justification for the inapplicability of the used test methods, and the lack of an adopted relevant
22 guidance and/or test guideline.

23 Parameters such as pH, ionic strength or suspended particulate matter influence the dissolution
24 rate of nanoforms and should be carefully evaluated and reported.

25 The following paragraphs are mainly focussed on environmental testing. Nevertheless, many of
26 the statements may be relevant for water and biological media as well.

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

30
31 As discussed above the determination of the dissolution rates depends on an appropriate
32 separation of particulates from the dissolved fraction and suitable time resolution. However, this
33 can be a drawback for a screening test based on the static batch mode, especially for rapidly
34 and fully dissolving nanoforms. Information on appropriate separation techniques for
35 nanomaterials can be found in ISO TR 19057. Based on OECD GD 318 the recommended method
36 is centrifugal ultrafiltration as it promotes a suitably rapid separation allowing for calculation of
37 dissolution rates. Still, care should be taken, when choosing filter cut-off to ensure no passage
38 of smaller solid fractions below the filter cut-off value. Also, build up of ions should be considered
39 when small filter cut-off values are chosen and similarly caution should be taken when using
40 NOM as it can block filters with smaller cut-off sizes.

41
42 For slowly dissolving nanoforms, the applicability of the batch method is mostly dependent on
43 the analytical power as the solubility limit may not be within the resolution of the analytical
44 method, i.e. staying under the limit of quantification or even limit of detection.

45
46 The dynamic method is based on a flow-through system and comprises the use of a test medium
47 delivered at constant flow rate through a compartment which entraps the nanoparticles, i.e.
48 using ultrafiltration membranes. Sensitivity can be partially compensated by adapting the low
49 flow rate and nanoform concentration.

50
51 For both methods, static batch mode and dynamic flow through mode, their applicability for
52 testing non-metallic nanoforms depends on the accuracy of the analytical method. In general,
53 most of the nanoforms tested are inorganic materials (mainly metal and metal oxide forms).
54 Other nanoforms such as organic or organometallic and carbon-based nanoforms have been less

tested and analytical methods may lack precision for quantification. The development of various analytical methods is ongoing (including methods for organic nanoforms) to increase the accuracy and sensitivity of the measurements and their applicability for measuring dissolution rates of nanoforms in water. Further specific considerations to the adaptation of analytical methods to nanoform testing (e.g. quantification of solid or dissolved fractions) is not yet fully developed.

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

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

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

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

OECD GD 318 also provides advice on how both static batch and dynamic methods can be adapted to measure dissolution rate in natural or artificial environmental media. According to OECD GD 318, the applicability of the dynamic method based on ISO TR 19057 for toxicological test media, i.e. in lung and gastro fluids, has already been demonstrated in [43] and [44]. In addition, the OECD WNT 1.5. project will provide a specific GD to determine dissolution in biological media and water [45]. While this is ongoing work, further toxicological considerations and advice on information regarding solubility and dissolution of nanoforms in biological media are described under Section 2.1.1 of this guidance and are to be followed.

Alternatively, OECD TG 105 is also considered to be potentially adaptable to determine dissolution rates. The use of the column elution method with continuous measurements of the dissolved fraction would allow the determination of a nanoform dissolution rate. To do so, nanoform adsorption to the substrate has 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 their apparent insolubility as quoted in Annex VII, Section 7.7, column 2 : *"must always be accompanied with robust technical and scientific justification comprising information on dissolution and dispersion stability of the nanoform(s) "* (see Section 1.2.1.1 & 1.2.2.2).

1.2.2 Partition coefficient n-octanol/water

1.2.2.1 Applicability of partition coefficient n-octanol/water

The n-octanol/water partition coefficient (K_{ow}) is defined as the ratio of the equilibrium concentrations of a dissolved substance in a two-phase system consisting of the largely immiscible solvents n-octanol and water. In a two-phase system, nanoparticles behave differently from organic molecules. Particles do not form solutions but colloidal dispersions, which are multiphase systems and thermodynamically unstable. Therefore, the fate of undissolved nanomaterials cannot be predicted by equilibrium partitioning [46], [47] as nanomaterials cannot reach thermodynamic equilibrium by distributing between two phases, water and n-octanol, due to their particulate nature.

OECD TGs recommended in the Section R.7.1.8.3 of the parent ECHA Guidance for partition coefficient n-octanol/water, i.e. OECD TG 107 [48], OECD TG 117 [49] and OECD TG 123 [50], are in most cases not applicable to nanoforms [20], [19], [51]. Results will be impacted by the presence of a colloidal suspension, which could be present if the manufactured nanomaterial does not completely dissolve [18], [19], [31].

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

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

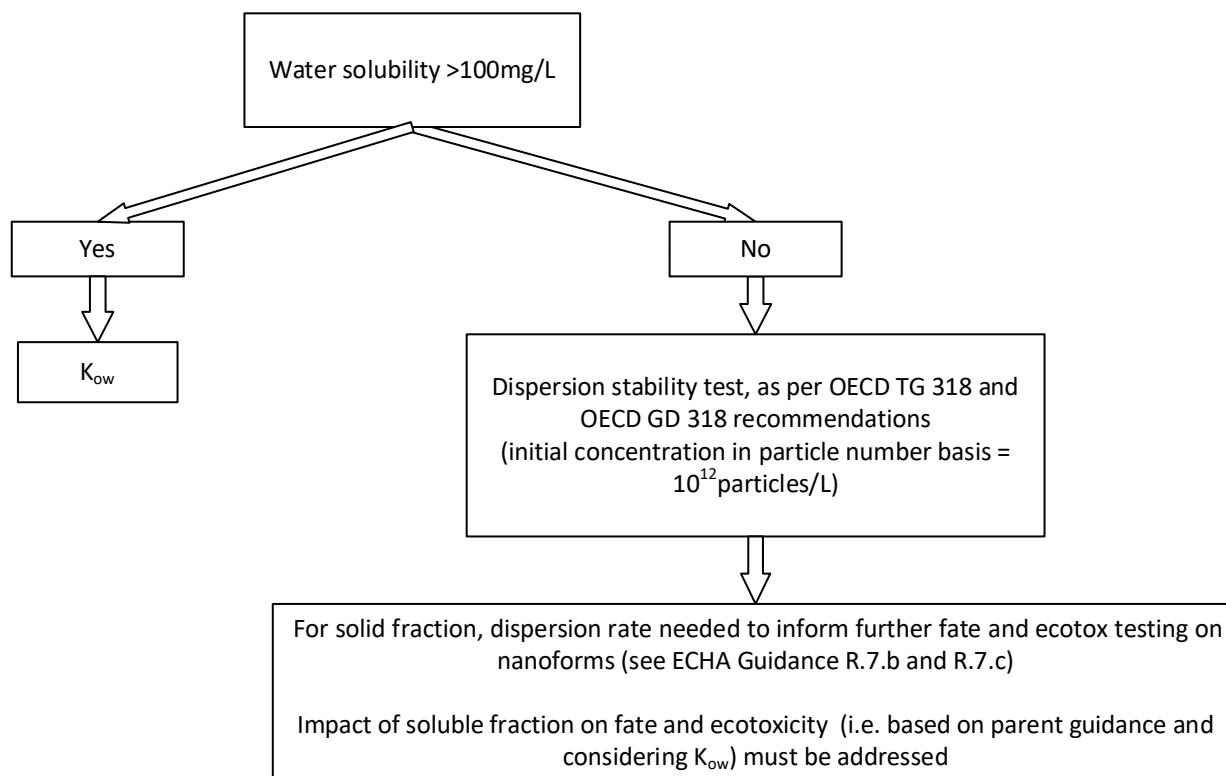


Figure 2: Scheme of the testing required to fulfill Annex VII, Section 7.8 information requirement of nanoforms of a substance and their impact on testing strategy

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

1.2.2.2 Dispersion stability

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

Furthermore, information on dispersion stability in relevant environmental media is relevant to help predict fate and transport of the nanoforms in the environment, as illustrated in **Figure 2**. However, information on dispersion stability can not be seen as a one-to-one replacement of K_{ow} and additional properties need to be considered as well.

Dissolution (rate), agglomeration, aggregation, deposition and attachment are considered informative properties to predict the environmental behaviour of the nanomaterials in the environment and organisms [46], [52], [53], [54]. In this line, OECD GD 318 [30] includes recent developments on dispersion stability measurements, considering both homoagglomeration, as per OECD TG 318 [55], and heteroagglomeration. These agglomeration parameters can be provided in addition to dispersion stability in order to justify behaviour and fate of the nanoforms and consequently some choice in the testing strategy.

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

environmental behaviour of nanomaterials.

Dispersion stability based on homoagglomeration

Agglomeration is the process by which two particles interact. In case the two particles are of the same kind, this process is called homoagglomeration. The OECD TG 318 [55] defines dispersion stability, accounting for agglomeration and sedimentation. As such, it can be used to design a testing strategy to determine homoagglomeration; i.e. particle-particle attachment of nanomaterials in ecotoxicological test media.

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

Testing in other environmental conditions is described in OECD GD 318 [30] where feasibility of testing in other test media, such as natural waters, is also explained.

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

- Tier 1 (screening test): measurements are done at two time points (0 and 6h, which represents test start and end) at relevant pH conditions, with natural organic matter (10 mg/L dissolved organic matter) and a range of electrolyte concentrations (0, 1 and 10 mM $\text{Ca}(\text{NO}_3)_2$). An additional measurement is done at the end of the test, after centrifugation of the sample. The centrifugation parameter is calculated in order to achieve a particle cut off value of 1 μm .
- Tier 2 (extended test): in addition to the condition range described in the screening test i.e pH and electrolyte range, presence and absence of natural organic matter is tested. When no organic matter is added, sodium bicarbonate (5 mM) has to be added as a buffering agent. The stability of the dispersion is measured hourly with sub-sampling over the test duration of 6 hours).

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

- High stability - if ≥ 90 % of the initial test concentration remains in dispersion for all test conditions or
- Low stability – if $\leq 10\%$ of the initial test concentration remains in dispersion for all test conditions.

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

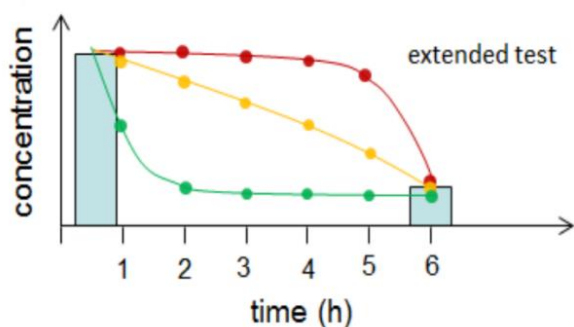


Figure 3: Schematic representation of exemplified dispersion stability results from an extended test (extracted from OECD TG 318). The hourly measurements revealing the sedimentation behaviour of the nanomaterial

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

- Material density (i.e. $>1\text{g/cm}^3$);
- Sensitivity of the analytical method which allowing quantification of the nanoforms from 100 to 10% of the particles present in the water column, i.e. LOQ lower or equal to 10% of initial mass concentration;

When applying the test with natural waters, careful removal of natural particles/colloids and microorganisms via filtration over a membrane of pore size $\leq 0.1\text{ }\mu\text{m}$ or via ultracentrifugation should be pursued in order not to compromise the test. Complex samples such as natural waters are considered to have a very specific hydro-chemical composition and if no pre-filtration or centrifugation is performed the results of the test will be rather representative of hetero-agglomeration (see heteroagglomeration considerations below).

To ensure data comparability media constituents interfering with agglomeration have to be reported (e.g. divalent cations and anions, pH and type of organic matter). The elements to be characterised are further discriminated under Section 3.0 and 3.1 of OECD GD 318.

OECD GD 318 provides further consideration when testing nanoform dispersion stabilitynamely:

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

medium. To reduce the impact of density on determining agglomeration, it is required to proceed with sample centrifugation, as described in OECD TG/GD 318.

Regarding quantification of dispersion stability, it is noted that the applicable analytical methods for nanoforms are limited only by their sensitivity. 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 a quantitative method. However, careful considerations of its applicability based on material properties is needed. This method provides indication of the maintenance of particle size and number in suspension therefore, nanoform stability can to some extent be monitored. Additionally, non-quantitative measurements such as electrophoretic mobility, i.e. Zeta potential, can also be used as an indicator of particle (in)stability. Further information on methods applicability can be found in "NanoDefine Technical Report D7.6" [22].

Dispersion stability based on heteroagglomeration

Heteroagglomeration, i.e. the interaction between two particles of different nature, is considered the most relevant type of agglomeration process to be investigated for fate evaluation, as it is performed under test conditions closer to natural settings.

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

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

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

There are currently constraints in assessing the n-octanol/water partition coefficient (K_{ow}) of the nanoforms, as explained in Section 1.2.2.1. Therefore, waiving n-octanol/water partition coefficient information requirement should always be accompanied by a robust technical and scientific justification on the applicability of the used test method (e.g. showing absence of water solubility or a low dissolution rate of the nanomaterial).

In case of partial solubility and dissolution rate of the nanoforms, K_{ow} can be considered and measured for the soluble fraction, at the same time than dispersion stability for the particulate fraction (see **Figure 2**).

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

waive the generation of further data on nanoform fate (e.g. column 2 adaptation of bioaccumulation information requirement) on its own (see ECHA Guidance R7c).

Currently there are no standardised methods for fate descriptors to predict the behaviour and transport of nanoforms in the environment and biological media as alternatives to n-octanol/water partition coefficient [46], [47]. Environmental fate descriptors for nanoforms are further discussed in Section 1.2.5 on adsorption/desorption, where state-of-the-art on attachment efficiency of nanomaterials is reported (e.g. OECD GD 342 [56]). Also, there is a list of the models and specific parameters under development, suggested as alternative methods to K_{ow} and K_{oc} as alternative fate descriptors, but sufficient validation is still pending (available in Appendix 1).

1.2.3 Granulometry

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

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

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

As with non-nanoforms, information on granulometry is relevant for the assessment of exposure to airborne particles/dusts, as well as for the performance of toxicity studies via the inhalation route. For the purpose of performing toxicity studies via the inhalation 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 1000 kg/m³ that has the same settling velocity as the particle in question. The mass median aerodynamic diameter (MMAD) is generally reported, and is the aerodynamic diameter where 50% of the particles by mass are below that size, and 50% of the particles are above that diameter. Note that this guidance is not intended to describe how to generate or characterise exposure conditions for the purpose of inhalation toxicity studies for nanomaterials. Some guidance on this subject can be found in ISO/TR 19601:2017 [57]: The generation of aerosols for the purpose of inhalation toxicity studies.

For reaching a conclusion on granulometry, it has to be taken into account that the potential release of particles into the workplace or environment is an important consideration in the design

and operation of many industrial processes and safe handling of substances. Release of particles may present hazard and could cause adverse health effects to humans and affect the environment. It is therefore important to obtain data about the propensity of substances to be released as particles, allowing risks to be evaluated, controlled and minimised. Measurement of the release of particles from powdered substances has similarities to the conventional measurement of the dustiness of a powder, but with significant differences in the methods and instrumentations suited to different particle size ranges.

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

1.2.3.1 Test methods for granulometry

The characterisation of particles requires very careful sampling and sample fractionation practises to be followed. ISO 14488:2007 [58] specifies methods for obtaining a test aliquot from a defined sample of particulate material (powder, paste, suspension or dust) that can be considered to be representative with a defined confidence level. Further information is available in Section 1.1.2 of this appendix on Sample Preparation.

The methods to measure the particle size distribution of the constituent particles, aggregates and agglomerates and/or mass median aerodynamic diameter must be such that they are applicable for nanoforms. The methods specified in the OECD TG 125 for particle size and particle size distribution of nanomaterials [59] and the method listed in **Table 1** can be used to measure the particle size and size distribution of nanoforms to fulfil the Granulometry endpoint. Also **Table 2** in Section 1.2.4 can be consulted for some methods to measure particles and fibres.

1 **Table 1: Methods of measuring airborne dispersed or nebulised particles**

Method and details	Material and size range	Data type
<p>Scanning Mobility Particle Sizer (SMPS) (ISO 15900:2020 [60]; ISO 10808:2010 [28]; ISO 28439:2011 [61])</p> <p>SMPS operates by charging particles and fractionating them based on their mobility when passing between electrodes. This method combines a Differential Mobility Analyser (DMA) and an Optical Particle Counter (OPC). SMPS detects and counts particles, and enables measurement of the particle size distribution and count median diameter of nano aerosols, up to 10^8 particles /cm³. This method also allows evaluation of particle surface area, mass dose, composition and dispersion to support effective analysis of inhalation toxicity testing results. SMPS also has useful application in relation to exposure estimation.</p> <p>Measurement with SMPS is the only currently available method that meets all of the following requirements in the size range below 100 nm: i) measurement of particle size distribution during particle exposures in a continuous manner with time resolution appropriate to check stability of particle size distribution and concentration; ii) measurement range of particle sizes and concentrations covers those of the particle aerosols exposed to the test system during the toxicity test; iii) particle size and concentration measurements are sufficiently accurate for particle toxicity testing and can be validated by ways such as calibration against appropriate reference standards; iv) resolution of particle sizing is sufficiently accurate to allow conversion from number-weighted distribution to surface area-weighted or volume-weighted distribution.</p> <p>However, SMPS is relatively slow and requires a scanning approach to measure different size intervals in series. This method is restricted to ambient temperatures below 35 °C (due to evaporation of butanol in the CPC) and requires aerosolisation of the sample. SMPS cannot distinguish between agglomerates and primary particles. For non-spherical particles (e.g. high aspect ratio nanomaterials), estimation of diameter and mass concentration by SMPS can result in significant error. Assembling data of measurements from SPMS and OPC to provide a whole picture</p>	<p>Particles in an aerosol</p> <p>Size range: ~1 nm – 1 µm</p>	<p>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.</p>

Method and details	Material and size range	Data type
<p>of particle size distribution is not appropriate, due to the different principles employed by the two methods [62]. 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</p>		
<p>Fast Mobility Particle Sizer (FMPS)</p> <p>FMPS enables determination of the size distribution of sub-micrometre 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.</p>	<p>Particles in an aerosol Size range: ~5 - 560 nm</p>	<p>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</p>
<p>Diffusion batteries</p> <p>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.</p> <p>The primary property measured is the diffusion coefficient of the particles and this has to be</p>	<p>Particles in an aerosol</p> <p>Size range: 0.005 – 0.1 μm</p>	<p>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.</p>

Method and details	Material and size range	Data type
<p>converted to particle diameter. The instrument needs to be operated with a particle counter (typically a continuous flow Condensation Particle Counter) in order to determine the number concentration before and after each diffusion stage. Inversion of the raw data to real size distribution is complex and the solutions of the equations do not give unambiguous results in the case of polydisperse aerosol size distributions.</p> <p>ISO/TR 27628:2007 [63] provides an informative description of this method.</p>		
<p>Optical Particle Counter (OPC)</p> <p>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.</p> <p>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 [62].</p> <p>ISO/TR 27628:2007 [63] provides an informative description of this method.</p>	<p>Particles in an aerosol</p> <p>Size range: 0.3 – 17 µm</p>	<p>Particle number concentration</p>
<p>Laser scattering/diffraction</p> <p>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.</p>	<p>Particles of all kind</p> <p>Size range: 0.06 - 100 µm</p>	<p>Particle size/size distribution, from which mass median diameter can be calculated, with knowledge of the density of the particles.</p>

Method and details	Material and size range	Data type
<p>The method is suitable to determine the distribution of particles of respirable and inhalable size. Laser diffraction assumes a spherical particle shape. Test products should therefore have no extreme aspect ratios, with a restriction of 3:1 for non-spherical particles. This method has limited applicability really suitable in the sub-100 nm range. In the range below several microns, results strongly depend on optical constants of particles.</p>		
<p>Light scattering aerosol spectrometer (LSAS)</p> <p>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/m³. The large measurement range of LSAS may result in high uncertainty in nanoscale measurements.</p> <p>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.</p>	<p>Particles in an aerosol Size range: 0.06 - 45 µm</p>	<p>Particle size/size distribution, from which mass median diameter can be calculated, with knowledge of the density of the particles</p>

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

1.2.3.2 Published data on granulometry

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

1.2.4 Dustiness

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

1.2.4.1 Type of property

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

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

- It is important when considering the potential workplace exposure;
- It is used as an input parameter for control banding and exposure modelling tools for nanomaterials;

- Knowledge on dustiness can be used to improve the product characteristics (e.g. create less dusty products) and help users of the products to choose products that potentially may lead to lower exposures for consumers.

1.2.4.2 Test methods for dustiness

There are currently no standardized methods for dustiness at OECD level. However, the OECD WNT project 1.8. [45] will provide a specific TG/GD: "Determination of the dustiness of manufactured nanomaterials". While this is ongoing work, CEN has published 5 standards (EN 17199: 1-5) for the testing of dustiness of materials that release or contain nanomaterials. EN 17199-1 [64] gives advice on the methodology and provides guidance to choose the most adequate test method. The other 4 standards, EN 17199-2 to 5 [65], [66], [67], [68], provide further details on the different test methods.

- Rotating drum (EN 15051-2 and also included in EN 17199-2) [65]
- Small rotating drum (EN 17199-4) [67]
- Continuous drop (EN 15051-3 and also included in EN 17199-3) [66]
- Vortex shaker (EN 17199-5) [68]

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

Method and details	Material and size range	MMAD
<p>Rotating drum method (EN 15051-2) [69] and small rotating drum method</p> <p>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 distribution of the aerosol generated. In addition, airborne nano-objects and structures can be collected for off-line (analytical) electron microscopy analysis.</p> <p>For the small rotating drum, a respirable dust cyclone collects the dust fractions onto a suitable media for gravimetric analysis.</p> <p>For the rotating drum, the determination of the inhalable, thoracic and respirable mass fractions of the released dust is carried out separately according to EN 15051-1 [70] and EN 15051-2 [69].</p> <p>The small rotating drum requires smaller amounts of bulk material for testing (2 to 6 g) compared to the rotating drum method.</p>	<p>Dry powders/granulates/friable products</p> <p>Size range: 0.5-10,000 µm</p>	<p>MMAD can be determined via an appropriate coupled analytical technique.</p>
<p>Continuous drop method (EN 15051-3) [71]</p> <p>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.</p> <p>This method is suitable to determine the distribution of particles of respirable or inhalable size.</p> <p>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 [72].</p>	<p>Dry powders/granulates/friable products</p> <p>Size range: 0.5-10,000 µm</p>	<p>MMAD can be determined via an appropriate coupled analytical technique.</p>

Method and details	Material and size range	MMAD
<p>Vortex shaker (EN 17199-5) [68]</p> <p>The vortex shaker method consists of especially designed cylindrical container that is continuously shaken according to a circular orbital motion, and in which a small volume (0.5 mL) of the test sample is placed. The released aerosol is transferred to the sampling and measurement section. The aerosol real time instruments measures time-resolved particle concentrations and the time-resolved size distribution of the aerosol generated within the vortex shaker. In addition, airborne nano-objects⁴ 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.</p>	<p>Dry Powders</p> <p>Size range: 10 nm-1000 nm</p>	

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

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

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

⁴ Nano-object: material with one, two or three external dimensions in the nano scale [source EN 17199-1:2019]

1.2.4.3 Exposure based waiver for dustiness

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

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

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

1.2.5 Adsorption/desorption

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

Adsorption is temporary (reversible) or permanent bonding between the substance and a surface. With regards to nanoforms, the distribution coefficient between solid phase and a liquid phase (K_d) very often must be based on actual testing since estimations of K_d derived from the organic carbon-water partition coefficient (K_{oc}) and the octanol-water partition coefficient (K_{ow}) is not applicable for most nanoforms, i.e. non-highly dissolving nanoforms (see Section 1.2.1). K_d measurement is based on the assumption of a thermodynamic equilibrium between a liquid and a solid phase. Equilibrium partitioning does not apply to undissolved nanomaterials [51], [46], [47], [20] as described in Section 1.2.2. Nanomaterial dispersions can be kinetically stable for a long period of time (typically through electrostatic or steric stabilization) but they will never reach thermodynamic equilibrium and consequently cannot be equilibrated with an additional phase [47], [73] [74].

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

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

Because of the inability to accurately quantify the physico-chemical forces contributing to particle attachment, this step is typically described by an empirical parameter termed the particle attachment efficiency (α) that needs to be determined in agglomeration (heteroagglomeration) or deposition experiments [46], [74].

OECD TG 106 Adsorption – Desorption Using Batch Equilibrium Method [75] is partially inadequate when the substance in question has a low dissolution rate, i.e. is present as a

dispersion, because it is currently not possible to differentiate between adsorbed or aggregated/agglomerated particles settled during the centrifugation step, and a new TG needs to be developed ([19], [76]). However, if it is shown that a nanomaterial has a high dissolution rate, it can be assessed in the same way as non-nanoforms of substances and the parent guidance will apply (as explained under Section 1.1.2.1 and Section 1.2.1).

1.2.5.1 Relevant method to measure adsorption/desorption of nanoforms

OECD TG 312 Leaching in Soil Columns [77] allows to study the mobility and leaching of the test substance into deeper soil layers or ground water. While it is agreed that the OECD TG 312 is generally applicable for the testing of nanoforms, a GD using this TG to test nanomaterials was established and published on 28 July 2021 OECD GD 342 [56].

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

OECD TG 312, implies thermodynamic processes where non-nanoforms of substances often will reach an equilibrium [77]. As such processes are not applicable for nanoforms, attachment efficiency for heteroagglomeration (α_{hetero}) can be calculated instead ([56]).

α_{hetero} expresses the probability that nanomaterials will attach when they collide with the soil grain surface and takes into account random effects caused by the way the soil matrix happens to be structured ([78]). A quantitative estimation of α can be obtained where a continuous nanomaterial input is applied into the column transport test and the nanomaterial concentration is monitored over time in the eluate as it is the case within TG 312. However, it needs to be noted that the determination of α_{hetero} is based on the "clean bed" assumption, which is valid only during the early stages of the deposition process, when low particle loadings are applied and no significant repulsion between particles and the porous medium is present. Outside these settings more complex mechanisms can influence the particle transport (e.g. blocking, ripening) and with α_{hetero} not being able to accurately describe the system anymore, leading to misinterpretation and misuse of the data.

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

The selection of test soils has to relate to environmental relevance rather than to properties, and at least two soils differing either in pH, organic carbon content, clay content and/or texture should be considered. Generally, soils with high clay content are to be avoided because here particle transport occurs predominantly in macropores [79] making experiments with saturated, stacked soil cores unrepresentative for nanoform transport rates.

This reduction of number of soils to two from the parent TG is based on reasons of practicability. Because soils with high clay content (soil 1 in OECD TG 312) tend to block during leaching (nanoforms strongly attach to the clay minerals preventing break through [80], [81], [82]) and sandy soils with high carbon content (soil 5 in OECD TG 312) are only limited available. In case natural soils are used a control experiment with soils not previously exposed to nanomaterials has to be conducted to determine the background of naturally occurring nanoforms.

To account for more realistic conditions of nanomaterial mobility in soils for which a considerably longer residence time is expected, flow rates of 2-3 L·m⁻²·h⁻¹ are to be used, avoiding an artificial

break through and posing as a realistic worst-case scenario. As a leaching solution mimicking artificial rain fall, aqueous solutions of 0.005 M KCl or NaCl should be used. For nanoforms reacting with chloride (e.g. silver that would precipitate and render them unavailable for the chemical analysis), other suitable anions such as NO₃ (KNO₃) have to be used. Furthermore, the use of divalent salts such as CaCl₂ will not provide a worst case scenario test as nanomaterials homoaggregates are generally less mobile in the presence of Ca²⁺. The chosen monovalent salt should not react with the nanomaterial, e.g. accelerating its dissolution and the stability of the nanomaterial suspension should be measured prior to the column test. To mimic conditions where the soil pore water is dominated by divalent cations a solution of 0.005 M CaCl₂ can be used according to GD 342. However, it has to be taken into account that this will not work for nanomaterials reacting with chloride such as silver. Furthermore, it does not present a worst case with respect to mobility.

The advice provided here is not applicable to nanomaterials with high dissolution rates (see Section 1.1.2., 1.2.1). For those nanomaterials, it might result in testing the adsorption behaviour of the dissolved fraction and not the nanoform. The advice is also not applicable for nanomaterials with low dispersion stability in aqueous media (with definitions given in OECD TG 318 (2017) [55]). Testing of such nanomaterials will result in a not-representative and not-reproducible addition of test material to the soil column. Guidance on how to determine dispersion stability/dissolution rate of the nanoforms in environmentally relevant conditions can be gained from OECD TG 318. Information on the use of the data for further environmental testing and assessment strategies can be found in GD 318 [30]. Testing of these parameters has to be performed before applying OECD TG 312, GD 342.

1.2.5.2 Alternative fate descriptors to adsorption/desorption of nanoforms

A list of available models to predict alternative fate descriptors for nanomaterials is available in **Appendix 1**. The OECD published results of a study where 10 nano-specific environmental exposure tools and models were tested for functionality and accuracy. Three of the models mentioned in Appendix 1 – namely nanoFATE, simplexbox4nano and nanoDUFLOW are part of this report and details on the functional assessment as well as the outcome of the statistical analysis can be found there [83].

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

1.2.5.3 Waiving of adsorption/desorption to nanoforms

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

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

Appendix 1 Models for fate and exposure of nanomaterials

There is on-going research and development of modelling tools to assess the fate of nanomaterials. The list of methods given below is not exhaustive and includes methods based on attachment efficiency and dissolution rate of nanomaterials. More information on these methods that may be used to predict fate and transport of nanomaterials in the environment and organisms can be found at [84]. 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	[85]
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.	[86]

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 <i>et al.</i> , [54] a river model was used.	[54]
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: [87]
NanoFASE	Water-Soil-Organism model, a complex multimedia spatiotemporal model predicts the fate and bio-uptake, across space and in time, of nanomaterials entering the soil and aquatic environments. It works by coupling submodels for environmental compartments: soils, rivers, bed sediments, lakes, estuaries and the sea, and simulating the transport of nanomaterials between these compartments of nanomaterials in different forms and states; useful for identifying accumulation hotspots and studying the temporal dynamics of NM concentrations.	Spatiotemporal distribution of nanomaterials (NM) across multiple environmental compartments, making it distinct from lower-tier screening level models, such as SimpleBox4nano,	http://nanofase.eu/show/element_268	

Model	Overview	Output	Link to the model tools	References
LOTOS-Euros	A long-range (regional scale) spatiotemporal atmospheric substance transport and deposition model; open source	Wide range of applications such as air quality forecast, emissions, depositions etc.	https://lotos-euros.tno.nl/publications/model-documentation/	Open source, see link; [88]

References

- [1] ECHA, "Appendix for nanoforms applicable to the Guidance on Registration and Substance Identification," [Online]. Available: <https://echa.europa.eu/guidance-documents/guidance-on-reach>.
- [2] European Parliament and the Council, "REGULATION (EC) No 1907/2006 OF THE EUROPEAN PARLIAMENT AND OF THE COUNCIL," 2006. [Online]. Available: <https://eur-lex.europa.eu/legal-content/EN/TXT/?uri=CELEX%3A02006R1907-20220108&qid=1644831475630>.
- [3] ECHA, "Guidance on information requirements and chemical safety assessment Chapter R.7a: Endpoint specific guidance," [Online]. Available: <http://echa.europa.eu/guidance-documents/guidance-on-information-requirements-and-chemical-safety-assessment>.
- [4] ECHA, "Guidance on information requirements and chemical safety assessment Chapter R.6: QSARs and grouping of chemicals," [Online]. Available: <http://echa.europa.eu/guidance-documents/guidance-on-information-requirements-and-chemical-safety-assessment>.
- [5] European Commission, "Official Journal of the European Union - Commission Recommendation of 10 June 2022 on the definition of nanomaterial (2022C 229/01)," 2022. [Online]. Available: [https://eur-lex.europa.eu/legal-content/EN/TXT/?uri=CELEX:32022H0614\(01\)](https://eur-lex.europa.eu/legal-content/EN/TXT/?uri=CELEX:32022H0614(01)). [Accessed August 2022].
- [6] ISO, "ISO/TS 80004-6:2013(en) Nanotechnologies - Vocabulary - Part 6: Nano-object characterization," 2013. [Online]. Available: <http://www.iso.org/iso/home.html>.
- [7] EFSA, A. Hardy, D. Benford, T. Halldorsson, M. Jeger, H. Knutsen, S. More, H. Naegeli, H. Noteborn, C. Ockleford, A. Ricci, G. Rychen, J. Schlatter, V. Silano, R. Solecki, D. Turck, M. Younes, Q. Chaudhry, F. Cubadda, D. Gott, A. Oomen, W. S. M. Karamitrou, R. Schoonjans and A. Mortensen, "Guidance on risk assessment of the application of nanoscience nanotechnologies in the food and feed chain: Part 1, human and animal health.," *EFSA Journal*, vol. 16, no. 7, 2018.
- [8] M. Elimelech, J. Gregory, X. Jia and R. Williams, "Particle Deposition & Aggregation: Measurement, Modelling and Simulation.," *Butterworth-Heinemann, Woburn.*, 1998.
- [9] K. T. Semple, K. J. Doick, K. C. Jones, P. Burauel, A. Craven and H. Harms, "Defining bioavailability and bioaccessibility of contaminated soil and sediment is complicated.," *Environmental Science & Technology*, pp. 38(12), 228A-231A, 2004.
- [10] OECD, "Assessment of Biodurability of Nanomaterials and their Surface ligands. Series on the Safety of Manufactured Nanomaterials No. 86 - ENV/JM/MONO(2018)11," 2018. [Online]. Available: [https://www.oecd.org/officialdocuments/publicdisplaydocumentpdf/?cote=env/jm/mono\(2018\)11&doclanguage=en](https://www.oecd.org/officialdocuments/publicdisplaydocumentpdf/?cote=env/jm/mono(2018)11&doclanguage=en).
- [11] OECD, "Guidance Document on Inhalation Toxicity Testing. Series on Testing and Assessment No. 39 - ENV/JM/MONO(2009)28/REV1," 2018. [Online]. Available: [https://www.oecd.org/officialdocuments/publicdisplaydocumentpdf/?cote=env/jm/mono\(2009\)28/rev1&doclanguage=en](https://www.oecd.org/officialdocuments/publicdisplaydocumentpdf/?cote=env/jm/mono(2009)28/rev1&doclanguage=en).
- [12] R. Hogg, T. Healy and D. Fuerstenau, "Mutual coagulation of colloidal dispersions.," *Transactions of the Faraday Society*, Vols. 62, pp 1638-1651., 1966.
- [13] A. Praetorius, N. Tepe and F. Von der Kammer, "Report on Driving Forces of NM Behaviour in Natural Waters for Agglomeration and Transformation," 2017.
- [14] K. Driscoll and P. Borm, "Expert workshop on the hazards and risks of poorly soluble low toxicity particles.," *Inhalation Toxicology*, vol. 32, no. 2, pp. 53-62, 2020.
- [15] European Food Safety Authority SCIENTIFIC COMMITTEE AND EMERGING RISKS UNIT, "EFSA GUIDANCE ON TECHNICAL REQUIREMENTS FOR REGULATED FOOD AND FEED PRODUCT APPLICATIONS TO ESTABLISH THE PRESENCE OF SMALL PARTICLES INCLUDING NANOPARTICLES," 2021. [Online]. Available:

<https://www.efsa.europa.eu/en/efsajournal/pub/6769>.

- [16] European Parliament and the Council, "Recital 12 of the REACH amended Regulation (EU 2018/1881)," 2018. [Online]. Available: <https://eur-lex.europa.eu/legal-content/EN/TXT/PDF/?uri=CELEX:32018R1881&from=EN>.
- [17] "ACEnano," [Online]. Available: <https://acenano.eu.auth0.com/login?state=hKFo2SB4QnJUzjgzN3hSbUZjRGd6MHBQa3ZWdXhsYThuRmFrUaFup>.
- [18] OECD, "Guidance on Sample Preparation and Dosimetry for the Safety Testing of Manufactured Nanomaterial. Series on the Safety of Manufactured Nanomaterials No. 36 - ENV/JM/MONO(2012)40," 2012. [Online]. Available: [https://www.oecd.org/officialdocuments/publicdisplaydocumentpdf/?cote=env/jm/mono\(2012\)40&doclanguage=en](https://www.oecd.org/officialdocuments/publicdisplaydocumentpdf/?cote=env/jm/mono(2012)40&doclanguage=en).
- [19] OECD, "OECD Series on Testing and Assessment, No. 194. Guidance on grouping of chemicals, second edition ENV/JM/MONO(2014)4," 2014. [Online]. Available: <http://www.oecd.org/chemicalsafety/testing/seriesontestingandassessmentnon-testingmethodseggsarandgrouping.htm>.
- [20] OECD, "Guidance manual for the testing of manufactured nanomaterials: OECD's sponsorship programme; First revision. Series on the safety of manufactured nanomaterials, ENV/JM/MONO(2009)20/REV," 2009. [Online]. Available: [https://www.oecd.org/officialdocuments/publicdisplaydocumentpdf/?cote=env/jm/mono\(2009\)20/rev&doclanguage=en](https://www.oecd.org/officialdocuments/publicdisplaydocumentpdf/?cote=env/jm/mono(2009)20/rev&doclanguage=en).
- [21] OECD, "Preliminary Review of OECD Test Guidelines for their Applicability to Manufactured Nanomaterials. Series on the safety of manufactured nanomaterials, ENV/JM/MONO(2009)21," 2009. [Online]. Available: <http://www.oecd.org/science/nanosafety>.
- [22] The NanoDefine Consortium, "The NanoDefine Methods Manual - NanoDefine Technical Report D7.6," 2015. [Online]. Available: http://www.nanodefine.eu/publications/reports/NanoDefine_TechnicalReport_D7.6.pdf.
- [23] OECD, "Testing Programme of Manufactured Nanomaterials," [Online]. Available: <http://www.oecd.org/chemicalsafety/nanosafety/testing-programme-manufactured-nanomaterials.htm>.
- [24] OECD, "Series on the Safety of Manufactured Nanomaterials- No. 40. Addendum to Ecotoxicology and Environmental Fate of Manufactured Nanomaterials: Test Guidelines. Expert Meeting Report ENV/JM/MONO(2014)1/ADD," [Online]. Available: <http://www.oecd.org/science/nanosafety/publications-series-safety-manufactured-nanomaterials.htm>.
- [25] OECD, "Guidance document on aquatic and sediment toxicological testing of nanomaterials - Series on Testing and Assessment (OECD GD 317) - ENV/JM/MONO(2020)8," 2021. [Online]. Available: [https://www.oecd.org/officialdocuments/publicdisplaydocumentpdf/?cote=env/jm/mono\(2020\)8&doclanguage=en](https://www.oecd.org/officialdocuments/publicdisplaydocumentpdf/?cote=env/jm/mono(2020)8&doclanguage=en).
- [26] ISO, "ISO 14887:2000. Sample preparation - Dispersing procedures for powders in liquids," 2000. [Online]. Available: <http://www.iso.org/iso/home.html>.
- [27] N. Hartmann, K. Jensen, A. Baun, K. Rasmussen, H. Rauscher, R. Tantra, D. Cupi, D. Gilliland, F. Pianella and J.-M. Riego Sintes, "Techniques and protocols for dispersing nanoparticle powders in aqueous media – is there a rationale for harmonization?," *Journal of Toxicology and Environmental Health, Part B Critical Reviews*, vol. 18, no. 6, pp. 299-326, 2015.
- [28] ISO, "ISO 10808:2010. Nanotechnologies - Characterization of nanoparticles in inhalation exposure chambers for inhalation toxicity testing," 2010. [Online]. Available: <http://www.iso.org/iso/home.html>.
- [29] E. J. Petersen, S. A. Diamond, A. Kennedy, G. Goss, K. Ho, J. Lead, S. Hanna, N. Hartmann, K. Hund-Rinke, B. Mader, N. Manier, P. Pandard, E. Salinas and P. Sayre, "Adapting OECD Aquatic Toxicity Tests for Use with Manufactured Nanomaterials: Key Issues and Consensus Recommendations," *Environmental Science & Technology*, vol.

49, no. 16, p. 9532–9547, 2015.

- [30] OECD, "Guidance document for the testing of dissolution and and dispersion stability of nanomaterials and the use of the data for further environmental testing and assessment strategies (OECD GD No. 318)," 2021. [Online]. Available: [https://www.oecd.org/officialdocuments/publicdisplaydocumentpdf/?cote=env/jm/mono\(2020\)9&doclanguage=en](https://www.oecd.org/officialdocuments/publicdisplaydocumentpdf/?cote=env/jm/mono(2020)9&doclanguage=en).
- [31] ECHA, "Appendix R.6-1 for nanomaterials applicable to the Guidance on QSARs and Grouping," [Online]. Available: <http://echa.europa.eu/guidance-documents/guidance-on-information-requirements-and-chemical-safety-assessment>.
- [32] J. Cheng, E. Flahaut and S. Cheng, "Effect of carbon nanotubes on developing zebrafish (*Danio rerio*) embryos," *Environmental Toxicology Chemistry*, vol. 26, no. 4, p. 708–716, 2004.
- [33] M. Dobrovolskaia, B. Neun, J. Clogston, J. Grossman and S. McNeil, "Choice of method for endotoxin detection depends on nanoformulation," *Nanomedicine*, vol. 9, no. 12, pp. 1847–1856, 2014.
- [34] J. Beltran-Huarac, Z. Zhang, G. Pyrgiotakis and et al., "Development of reference metal and metal oxide engineered nanomaterials for nanotoxicology research using high throughput and precision flame spray synthesis approaches," *Nanoimpact.*, vol. 10, no. DOI: 10.1016/j.impact.2017.11.007., pp. 26–37, 2018.
- [35] US FDA C, CMV, CDRH and ORA, "Guidance for Industry: Pyrogen and Endotoxins Testing: Questions and Answers.," 2012. [Online]. Available: <http://www.fda.gov/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/ucm314718.htm>.
- [36] Council of Europe, "The European Pharmacopoeia general method 2.6.30 Monocyte-activation test allows the MAT to serve as a full replacement for the RPT after product-specific validation," 28 June 2021. [Online]. Available: <https://www.edqm.eu/en/news/european-pharmacopoeia-put-end-rabbit-pyrogen-test>.
- [37] Council of Europe, "Recombinant factor C: new Ph. Eur. chapter available as of 1 July 2020," 01 July 2020. [Online]. Available: <https://www.edqm.eu/en/news/recombinant-factor-c-new-ph-eur-chapter-available-1-july-2020>.
- [38] ISO, "ISO 29701:2010. Nanotechnologies - Endotoxin test on nanomaterial samples for in vitro systems - Limulus amebocyte lysate (LAL) test," 2010. [Online]. Available: <http://www.iso.org/iso/home.html>.
- [39] C. Jones and D. Grainger, "In vitro assessments of nanomaterial toxicity," *Advanced Drug Delivery Reviews*, vol. 61, no. 6, p. 438–456, 2009.
- [40] OECD, "Test No. 105: Water Solubility, OECD Guidelines for the Testing of Chemicals, Section 1," [Online]. Available: http://www.oecd-ilibrary.org/environment/oecd-guidelines-for-the-testing-of-chemicals-section-1-physical-chemical-properties_20745753.
- [41] OECD, "Series on Testing and Assessment. No. 29. Guidance Document on Transformation/Dissolution of Metals and Metal Compounds in Aqueous Media. ENV/JM/MONO(2001)9," 2001. [Online]. Available: <http://www.oecd.org/chemicalsafety/testing/seriesontestingandassessmenttestingforenvironmentalfate.htm>.
- [42] ISO, "ISO/TR 19057:2017(en) Nanotechnologies - Use and application of acellular in vitro tests and methodologies to assess nanomaterial biodurability," 2017. [Online]. Available: <http://www.iso.org/iso/home.html>.
- [43] J. Koltermann-Juelly, J. Keller, A. Vennemann, K. Werle, P. Müller, L. Ma-Hock, R. Landsiedel, M. Wiemann and W. Wohlleben, "Abiotic dissolution rates of 24 (nano)forms of 6 substances compared to macrophage-assisted dissolution and in vivo pulmonary clearance: Grouping by biodissolution and transformation," *NanoImpact*, vol. 12, pp. 29–41, 2018.
- [44] P. Bove, M. Malvindi, S. Kote, R. Bertorelli, M. Summa and S. Sabella, "Dissolution test for risk assessment of nanoparticles: a pilot study," *Nanoscale*, vol. 9, pp. 6315–26,

2017.

- [45] OECD, "Work plan for the Test Guidelines Programme (TGP)," June 2020. [Online]. Available: https://www.oecd.org/chemicalsafety/testing/Test_Guidelines_Workplan_2020.pdf.
- [46] A. Praetorius, N. Tufenkji, K.-U. Goss, M. Scheringer, F. von der Kammer and M. Elimelech, "The road to nowhere: equilibrium partition coefficients for nanoparticles," *Environmental Science: Nano*, vol. 1, pp. 317-323, 2014.
- [47] G. Cornelis, "Fate descriptors for engineered nanoparticles: the good, the bad, and the ugly," *Environmental Science: Nano*, vol. 2, pp. 19-26, 2015.
- [48] OECD, "Test No. 107: Partition Coefficient (n-octanol/water): Shake Flask Method," 1995. [Online]. Available: https://www.oecd-ilibrary.org/environment/test-no-107-partition-coefficient-n-octanol-water-shake-flask-method_9789264069626-en.
- [49] OECD, "Test No. 117: Partition Coefficient (n-octanol/water), HPLC Method," 2004. [Online]. Available: https://www.oecd-ilibrary.org/environment/test-no-117-partition-coefficient-n-octanol-water-hplc-method_9789264069824-en.
- [50] OECD, "Test No. 123: Partition Coefficient (1-Octanol/Water): Slow-Stirring Method," 2006. [Online]. Available: https://www.oecd-ilibrary.org/environment/test-no-123-partition-coefficient-1-octanol-water-slow-stirring-method_9789264015845-en.
- [51] K. Rasmussen, M. Gonzalez, P. Kearns, J. Riego Sintes, F. Rossi and P. Sayre, "Review of achievements of the OECD Working Party on Manufactured Nanomaterials' Testing and Assessment Programme. From exploratory testing to test guidelines," *Regulatory Toxicology and Pharmacology*, vol. 74, p. 147-160, 2016.
- [52] S. Ottofuelling, F. Von Der Kammer and T. Hofmann, "Commercial titanium dioxide nanoparticles in both natural and synthetic water: comprehensive multidimensional testing and prediction of aggregation behavior," *Environmental Science and Technology*, vol. 45, p. 10045-10052, 2011.
- [53] S. Sørensen, R. Hjorth, C. Delgado, N. Hartmann and A. Baun, "Nanoparticle ecotoxicity – physical and/or chemical effects?," *Integrated Environmental Assessment and Management*, vol. 11, no. 4, p. 719-728, 2015.
- [54] A. Praetorius, M. Scheringer and K. Hungerbühler, "Development of Environmental Fate Models for Engineered Nanoparticles—A Case Study of TiO₂ Nanoparticles in the Rhine River," *Environmental Science & Technology*, vol. 46, no. 12, p. 6705-6713, 2012.
- [55] OECD, "Test No. 318: Dispersion Stability of Nanomaterials in Simulated Environmental Media," 2017. [Online]. Available: https://www.oecd-ilibrary.org/environment/test-no-318-dispersion-stability-of-nanomaterials-in-simulated-environmental-media_9789264284142-en.
- [56] OECD, "Guidance document on testing nanomaterials using OECD TG No. 312 "Leaching in soil columns" - Series on Testing and Assessment No. 342 - ENV/CBC/MONO(2021)17," 2021. [Online]. Available: [https://www.oecd.org/officialdocuments/publicdisplaydocumentpdf/?cote=ENV/CBC/MONO\(2021\)17&docLanguage=En](https://www.oecd.org/officialdocuments/publicdisplaydocumentpdf/?cote=ENV/CBC/MONO(2021)17&docLanguage=En).
- [57] ISO, "ISO/TR 19601:2017(en) - Nanotechnologies - Aerosol generation for air exposure studies of nano-objects and their aggregates and agglomerates (NOAA)," 2017. [Online]. Available: <http://www.iso.org/iso/home.html>.
- [58] ISO, "ISO 14488:2007. Particulate materials - Sampling and sample splitting for the determination of particulate properties," 2007. [Online]. Available: <http://www.iso.org/iso/home.html>.
- [59] OECD, "Test No. 125: Nanomaterial Particle Size and Size Distribution of Nanomaterials, OECD Guidelines for the Testing of Chemicals, Section 1," 2022. [Online]. Available: https://www.oecd-ilibrary.org/environment/test-no-125-nanomaterial-particle-size-and-size-distribution-of-nanomaterials_af5f9bda-en.
- [60] ISO, "ISO 15900:2020. Determination of particle size distribution - Differential electrical mobility," 2020. [Online]. Available: <http://www.iso.org/iso/home.html>.
- [61] ISO, "ISO 28439:2011. Workplace atmospheres - Characterization of ultrafine

aerosols/nanoaerosols - Determination of the size distribution and number concentration using differential electrical mobility analysing systems," 2011. [Online]. Available: <http://www.iso.org/iso/home.html>.

- [62] L. Ma-Hock, A. O. Gamer, R. Landsiedel, E. Leibold, T. Frechen, B. Sens, M. Linsenbuehler and B. van Ravenzwaay, "Generation and characterization of test atmospheres with nanomaterials," *Inhalation Toxicology*, vol. 19, no. 10, pp. 833-848, 2007.
- [63] ISO, "ISO/TR 27628:2007. Workplace atmospheres - Ultrafine, nanoparticle and non-structured aerosols - Inhalation exposure characterization and assessment," 2007. [Online]. Available: <http://www.iso.org/iso/home.html>.
- [64] CEN, "EN 17199-1:2019. Workplace exposure - Measurement of dustiness of bulk materials that contain or release respirable NOAA and other respirable particles - Part 1: Requirements and choice of test methods," 2019. [Online]. Available: <https://www.cen.eu/Pages/default.aspx>.
- [65] CEN, "EN 17199-2:2019. Workplace exposure - Measurement of dustiness of bulk materials that contain or release respirable NOAA or other respirable particles - Part 2: Rotating drum method," 2019. [Online]. Available: <https://www.cen.eu/Pages/default.aspx>.
- [66] CEN, "EN 17199-3:2019. Workplace exposure - Measurement of dustiness of bulk materials that contain or release respirable NOAA or other respirable particles - Part 3: Continuous drop method," 2019. [Online]. Available: <https://www.cen.eu/Pages/default.aspx>.
- [67] CEN, "EN 17199-4:2019. Workplace exposure - Measurement of dustiness of bulk materials that contain or release respirable NOAA or other respirable particles - Part 4: Small rotating drum method," 2019. [Online]. Available: <https://www.cen.eu/Pages/default.aspx>.
- [68] CEN, "EN 17199-5:2019. Workplace exposure - Measurement of dustiness of bulk materials that contain or release respirable NOAA or other respirable particles - Part 5: Vortex shaker method," 2019. [Online]. Available: <https://www.cen.eu/Pages/default.aspx>.
- [69] CEN, "EN 15051-2:2013. Workplace exposure - Measurement of the dustiness of bulk materials - Part 2: Rotating drum method," 2013. [Online]. Available: <https://www.cen.eu/Pages/default.aspx>.
- [70] CEN, "EN 15051-1:2013. Workplace exposure - Measurement of the dustiness of bulk materials - Part 1: Requirements and choice of test methods," 2013. [Online]. Available: <https://www.cen.eu/Pages/default.aspx>.
- [71] CEN, "EN 15051-3:2013. Workplace exposure - Measurement of the dustiness of bulk materials - Part 3: Continuous drop method," 2013. [Online]. Available: <https://www.cen.eu/Pages/default.aspx>.
- [72] T. Schneider and K. Jensen, "Combined single-drop and rotating drum dustiness test of fine to nanosize powders using a small drum," *The Annals of Occupational Hygiene*, vol. 52, no. 1, pp. 23-34, 2008.
- [73] N. Hartmann, L. Skjolding, S. Hansen, J. Kjølholt, F. Gottschalck and A. Baun, *Environmental fate and behaviour of nanomaterials- New knowledge on important transformation processes- Environmental Project No. 1594*, The Danish Environmental Protection Agency., 2014.
- [74] L. Barton, M. Therezien, M. Auffan, J. Bottero and M. Wiesner, "Theory and Methodology for Determining Nanoparticle Affinity for Heteroaggregation in Environmental Matrices Using Batch Measurements," *Environmental Engineering Science*, vol. 31, no. 7, pp. 421-427, 2014.
- [75] OECD, "Test No. 106: Adsorption -- Desorption Using a Batch Equilibrium Method," 2000. [Online]. Available: https://www.oecd-ilibrary.org/environment/test-no-106-adsorption-desorption-using-a-batch-equilibrium-method_9789264069602-en.
- [76] T. Kuhlbusch, C. Nickel, B. Hellack, S. Gartiser, F. Flach, A. Schiwy, H. Maes, A.

Schäffer, L. Erdinger, S. Gabsch and M. Stintz, "Fate and behaviour of TiO₂ nanomaterials in the environment, influenced by their shape, size and surface area, Federal Environment Agency (Umweltbundesamt), Report No. (UBA-FB) 001577," 2012, p. 166.

- [77] OECD, "Test No. 312: Leaching in Soil Columns, OECD Guidelines for the Testing of Chemicals, Section 3," [Online]. Available: https://www.oecd-ilibrary.org/environment/test-no-312-leaching-in-soil-columns_9789264070561-en.
- [78] N. Tufenkji and M. Elimelech, "Deviation from the Classical Colloid Filtration Theory in," *Langmuir*, vol. 20, pp. 10818-10828, 2004.
- [79] J. Ryan and M. Elimelech, "Colloid mobilization and transport in groundwater," *Colloids and Surfaces A: Physicochemical and Engineering Aspects*, vol. 107, pp. 1-56, 1996.
- [80] G. Cornelis, L. Pang, C. Doolette, J. Kirby and M. McLaughlin, "Transport of silver nanoparticles in saturated columns of natural soils.," *Science of The Total Environment*, Vols. 463-464, pp. 120-130, 2013.
- [81] G. Cornelis, C. Doolette, M. Thomas, M. McLaughlin, J. Kirby, D. Beak and C. D., "Retention and Dissolution of Engineered Silver Nanoparticles in Natural Soils.," *Soil Science Society of America Journal*, vol. 76, pp. 891-902, 2012.
- [82] G. Cornelis, B. Ryan, M. McLaughlin, J. Kirby, D. Beak and D. Chittleborough, "Solubility and batch retention of CeO₂ nanoparticles in soils.," *Environmental Science & Technology*, vol. 45, no. 7, pp. 2777-2782, 2011.
- [83] OECD, "Evaluation of Tools and Models Used for Assessing Environmental Exposure to Manufactured Nanomaterials - Series on the Safety of Manufactured Nanomaterials No.98 - Series on Testing and Assessment No.345," ENV/CBC/MONO(2021)23 - 2021. [Online]. Available: [https://www.oecd.org/officialdocuments/publicdisplaydocumentpdf/?cote=ENV-CBC-MONO\(2021\)23%20&doclanguage=en](https://www.oecd.org/officialdocuments/publicdisplaydocumentpdf/?cote=ENV-CBC-MONO(2021)23%20&doclanguage=en).
- [84] Y. Cohen, R. Rallo, R. Liu and H. Liu, "In Silico Analysis of Nanomaterials Hazard and Risk," *Acc Chem Res.*, vol. 46, no. 3, p. 802-812, 2013.
- [85] J. A. J. Meesters, A. A. Koelmans, J. T. K. Quik, A. J. Hendriks and D. van de Meent, "Multimedia Modeling of Engineered Nanoparticles with SimpleBox4nano: Model Definition and Evaluation," *Environmental Science & Technology*, vol. 48, no. 10, pp. 5726-5736, 2014.
- [86] J. T. Quik, J. J. de Klein and A. A. Koelmans, "Spatially explicit fate modelling of nanomaterials in natural waters," *Water Research*, vol. 80, p. 200-208, 2015.
- [87] K. Garner, S. Suh and A. Keller, "Assessing the risk of engineered nanomaterials in the environment: development and application of the nanoFATE model.," *Environ. Sci. Technol.*, vol. 51, no. 10, pp. 5541-5551, 2017.
- [88] C. Svendsen, L. Walker, M. Matzke, E. Lahive, S. Harrison, A. Crossley, B. Park, S. Lofts, I. Lynch, S. Vazquez-Campos, R. Kaegi, A. Gogos, C. Asbach, G. Cornelis, F. von der Kammer, N. van den Brink, C. Mays and D. Spurgeon, "Key principles and operational practices for improved nanotechnology environmental exposure assessment.," *Nature Nanotechnology*, vol. 15, pp. 731-742, 2020.

EUROPEAN CHEMICALS AGENCY
TELAKKAKATU 6, P.O. BOX 400,
FI-00121 HELSINKI, FINLAND
ECHA.EUROPA.EU