Draft GRACIOUS framework for grouping and read-across of nanomaterials for regulatory risk assessment and safe-by-design

For circulation

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Abstract

The GRACIOUS framework aims to support practical application of grouping of nanomaterials/nanoforms in a risk assessment context, in order to meet the needs of various stakeholders, particularly regulators and industry. To ascertain alignment to the present EU legislation and to make use of existing approaches, a state-of-the-art overview is provided and used in further development of the draft GRACIOUS framework. The framework provides a structure to develop and refine hypotheses that outline why specific nanomaterials/nanoforms can be grouped, taking into account the purpose or context of the user (targeted testing, regulatory, precautionary or safe-by-design). The scientifically based hypotheses comprise combinations of physicochemical, fate and kinetics, and hazard aspects that are relevant for risk assessment at different life cycle stages. The hypothesis can be developed and refined at 3 levels, which may include the development of a read-across justification. Each level is linked to a corresponding Integrated Approach to Testing and Assessment (IATA) in order to guide the work needed to justify the proposed grouping. The basic information needed as a starting point is indicated. A number of hypotheses are provided as examples, and the relation of the framework to safe-by-design via the Stage-Gate innovation process is outlined. In this manner, the GRACIOUS Framework aims to facilitate scientifically sound risk assessment for NMs different from the case-by-case testing and risk assessment paradigm.

Invitation

Stakeholders are invited to provide feedback on the following document. Comments are invited in any form, including:

- Completion of a questionnaire
- Comments written on the document
- E-mail.
- Please return your comments to: f.murphy@hw.ac.uk and s.stoycheva@yordasgroup.com

1 Introduction

Manufacturing and functionalising of materials at the nanoscale leads to a whole array of nanomaterials (NMs) / nanoforms (NFs) (see text box[1]) varying not only in chemical composition, but also in e.g. size, morphology and surface characteristics. Apart from expected benefits, distinctive properties of NFs may also affect environmental and human health. Risk assessment requires sufficient information for each NF, but testing every unique NF for their potential adverse effects would be highly resource demanding. More efficient ways to obtain risk information are needed, and this could be achieved by applying grouping and read-across approaches to NFs. GRACIOUS will address this urgent need by providing a framework for grouping and read-across based on physicochemical, release, exposure, environmental fate, toxicokinetic and toxicological information.

Nanomaterials (NMs) and nanoforms (NFs)

A nanomaterial is defined only by its size according to the EC Recommendation 2011/696/EU (EU, 2011). Under REACH, a nanoform is a form of a substance, which fulfils the EC recommended definition of nanomaterial (EU, 2018). In addition to size, nanoforms are characterised by shape and surface chemistry (EU, 2018). These properties may be described by ranges of values with clear boundaries. Different nanoforms may or may not show different exposure, fate/toxicokinetic behaviour and toxicity. As the draft GRACIOUS framework for grouping and read-across of nanomaterials is developed for the purposes of regulatory risk assessment and safe-by-design, the term nanoform is used in the remainder of the manuscript. The terminology is further explained in Chapter 2 and Appendix A.

This document is a first draft of the GRACIOUS framework. Relevant stakeholders (e.g. regulators, industries, SMEs) will be engaged from the start of the project to advance these early concepts and include other relevant developments into an improved and usable Framework. This dialogue with stakeholders will ensure that the Framework will be relevant to the objectives of these key stakeholders.

The document provided is highly detailed, including background information to demonstrate the evidence base on which the framework is developed. Once the framework detail is finalised, an abbreviated version of the framework will be generated, allowing a more user friendly guidance document to evolve.

The scientific foundations for the application of grouping and read-across to NFs have been established in a number of conceptual schemes as developed in the EU-funded projects MARINA (Oomen et al., 2015), NANoREG (Dekkers et al., 2016), NanoReg2, GUIDEnano (Park et al., 2018) and ITS-NANO (Stone et al., 2013), and in the ECETOC Nano Task Force (Arts et al., 2015). In addition, European regulatory bodies and related expert committees have provided recommendations on how to identify NFs and apply grouping and read-across to NFs of the same substance in the context of REACH (ECHA, 2013; ECHA et al., 2016; ECHA, 2017b, c). One of the major conclusions of these activities is that future nanospecific grouping and read-across strategies should be hypothesis-driven and must consider not only intrinsic properties and (eco)toxicological effects, but also extrinsic (system-dependent) descriptors of exposure, toxicokinetics and environmental fate.

[1] These definitions may be revisited for a next version of the GRACIOUS Framework to ensure alignment with regulatory developments.

1.1 The potential use and application of grouping for NMs/NFs

As can be seen from the illustrative example in the text box, the purpose and needs of the user with respect to grouping should be clear from the beginning. This also means that one should consider what the consequences would be of placing a NF inside or outside a specific group, and whether it should be included in multiple groups.

An example to illustrate the importance to consider the purpose of grouping

When in a shop, one can group fruit and vegetables, or group according to colour, size, or storage life, etc. Whether such a grouping is useful, depends on the purpose and needs of the user.

Similarly for NFs, the purpose and needs of the user should be clear as grouping for the purpose of grouping has little added value.

For risk assessment in a regulatory context, grouping can serve several purposes:

- **To facilitate targeted testing or targeted risk assessment.** If it is known that one or more aspects (e.g. a physicochemical property) of a material may inform exposure, fate, kinetic behaviour or a specific hazard, this knowledge can be used to target information gathering/ testing for risk assessment, or to highlight specific points of interest when assessing the risk. The latter may e.g. be relevant for a substance evaluation under REACH, where one may focus specifically on certain aspects, such as human inhalation risks or hazards for the aquatic environment. A number of similar materials sharing known exposure, fate, kinetic or hazard information may be seen as an initial group and a starting point for hypothesis formulation.
- **To fill a data gap in a regulatory dossier.** When a regulatory dossier on a chemical is submitted to a regulatory agency, it may be possible to provide the requested information by grouping chemicals based on similarity and by applying read-across, i.e. use information from other (groups of) similar chemicals to predict required information and fill data gaps. REACH is the regulatory framework that is the most advanced legislation with regard to grouping and read-across, where it is specifically mentioned in the legal text as an option to fulfil information requirements (Annex XI; EC, 2006), see also Appendix B/B.1. Also other legal frameworks in the EU and international organisations, such as the Organisation for Economic Co-operation and Development (OECD), apply or discuss grouping and read-across for chemicals and nanomaterials (e.g. OECD, 2014, 2016b), see Appendix B.
- **To develop precautionary measures.** Based on the known information on exposure, fate, kinetic behaviour or hazard of similar materials, precautionary measures can be taken for a new material for which that information is not available, e.g. by reducing or preventing exposure.
- **To steer safe innovation/safe-by-design.** For a new material under development, information available on similar materials or relationships with e.g. physicochemical properties can provide an indication of potential issues with exposure, fate, kinetic behaviour, or hazard. This provides an opportunity to exploit this information to steer safe innovation/safe-by-design. Also knowledge on the likelihood to use grouping and read-across later in the innovation process is relevant, as targeted testing and read-across approaches will likely reduce resources and be less time-consuming than case-by-case testing to satisfy regulatory information requirements to obtain market approval under a specific law.

Grouping may also be used to improve scientific understanding. For example, modelling (e.g. quantitative structure-activity relationships, QSARs) of the behaviour of NFs (fate/toxicokinetic behaviour, effects) can lead to new insights that can in turn lead to establishing new groups of NFs and to new read-across options. Where the scientific understanding increases the possibilities of grouping of NFs increase, and vice versa, identifying possibilities for grouping may increase scientific understanding. This scientific knowledge and understanding can be used in regulation, for targeted testing, safe-by-design, etc.

1.2 Aim of the framework

The GRACIOUS framework aims to support practical grouping of NFs for risk assessment and risk decision making, meeting the needs of various stakeholders, particularly regulators and industry. Application of the Framework will support making better use of grouping and read-across for the purpose of risk assessment.

According to the Second Regulatory Review on Nanomaterials by the European Commission (EC, 2012), conclusion of a chemical safety assessment should cover all forms of a substance in a registration dossier. "*Where data from one form of a substance are used in demonstration of the safe use of other forms, a scientific justification should be given on how, applying the rules for grouping and read-across, the data from a specific test or other information can be used for the other forms of the substance. Similar considerations apply to exposure scenarios and the risk management measures."*

To facilitate achievement of this aim, the GRACIOUS Framework will develop robust scientific arguments that can support justifying grouping of NFs and related read-across cases and in this way help industry and competent authorities reduce the testing burden of a case-by-case risk assessment of NFs. The GRACIOUS Framework thereby aims to improve the efficiency of information gathering for NFs. In addition, it will support decision making for safe innovation/safe-by-design of nano-enabled products (NEPs).

1.3 Framework overview

The groups generated by the GRACIOUS Framework are based on scientific hypotheses related to combinations of physicochemical, fate and kinetics, and hazard endpoints that are relevant for the use of grouping and read-across in risk assessment and decision making. To generate the knowledge and information needed to assess if a NF fits into a group related to a hypothesis, Integrated Approaches to Testing and Assessment (IATAs)[2] will be developed to incorporate all domains of relevance for risk assessment, namely: (i) "uses in the lifecycle that lead to environmental release and human exposure", (ii) "what they are: physicochemical identity", (iii) "where they go: environmental fate, uptake and toxicokinetics", and iv) "what they do: human and environmental toxicity". The Framework will reduce, refine and replace (where possible) the need for animal testing by supporting the use of grouping, read-across, (*in silico*) modelling (e.g. QSARS, including modelling of fate and exposure), and of *in vitro* tests.

The Framework is based on existing approaches on grouping and read-across (Arts et al., 2015; ECHA, 2017b) and aims to align to regulatory terminology and EU legislation. In Appendix A and Appendix B further information can be found on these backgrounds.

The Framework (and the IATAs) will be delivered both as a guiding Background Document and a (set of) software module(s) designed for practical application to i) help industries and regulators assess environmental and human health risks of existing NFs cost-effectively; ii) facilitate business decisions concerned with developing new NEPs, i.e. inform safe-by-design practices.

In order to ensure sustainability, the Framework will be open for easy integration of new knowledge, as well as modular and sufficiently flexible to accommodate new insights. This will enable it to evolve and mature within the current regulatory risk assessment practices to meet the needs of both regulators and industry, and to become part of the future nanotechnology regulation and governance.

[2] IATA is preferred over the earlier used term ITS, as IATA is nowadays more commonly used in a regulatory context (REACH, OECD) and it is considered that the term IATA better describes the intended purpose in that it includes a combination of different types of testing as well as assessment approaches.

1.4 How to read this document?

This deliverable contains background information on the terminology, how grouping and read-across can potentially be used in EU legislation and existing grouping approaches for nanomaterials, as well as an outline of the draft GRACIOUS Framework for grouping and read-across of nanomaterials. After the present introductory Chapter 1 in which the need, possible applications, aim and general overview of the GRACIOUS Framework are described, Chapter 2 contains the background information. Additional supporting information on the terminology and on how nanomaterials and grouping and read-across are addressed in different EU legislations can be found in the Appendices. Chapter 3 describes the development of the draft Framework in general, while a more detailed description of the different parts of the framework can be found in Chapter 4, including a brief description of a number of hypotheses. Chapter 5 addresses elements for quality evaluation to assess if a hypothesis is sufficiently justification. An inventory of relevant tools, methods and protocols is presented in Chapter 6, e.g. for physicochemical characterization, exposure assessment, *in silico*, *in vitro* and *in vivo* toxicity, kinetics and similarity. The last Chapter (Chapter 7) describes how the draft GRACIOUS framework will be further developed within the project.

2 State of the art

The present Chapter describes key terms used within GRACIOUS, aligning as far as possible to existing definitions (section 2.1). More detailed information on the definitions of these key terms is provided in Appendix A. Also an overview of existing grouping approaches if provided (section 2.2). An overview of EU legislation addressing NM and/or NF and the application of grouping and read-across therein is provided in Appendix B.

2.1 Terminology

In 2016, the European Commission's Joint Research Centre (JRC) published a report describing the harmonised terminology for the nanosafety field, which was developed in NANoREG (Gottardo et al., 2017) and subsequently refined in NanoReg2 (Hernandez and Noorlander, 2016). The list includes terms with international regulatory relevance (e.g. defined and used by OECD) or a specific meaning at European level (e.g. chemical safety assessment, nanomaterial, nanoform).

Seven terms have been identified as key in GRACIOUS, namely nanomaterial, nanoform, grouping, read-across, classification, safe-by-design, and representative test material/benchmark material. For each term, the NANoREG harmonised definition, when existing, has been used as a basis and updated in view of new developments, for example the European Chemicals Agency (ECHA) appendix providing best practices for NFs (ECHA, 2017c) and the revised REACH Annexes containing provisions for NFs (EU, 2018). For the term nanomaterial, GRACIOUS also refers to the definition provided by the EC in the Recommendation 2011/696/EU (EU, 2011) (please note that this is also under review by the EC) and considers the existing definitions in the EU legal acts (for instance, in the Cosmetic Products Regulation and Novel Food Regulation).

Table 1 provides short definitions for each of the seven GRACIOUS key terms; more comprehensive information can be found in Appendix A of the present manuscript. Some terms (for instance, nanomaterial and nanoform) are under discussion and their regulatory definitions may change in the near future. If this occurs, Table 1 will be updated accordingly. Moreover, other terms may be added during the project.

Table 1: Definitions for the GRACIOUS key terms and their regulatory references. More comprehensive information on these terms can be found in Appendix A.

KEY TERM	PROPOSED DEFINITION
NANOMATERIAL (NM)	According to the definition published by the European Commission (EC) in the Recommendation 2011/696/EU (EU, 2011), a nanomaterial is: "A natural, incidental or manufactured material containing particles, in an unbound state or as an aggregate or as an agglomerate and where, for 50 % or more of the particles in the number size distribution, one or more external dimensions is in the size range 1 nm - 100 nm. […]. By derogation from the above, fullerenes, graphene flakes and single wall carbon nanotubes with one or more external dimensions below 1 nm should be considered as nanomaterials."
NANOFORM (NF)	According to the latest revision of the REACH Annexes (EU, 2018), a nanoform is a form of a substance that meets the requirements of the European Commission (EC) Recommendation 2011/696/EU on the definition of the term nanomaterial (EU, 2011) that is further characterised by its number based particle size distribution with indication of the number fraction of constituent particles in the size range within 1 nm – 100 nm, its shape and surface area, and a description of its surface functionalisation or treatment and identification of each agent.

KEY TERM	PROPOSED DEFINITION
GROUPING	The Organisation for Economic Co-operation and Development (OECD) defines grouping as the general approach for considering more than one chemical at the same time (OECD, 2014). • According to OECD (2014), the rationale underpinning grouping may be based on: • Common functional group(s); • Common constituents or chemical classes, similar carbon range numbers; • A common mode or mechanism of action or adverse outcome pathway; • The likelihood of common precursors and/or breakdown products via physical or biological processes that result in structurally similar chemicals; • An incremental and constant change across the category. At European level, Annex XI to REACH addresses grouping and read-across between different substances and establishes that structural similarity is a prerequisite for any grouping and read-across approach aimed to fulfil the standard information requirements. ECHA released guidance on how to apply grouping and read-across to nanoforms of the same substance (ECHA, 2017b). Annex XI to REACH was recently revised to include specific provisions for nanoforms and extend the applicability of the concept of grouping and read-across to different nanoforms of the same substance (EU, 2018).
READ-ACROSS	The OECD defines read-across as a technique to fill in data gaps where the test information concerning a certain endpoint for one chemical, referred to as source chemical, is used to predicted the test information concerning the same endpoint for another chemical, referred to as target chemical, which is considered to be similar based on a scientific justification (OECD, 2014). At European level, Annex XI to REACH addresses grouping and read-across between different substances and establishes that structural similarity is a prerequisite for any grouping and read-across approach aimed to fulfil the standard information requirements. ECHA released guidance on how to apply grouping and read-across to nanoforms of the same substance (ECHA, 2017b). Annex XI to REACH was recently revised to include specific provisions for nanoforms and extend the applicability of the concept of grouping and read-across to different nanoforms of the same substance (EU, 2018).
CLASSIFICATION	Neither the United Nations Globally Harmonised System (UN, 2017), nor its European implementation in the CLP regulation (EC, 2008) formally defines the term 'classification'. At European level, however, the European Chemicals Agency (ECHA) defines the hazard classification of a substance or a mixture as the assignment of a standardised description to its physical, health or environmental hazard (ECHA, 2015b). Examples of classification include sensitizer or carcinogen.
SAFE-BY-DESIGN*	According to NANoREG (Gottardo et al., 2017) and NanoReg2 (Hernandez and Noorlander, 2016), the Safe-by-Design (SbD) concept aims at reducing the risks of a NM or nano-enabled product for human health and the environment, and associated uncertainties, starting from an early stage of the innovation process.

KEY TERM	PROPOSED DEFINITION
REPRESENTATIVE TEST MATERIAL/ BENCHMARK MATERIAL	A representative test material is a material from a single batch, which is sufficiently homogeneous and stable with respect to one or more specified properties, and which implicitly is assumed to be fit for its intended use in the development of test methods which target properties other than the properties for which homogeneity and stability have been demonstrated (Roebben et al., 2013). All materials used in GRACIOUS as benchmark or reference materials are in fact representative test materials. It is proposed that, if the use of the term benchmark material cannot be avoided, it should be regarded as having the identical meaning of representative test material within the context of GRACIOUS.

* Safe-by-design is considered to be the same as safer-by-design in the present document. This may be updated when international consensus on the term is reached.

2.2 Existing grouping approaches for NMs and/or NFs

For chemicals substances in general, *in silico* methods (including QSAR and grouping for read-across) have been used for several decades now to gain efficiencies and improving animal welfare in regulatory hazard assessments. As a consequence in several jurisdictions guidance was developed for its regulatory use (e.g. US HPV Challenge Program in 1998, or more recently the European REACH Regulation). Based on these regulatory developments, OECD published its first Guidance on Grouping of Chemicals in 2007 (OECD, 2007b) and in the context of REACH, ECHA published the Guidance on Grouping of Chemicals (ECHA, 2008) and the Read-Across Assessment Framework (updated in 2017; ECHA, 2017d). Neither of these, however, specifically mentions the grouping and read-across of NFs. In 2014 OECD published a second edition of its Guidance on Grouping of Chemicals (OECD, 2014), but although nanomaterials are mentioned, OECD concludes in this document that “at present, it seems premature to develop guidance on grouping specifically for nanomaterials”.

In recent years, however, several approaches for grouping and read-across of NFs have been developed, which form the starting point for the GRACIOUS framework. In this section, we provide an overview of the existing grouping frameworks for NFs, and, where available, the case studies to which they were applied.

The US National Institute on Occupational Safety and Health (NIOSH) proposed a framework where NMs are grouped according to the mechanism causing toxic action, and identify four groups depending on surface reactivity, shape and solubility: higher solubility particles that can reach systemic tissues (toxic ions reach systemic tissue); poorly soluble, low toxicity particles; poorly soluble, high toxicity particles (same as above but with reactive surface); fibrous particles for which the toxicity is related to biopersistence and genotoxicity (Kuempel et al., 2012).

The US-Canada Regulatory Cooperation Council (RCC) defined seven classes of NMs according to chemical composition: carbon nanotubes (CNTs); inorganic carbon; metal and metalloid oxides; metals, metal salts and metalloids; semiconductor quantum dots; organics and other classes. In addition, toxicologically relevant physicochemical properties were identified for each of the classes to support (sub-)classification (RCC, 2013a, b). The FP7 project ITS-NANO suggested that any approach adopted for grouping should take into account the changes occurring during the lifecycle (LC) of NMs (Stone et al., 2013). Key aspects are physicochemical (PC) characteristics of NMs (chemical composition, size, specific surface area -SSA, etc.), their behaviour and effects (reactive oxygen species - ROS generation, electron transfer, photoreactivity, etc.) and their fate (e.g. hydrophobicity, agglomeration, zeta potential). The FP7 research project MARINA added to this that grouping should be supported by information on kinetics (uptake, distribution, biopersistence) and early and apical biological effects (Oomen et al., 2015).

These aspects related to the LC and exposure to a NF are taken into account in the framework proposed by ECETOC task force on NMs (Arts et al., 2015) that defines a three-tier approach (DF4nanoGrouping) to group NMs for inhalation exposure in one of four main groups identified depending on persistence, shape, surface reactivity, and solubility. Four groups are identified accordingly: soluble NMs, biopersistent with high aspect ratio NMs, passive NMs, and active NMs. Tier 0 precedes of the DF4NanoGrouping consists in collecting intrinsic physicochemical properties supporting identification of the NM. This information feeds the assignment of the NM to one of the four groups in Tier 1. Tier 2 sharpens this assignment to the groups of biopersistent high aspect ratio, passive, or active NMs by using information on system-dependent properties. For each Tier 2 parameter above a defined cut-off, the NM is assigned to different sub-groups of active NMs (non-compensating, binary decision logic). Toxicological information is then optionally used in Tier 3 to corroborate the assignment of the NM to a group and to support sub-grouping of active NMs depending on the outcome of short term *in vivo* studies. DF4NanoGrouping supports read-across within each group, consisting of NMs with similar physicochemical and activity properties. For instance, group 1 may allow read-across between soluble NMs of the same chemical composition (also from non-NF), group 2 for biopersistent and high aspect ratio NMs like CNTs, group 3 for non-fibrous passive NMs, and group 4 between reactive NMs. Applicability of the framework is addressed by Arts et al. (2016), where 24 NMs of different composition (carbonaceous, metal oxides and sulphates, amorphous silica, organic pigments) are assigned to one of the four pre-defined groups. The testing strategy supported by DF4NanoGrouping provides that when a NM is assigned to the group of active NMs, the specific hazards may be addressed by *in vivo* experiments.

On the other hand, RIVM proposed a grouping approach that substantiates a hypothesis on the behaviour of the NM of interest depending on known information. It is a tiered approach where data are collected at different levels of complexity, and read-across is supported endpoint by endpoint according to similarities identified depending on the collected information (mainly based on physicochemical properties and behaviour in environmental or biological media) (Sellers et al., 2015). The 4-steps framework consists of (1) collection of existing data (physicochemical characterisation and behaviour of the NM in environmental and biological media), (2) hypothesis formulation (that may lead to experimental testing for the final assessment), (3) testing (3 tiers: PC properties, reactivity and *in vitro* toxicity, and *in vivo* toxicity), (4) assessment (do data support the hypothesis, or is there need of new data?). This approach does not aim primarily at assigning a NM to a predefined category, as hazard groups are eventually defined in a flexible manner after collection of information on physicochemical properties and toxicological endpoints. In this approach, the LC of products containing NMs is considered as a step for identifying exposure routes when addressing specific case studies.

ECHA guidance on information requirement and chemical safety assessment specific to the application of QSARs and grouping of chemicals to NFs (ECHA, 2017b) presents a framework where grouping is proposed according to similarity following the definition from REACH Annex XI (EC, 2006). ECHA guidance introduces properties beyond chemical composition to support the grouping hypothesis (e.g. aspect ratio, particle size, shape, or solubility), and highlights the importance of toxicokinetic studies in grouping, read-across, and for *in vitro* to *in vivo* extrapolation (ECHA et al., 2016; ECHA, 2017b). The framework proposed by ECHA was tested on two case studies, nano-TiO_2 and CNTs (Worth et al., 2017a). The two case studies were chosen as data-rich NFs, to illustrate how chemoinformatic techniques such as hierarchical clustering, principal components analysis, and random forest for variable selection can be used to support grouping and identify key physicochemical properties to predict the *in vitro* comet assay results of the target substances. The validity of the grouping hypothesis was tested through the application of chemoinformatic tools and random forest variable selection was applied to make the prediction to the two target substances. For the nano-TiO_2, two classes were identified and the property relevant to justify the grouping was the presence of coating, that acted by preventing the contact between the nano-TiO_2 and the cellular components.

Within the EU FP-7 GUIDEnano project (Park et al., 2018), a methodology was developed to systematically quantify the similarity between NFs that have been tested in toxicity studies and the NF

for which risk needs to be evaluated, for the purpose of extrapolating toxicity data between the two materials. The methodology is a first attempt to use current knowledge on NF property-hazard relationships to develop a series of pragmatic and systematic rules for assessing NF similarity. It takes into account the practical feasibility, being based on generally available NF characterization information. This methodology is part of the quantitative human and environmental hazard evaluation within the GUIDEnano tool and it is organized in the following way: Step 1: Use previously derived hazard values (e.g. derived non effect level – DNEL, predicted non effect concentration – PNEC) for the exposure-relevant NF or similar; Step 2: Where no previously derived hazard values are available, use conservative default hazard values for general NF categories; Step 3: Where conservative hazard values lead to a risk, identify data from individual toxicity studies with the exposure relevant NF or similar. Each study is then evaluated using criteria related to: a) similarity between the exposure-relevant NF and the tested material; b) quality of the data; and c) relevance of the study for each given endpoint. The evaluation of these three aspects results in an overall score and only studies with NFs that have a score above a defined threshold will be considered acceptable for the hazard assessment.

An in-depth overview on the available grouping frameworks, on the opinions on the properties that may justify or support grouping of NMs and on modelling approaches to grouping for read-across is available in Worth et al. (2017a).

The starting point for the draft GRACIOUS framework (Figure 1) integrates the industry (DF4NanoGrouping) and regulatory (ECHA, 2017b) grouping concepts that will be updated to incorporate further state-of-the art thinking (e.g. the NANoREG and NanoReg2 approaches to grouping and read-across). It is designed to follow the ITS-NANO recommendations by efficiently identifying grouping hypotheses and proposing IATAs to substantiate them, based on the latest scientific, technical, and regulatory knowledge. Both the DF4NanoGrouping and the ECHA concepts recommended that in order to acquire the data needed for grouping and read-across in a rational and cost-effective manner, the IATAs should be implemented in tiers of increasing specificity and complexity and would cover the following areas: i) “What they are: Physicochemical identity”, ii) “Lifecycle: human exposure and environmental release”, iii) “Where they go: Environmental fate, uptake and toxicokinetics”, and iv) “What they do: Human and environmental toxicity”. This is consistent with our plans to develop a series of IATAs covering all these areas to enable the generation of adequate information to substantiate a number of pre-identified grouping hypotheses.

3 Outline Draft framework

3.1 Starting point of the draft framework structure

The starting point for the draft GRACIOUS Framework as developed during the project proposal stage is presented in Figure 1, which integrates the state-of-the-art grouping concepts developed by industry (DF4NanoGrouping) (Arts et al., 2015) and for regulatory purposes by ECHA (ECHA, 2017b).

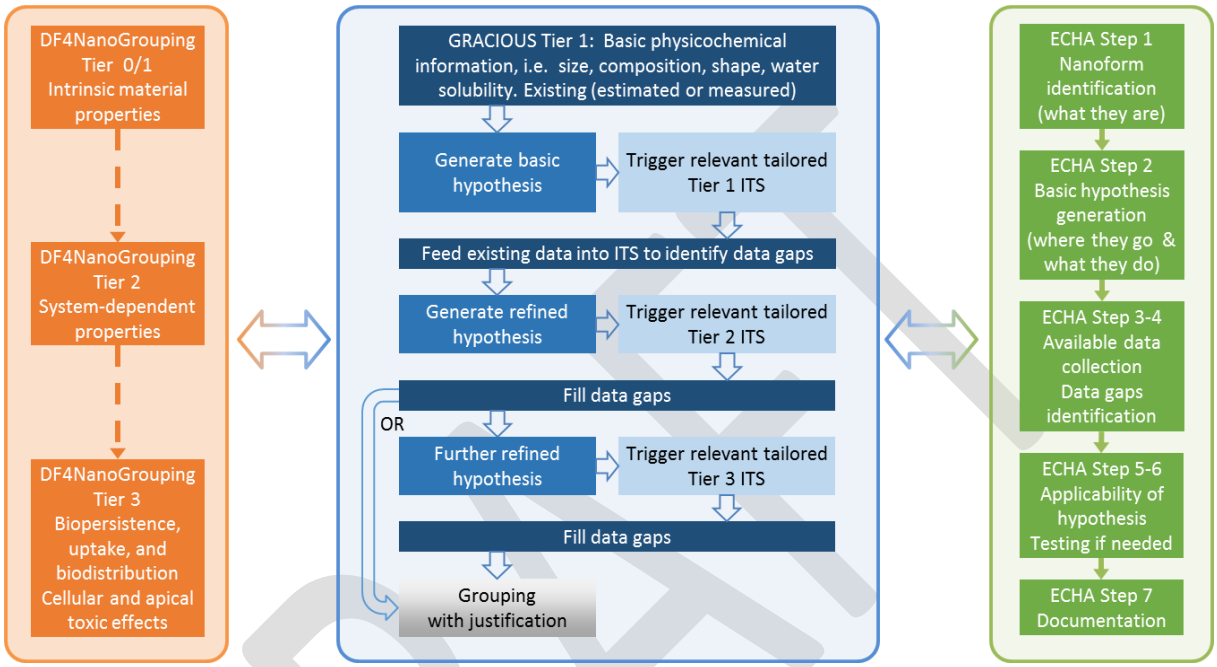

Figure 1. The starting point for the GRACIOUS Framework, derived from integrating industry (DF4NanoGrouping, in orange on the left) and regulatory (ECHA, in green on the right) state-of-the-art concepts. By using the 'functionality' of nanomaterials the DF4nanoGrouping aims to group nanomaterials by their specific mode-of-action that results in an apical toxic effect. The ECHA approach aims at building endpoint specific grouping and read-across hypotheses to establish why similarities/differences between the nanoforms in physicochemical properties allow for predicting a specific (eco)toxicological behaviour and determine the ranges of applicability of these hypotheses. The GRACIOUS Framework aims to integrate these approaches by establish hypotheses based on a limited set of intrinsic material properties and further refine these based on the 'functionality' of nanomaterials and further filling of data gaps.

This starting point has been further developed and elaborated in the first months of the project to a draft framework (see section 3.2 and Chapter 4) to be shared and discussed with various stakeholders.

The GRACIOUS framework aims to efficiently identify grouping hypotheses and propose IATAs to substantiate them based on the latest scientific, technical, and regulatory knowledge. As indicated in section 2.2, existing approaches recommend that the IATAs should be implemented in tiers of increasing specificity and complexity to acquire the data needed to justify grouping and read-across (Figure 1).

Early in the framework, the specific objectives or purpose of the grouping or read-across (e.g. regulatory or SbD) and the associated information requirements will be specified. Basic physicochemical data such as size, shape and solubility/dissolution rate, which are available at this stage will be used to initiate the hypothesis generation. This will enable an estimation of the quality, usefulness and completeness of the available data before it guides the user to options for grouping and read-across and the respective strategies to obtain additional information and refine the hypothesis, if required.

Figure 2 shows the starting point for aligning the Framework with the *Stage-Gate* Idea-to-Launch process as developed during the project proposal stage, to facilitate SbD via grouping. *Stage-Gate* reasoning will ensure that the GRACIOUS Framework is well-aligned with the SbD approaches proposed by the NANoREG (Noorlander et al., 2016; Gottardo et al., 2017) and NanoReg2 projects. Specifically, the first characterisation tier can be applied before the strategic decision *"Go to development"* (*Gate 3)*, whereas the second tier provides initial feedback during performance optimisation in the development *Stages*, without incurring too much cost. Therefore, the first two tiers enable the SbD principles of NANoREG (Noorlander et al., 2016), stopping the innovation process if profitability and/or the technical and/or commercial probability of success are compromised, or if the (uncertainties on) risks are considered unacceptable. Alignment with *Stage-Gate* thereby ensures the relevance of the Framework to business decision-making, regarding the design of safer NEPs and manufacturing processes.

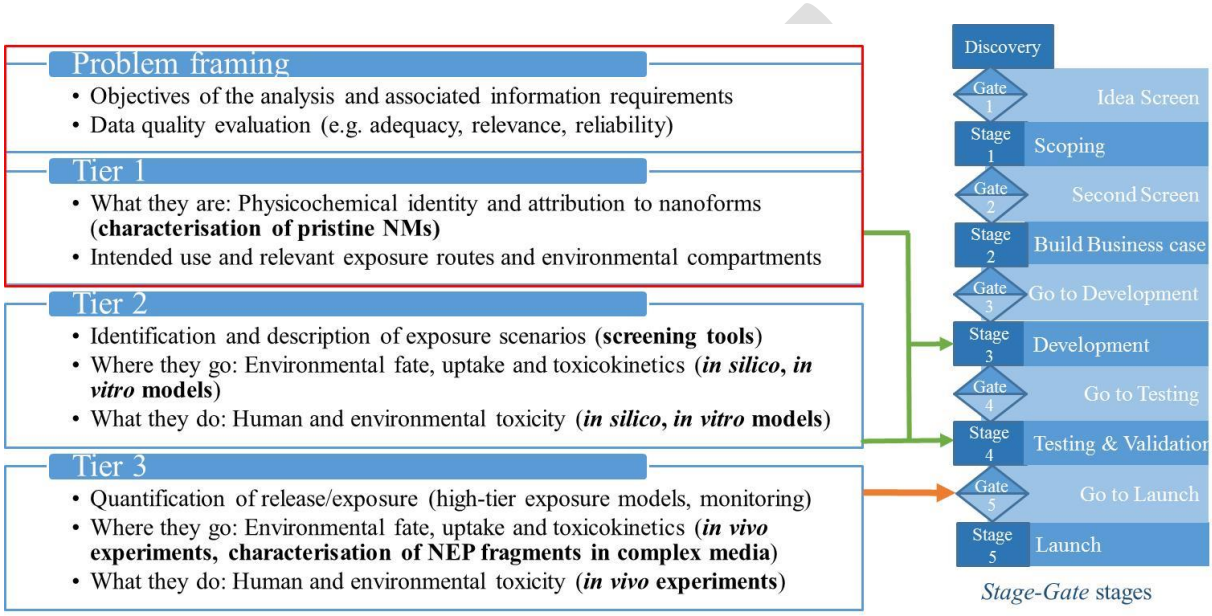

Figure 2. The starting point for the GRACIOUS Framework and its relation to SbD via the Stage-Gate innovation process (as developed during project proposal stage). The red box shows the stages for which data generation, beyond identification as nanomaterial, is not required.

3.2 Outline of the draft framework

The basic structure of the draft GRACIOUS Framework as developed in the first months of the project is presented in Figure 1. This is expanded in Figure 4 to provide more detail including the hypotheses and the consequences for NFs of falling within a group or not within a group. A further elaboration on different parts of the draft framework is provided in Chapter 4.

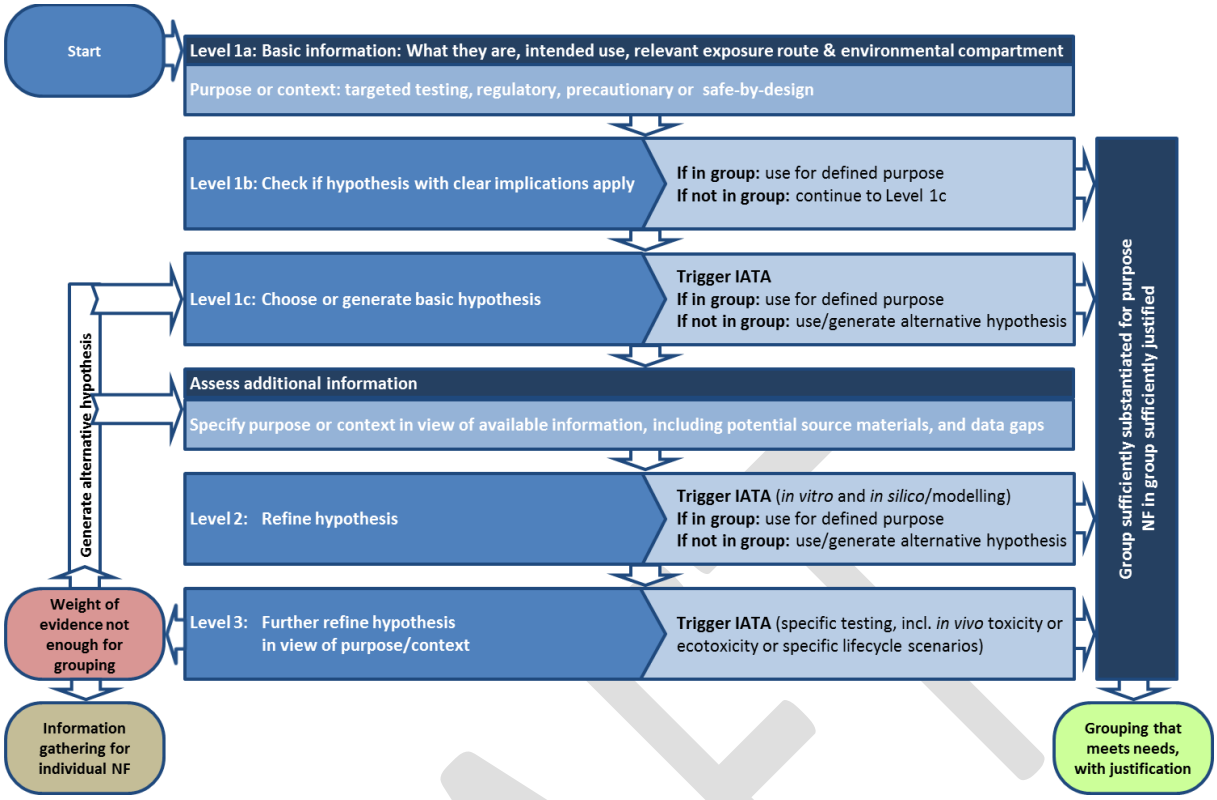

Figure 3 Structure of the draft GRACIOUS Framework. Basic information (Level 1a) may be used to easily assign a new NF to one of a few groups associated with a hypothesis for which clear implications apply (Level 1b). If this is not possible, a different basic hypothesis is chosen or generated (Level 1c). Where the required additional information is generated to (further) substantiate the group (while refining the hypothesis where necessary) and/or justify that the NF fits in the group (Levels 2 and 3). IATAs are used to guide the generation of additional information, generally with more complex and resource intense methods in later Levels of the framework. As soon as grouping is sufficiently justified, the framework is exited (box on the right). In case a Level 3 hypothesis cannot be justified, the processes is (partly) reiterated, or the required information is generated by testing of an individual NF (i.e. no grouping is applied).

In Level 1a, basic information is gathered for a (group of) NF(s) that enter(s) the framework (see section 4.2). With this information and considering the purpose of grouping, a quick screening is performed on the applicability of several well-known hypotheses with clear implications (Level 1b) for one or more endpoints. If one of these hypotheses is applicable, it can be used for the defined purpose provided that, in case of regulatory application, justification can be given for each of the endpoints it is used for (see also Section 4.3). If none of these hypotheses apply, a basic hypothesis is used or developed (Level 1c), which triggers a Level 1 IATA (if the available data do not already allow assessing if a NF fits into a group) (section 4.4). Based on the results of the IATA, the user will be able to identify whether the NF(s) fall within the group defined by the hypothesis. If not, an alternative hypothesis should be used. If the NF(s) fall within the group described in the hypothesis, the framework will help the user to determine if this group, including its potential consequences and implications, is sufficiently substantiated for the defined purpose (section 4.5). If the group is sufficiently substantiated, it can be used for the defined purpose. If the group is not sufficiently substantiated, the framework will guide the user to determine whether the grouping needs can be further specified and refined. For example, with the newly generated information from the Level 1 IATA, data gaps (in a regulatory dossier) and potential source materials (which can be both other NFs or non-nanomaterials) may be identified. This information is used to formulate a refined hypothesis in Level 2, triggering one or more Level 2 IATAs, that consist of *in silico* assessments, *in vitro* and/or invertebrate testing (section 4.6). The results are used to determine if the grouping is sufficiently justified to meet the needs of the user. If grouping is not sufficiently justified,

further refinement and testing in Level 3 IATAs, using more advanced *in vitro* and/or *in vivo* studies, can be performed (section 4.7), after which the framework will again aid the user to determine if the grouping is sufficiently substantiated for the purpose (section 4.8). In the different sections of Chapter 4, all the steps are described in more detail.

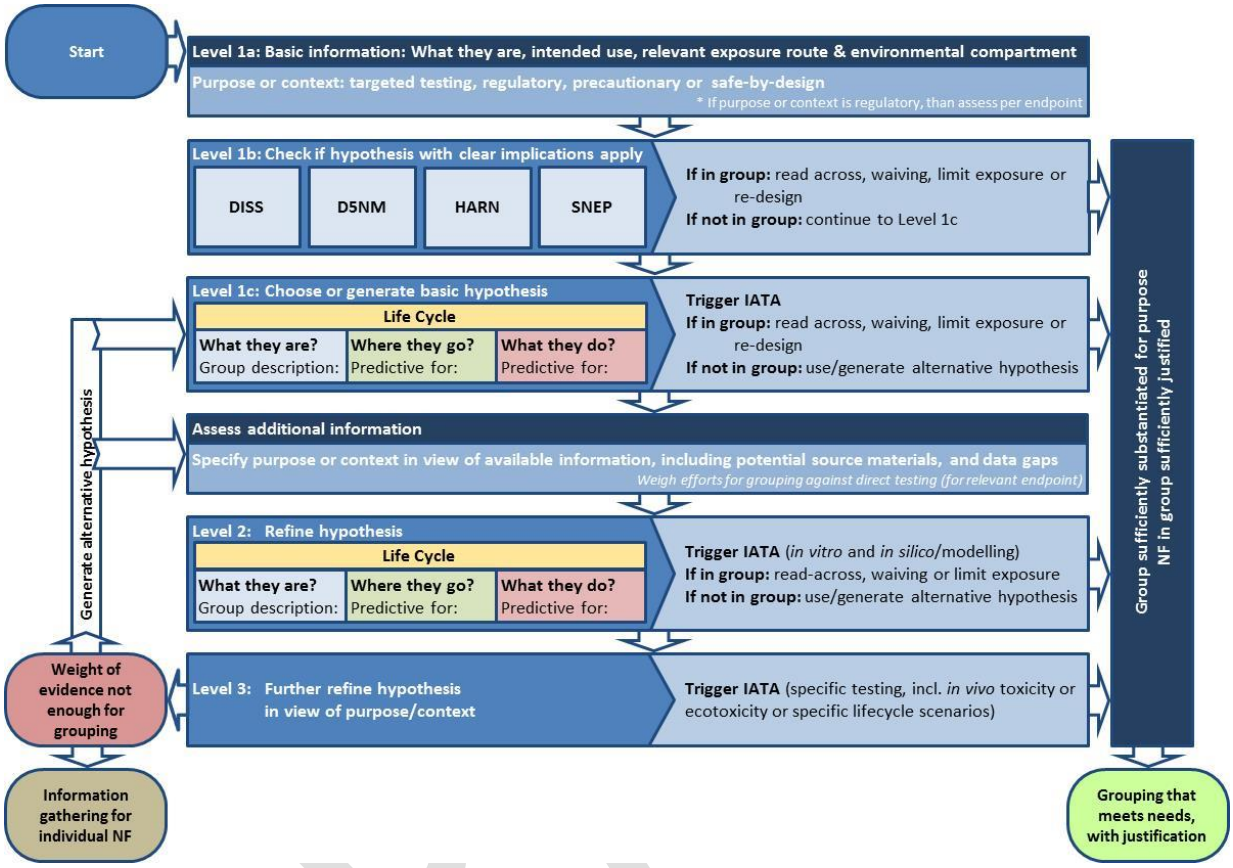

Figure 4. Detailed outline of draft GRACIOUS framework, elaborating on the structure as shown in Figure 3. Basic information (Level 1a) may be used to easily assign a new NF to one of a few groups associated with a hypothesis for which clear implications apply (Level 1b). If this is not possible, a different basic hypothesis is chosen or generated (Level 1c). Where the required additional information is generated to (further) substantiate the group (while refining the hypothesis where necessary) and/or justify that the NF fits in the group (Levels 2 and 3). Hypotheses are based on knowledge on the (expected) releases during the life cycle, and knowledge on characteristics of the NF (what they are), environmental fate and/or toxicokinetics (where they go), and reactivity (what they do) and what these can contribute to the prediction of a specific endpoint. IATAs are used to guide the generation of additional information, generally with more complex and resource intense methods in later Levels of the framework. As soon as grouping is sufficiently justified, the framework is exited (boxes on the right). In case a Level 3 hypothesis cannot be justified, the Levels are (partly) reiterated (boxes on the left) or the required information is generated for an individual NF (i.e. no grouping is applied and the Framework is exited).

Figure 4 has the same structure as Figure 3 with some additional information on the hypotheses and on the consequences for NF(s) of falling within the group or not within the group. Using the basic information from Level 1a and the purpose of the grouping, a quick screening is performed on the applicability of four well-known hypotheses with clear implications (Level 1b). The groups defined in these hypotheses with clear implications include:

- Quickly dissolving NFs: **DISS**,
- Dermal exposure to NFs larger than 5 nm: **D5NM**,
- Respirable biopersistent rigid High Aspect Ratio Nanomaterials: **HARN**,

- NFs incorporated into a Solid matrix Nano-Enabled Product: **SNEP**.

More details on these hypotheses are described in section 4.3 and Table 5.

If the NF(s) fall into one of these hypothesis groups, a check is imposed to assess whether this group is sufficiently substantiated for the defined purpose., For example, the group 'dermal exposure to NFs larger than 5 nm' may be sufficiently substantiated for regulatory decisions or be more indicative for use in targeted testing, precautionary measures and SbD. Within a regulatory context, possible consequences include read-across and waiving. Within a precautionary context a possible consequence is to limit the exposure, while for SbD the consequence is generally to re-design the NEP product or NF.

The generation of a basic or refined hypothesis consists of the description of the group and a prediction of where they are expected to go (environmental fate and human kinetics) and/or what they are expected to do (toxicity). Especially, for the group description, but also for the other parts of the hypothesis, information on the life cycle of the NF, including the intended use, expected release and potential exposure to the environment and human compartments, is used.

3.3 Using the framework for Safe-by-Design in the different *Stage-Gates*

Grouping and read-across can be used in all stages of the innovation process, although certain parts or levels of the draft GRACIOUS Framework are more useful in the early stages, whereas other parts are more useful in the later stages of the innovation process (see Figure 5). In the first two stages of the *Stage-Gate* Idea-to-Launch process, existing knowledge can be used to select NFs for which grouping or read-across for regulatory use is expected to be possible and efficient, e.g. to select a NF for which read-across to another (data rich) NF seems feasible. Level 1b and 1c hypotheses can facilitate in the selection of the NFs as well, where they are also useful in decisions to either "*Go on"* or reconsider building a business case (stage 2), developing (stage 3) or testing and validation (stage 4). The Level 2 refined hypotheses are probably most helpful in the development (stage 3) and testing and validation (stage 4) stages of the innovation process. Whereas the further refined hypothesis in Level 3 will be most helpful in weighing the health and environmental risks against other criteria in the decision to launch (stage 5).

In Figure 5, only the alignment of existing knowledge and the different hypotheses with the different stages within the innovation process are indicated, as the other parts of the framework are used to generate, refine or test the hypothesis. Alignment with *Stage-Gate* thereby ensures the relevance of the Framework to business decision-making, regarding the design of safer NEPs and manufacturing processes.

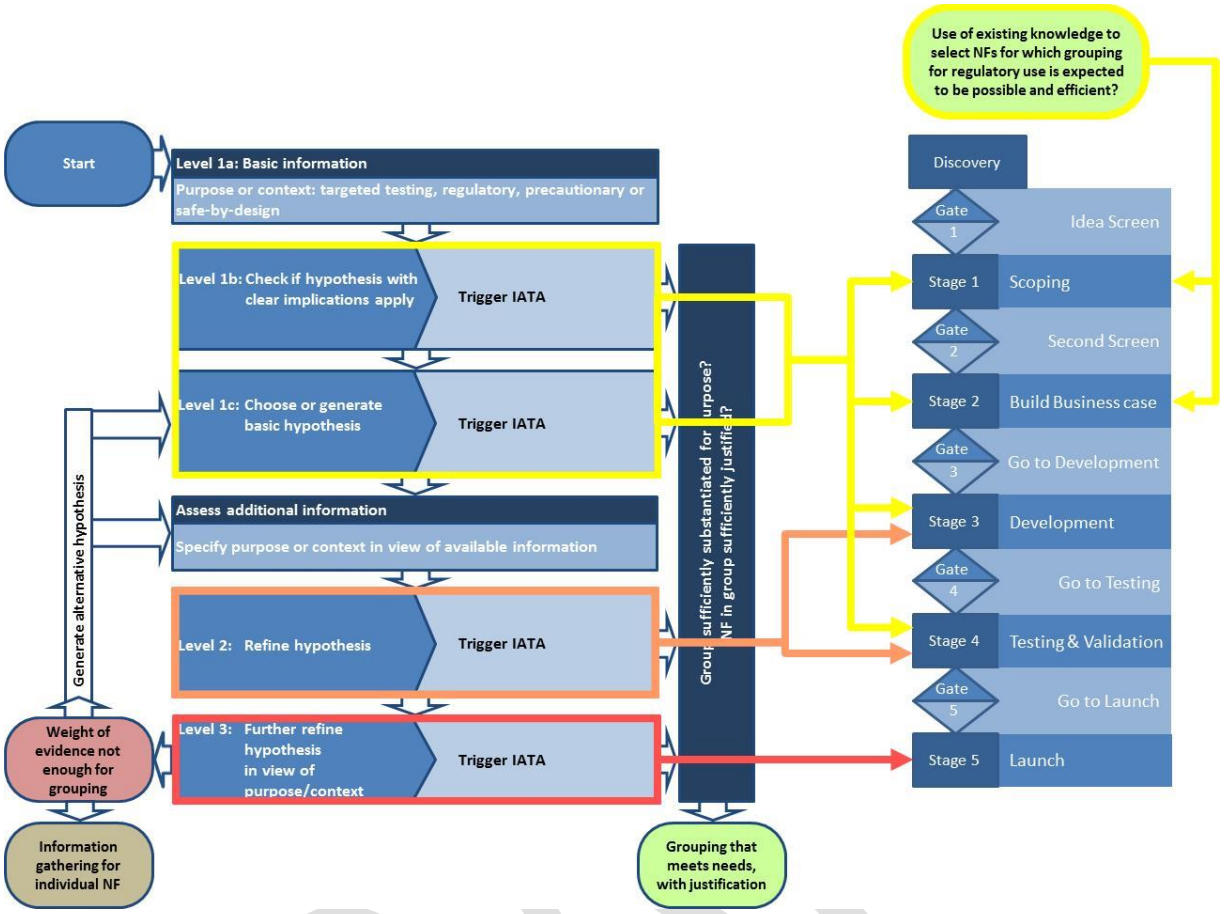

Figure 5. Outline for the relationship between the GRACIOUS Framework and SbD via the Stage-Gate innovation process. The yellow, orange and red boxes indicate which Level of the Framework is most helpful in the different Stages of the Stage-Gate Idea-to-Launch process.

3.4 General considerations on grouping and read-across

It should be noted that multiple hypotheses, i.e. related to different endpoints, can be applicable to the same NF. This also means that the group to which a specific NF belongs, can differ per grouping or read-across application. Read-across for hazard in REACH and other regulations is endpoint specific. In some cases the same justification for read-across may be applicable to more endpoints, as for example for quickly dissolving NFs. Which endpoints are applicable may be more straightforward and generalizable for the hypotheses with clearer implications (Level 1b), than for Level 1c and further refined hypotheses. In any case, the relevant endpoints should be indicated for each regulatory application of grouping and read-across. For other applications (targeted testing, development of precautionary measures and SbD), it may be relevant to consider which endpoints the grouping or read-across encompasses, as this may facilitate the development of a subsequent grouping or read-across justification for regulatory use, if applicable.

4 Elaboration on specific parts of the framework

Below, the different parts of the draft framework are elaborated in detail.

4.1 Input to the framework

For the purpose of grouping, either a single NF or a provisional group of NFs can be used as input to the framework. In the case of a provisional group of NFs, the data to be generated should allow for interpolation[3] of test results and thereby provide justification of the group. Such a provisional group may consist of several NFs that are likely to form a group, e.g. based on their similarity in physicochemical properties and intended use, for example for NFs that only differ in size, because the same production process is used under slightly different conditions. Yet, further data are needed to substantiate the group or to justify that a certain structure-activity relationship is valid. By identification of a provisional group, information can be gathered on a few NFs that can cover the entire group by interpolation of test results. Developing such a group of similar NFs may thus substantially increase the efficiency of information gathering for risk assessment of these NFs.

During the entire process users will need to consider if applying the framework to acquire the required information via grouping or read-across will be the most effective approach (i.e. saving time, costs and/or test animals). In particular, where the substantiation of grouping or read-across would require considerable testing, gathering the required information for each individual NF under consideration via direct testing may be more efficient in time or costs needed.

4.2 Framework Level 1a: Basic information

In order to identify or generate the initial Level 1 hypothesis, basic information on the NF(s) is essential, including:

- "What they are"
  - **Basic physicochemical information** on the pristine NF: targeted properties (when using the GRACIOUS framework for *Stage-Gate* screening of novel materials) or measured properties (for other purposes)
- "Uses in the lifecycle that lead to environmental release and human exposure"
  - **Intended use and physical form** (e.g. solid, suspension, aerosol) of the NF or NEP, including how the NF is incorporated into the matrix of the NEP (e.g. matrix-embedded or mixed powder).
  - **Anticipated relevant exposure route(s) to humans and/or relevant environmental compartment(s)**

The physicochemical characterization allows assessment of already available information on hazard (what they do) or fate/kinetics (where they go) of the NF (s), e.g. in literature and may inform on the need for grouping approaches or highlight specific (potential) endpoints of interest. The basic information is also needed to assess to which group in Level 1b the NF(s) belong and/or for generation of a grouping hypothesis in Level 1c. Finally, the basic information is used to initially identify similar materials for which information is available and that can be used either as a representative test material for testing and/or as a source material for read-across.

The precondition of the availability of basic physicochemical information on the material for grouping and read-across is in line with regulations such as REACH, where information on substance identity is

[3] Interpolation tries to find the values between two or more known data points.

required for read-across (ECHA, 2017d). Please note that this basic physicochemical information should in fact be available for any regulatory dossier to identify or characterise a NF, regardless of the application of grouping and read-across.

4.2.1 Basic physicochemical information

There are numerous sources of information on basic information requirements for NFs, including regulatory guidance (ECHA, 2017b, c), reviews (especially ProSafe on methods (Steinhäuser and Sayre, 2017) and on properties used in frameworks (Oomen et al., 2018)) and project documents (especially NANoREG and specifically for grouping nanoGRAVUR).

Within the GRACIOUS project, the following physicochemical properties are required as basic information (Level 1a) for the pristine material, consisting mainly of intrinsic properties:

- Composition incl. impurities and additives and endotoxin content
  - supported by crystallinity, and ion content of suspensions (both seen as a means to describe impurities)
- Constituent particle size distribution
- Constituent particle shape
- Chemical nature of the surface (this includes basic information on surface coatings and functionalisations when applicable; also referred to as surface chemistry)
- Specific surface area
- Water solubility

Further information on physicochemical characterisation that may inform the IATA is described below in section 4.4.1. Information on tools, methods and protocols to determine these physicochemical properties is provided in section 6.1.

4.2.2 Intended use (including physical form), relevant exposure route(s) and/or environmental compartment(s)

The initial step in the process of understanding the potential safety implications associated with each NEP is to provide a comprehensive pathway analysis along and beyond the product value chain. The basis of this pathway analysis will be the information available about the specific NF, the product and the processes that these two pass through during the whole life cycle of the NEP, see Figure 6.

A general diagram is proposed to generate conceptual maps for the life cycle of each NEP. This diagram will describe the case study considering the following life cycle stages:

1) NF synthesis
2) Incorporation of NF into the product (NEP manufacturing)
3) Use phase
4) Recycling and
5) End-of-life

These diagrams link life cycle stages, potential release scenarios (defined as those activities from which release is highly probable to occur), receptors (i.e. human, water, soil, air, biota) and technological compartments (waste treatment plants, incinerators, landfill sites).

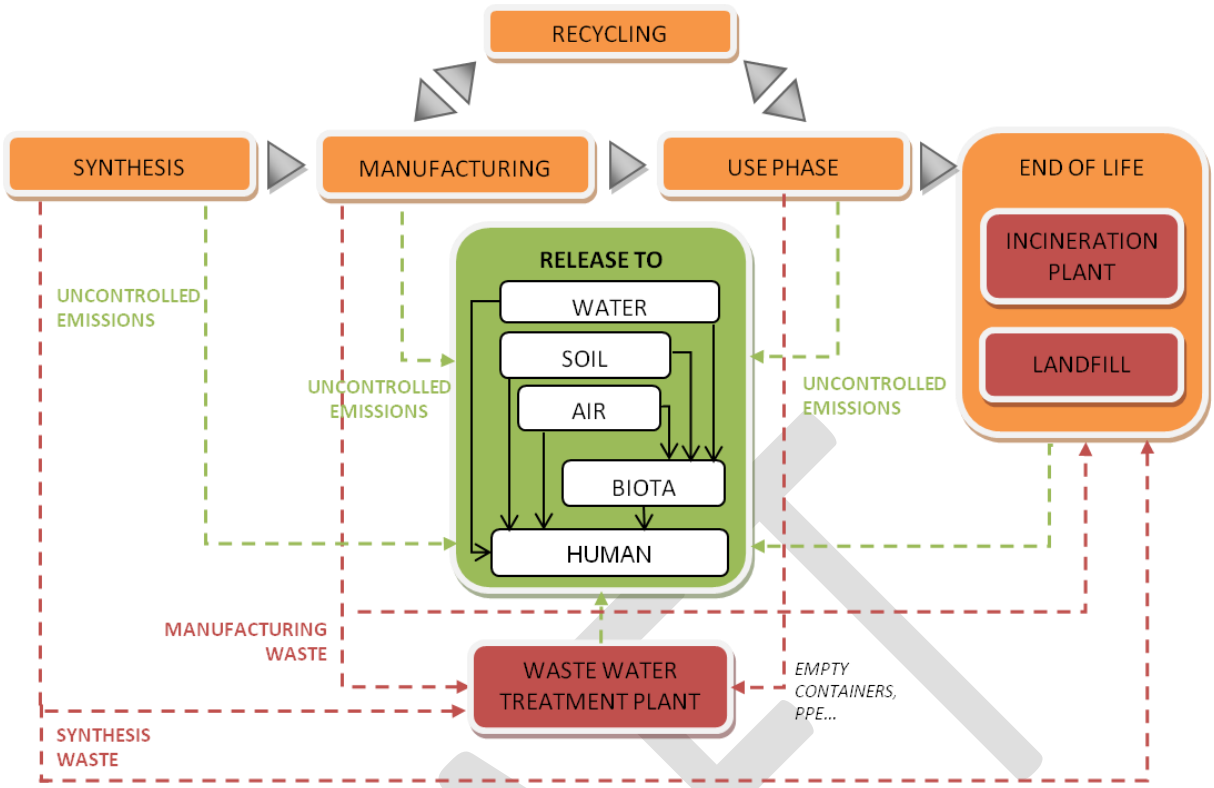

Figure 6: Product life cycle stages and potential release flows.

For the basic information in Level 1a of the Framework the aim is to provide information on the intended use, composition of the matrix and anticipated production process including physical state (e.g. powder, suspension, spray, paste, solid composite), release potential, (most) relevant environmental compartment(s), anticipated release form (single particles, agglomerates, attached or bonded to other substances, etc.) and most relevant human and environmental exposure routes. ECHA has developed a system to describe uses, the so-called Use Descriptor System (UDS; ECHA, 2015a). It will be investigated how this system can be used in the identification of intended uses.

4.3 Framework Level 1b: Check if hypotheses with clear implications apply

Some hypotheses, once applicable to the situation, the intention of the user, and once they are sufficiently justified, lead to grouping that, with a limited set of information, can be substantiated for a broader range of endpoints. Examples of groups with potentially clear implications are described in Table 5. For example, for a NF that falls within the group “quickly dissolving NFs”, read-across from the corresponding solute (ions, molecules) should be possible for any endpoint, including human health and environmental endpoints. If such a read-across approach is to be used in a regulatory framework, an argument should be developed that read-across is scientifically correct. Information on the dissolution rate in relevant media may be sufficient.

To aid the user and increase the efficiency in coming to a suitable grouping, Level 1b therefore directs the user to the options in Table 2 at an early stage in the framework. Each hypothesis is linked to a corresponding Integrated Approach to Testing and Assessment (IATA) in order to guide the work needed to justify the proposed grouping. If these hypotheses with clear implications do not apply, the user proceeds to Level 1c, in which a basic hypothesis (other than the ones in Level 1a) is generated.

Table 2: Non-exhaustive list of examples for Level 1b hypotheses that can lead to a grouping that can relatively quickly be substantiated sufficiently to meet the needs of the user. For each hypothesis, the potential consequences of the material fitting into the group are given, along with suggested relevant IATA(s).

Group description and hypothesis	Potential implications/consequences	Relevant testing (in IATA where appropriate)
Quickly dissolving NFs (DISS): NF will quickly transform to the ionic or molecular form and have the same fate, kinetic and toxicity profile as the ionic or molecular form. *Scientific rationale:* Exposure to and uptake of the NF is negligible.	*Regulatory:* Read-across to the ionic or molecular form may be possible (in subsequent Level).	• Dissolution rate and transformation in water and relevant media.
Respirable biopersistent rigid High Aspect Ratio NFs (HARN): NF will translocate to the pleural membrane and lead to frustrated phagocytosis (uptake and clearance) by macrophages (immune cells) that subsequently can cause mesothelioma (cancer of pleural cavity around lungs). *Scientific/clinical rationale:* Mesothelioma.	*Precautionary approaches or safe-by-design:* Prevent/minimize exposure, or modify the NF/NEP to reduce hazard. *Targeted testing:* Testing to assess concerns. *Regulatory:* Read-across to asbestos (Level 1), or another rigid HARN (in subsequent Level) may be possible.	• Dissolution in fluids representative of lung lining and lysosomal fluid. • *In vitro* assessment of frustrated phagocytosis. • *In vitro* assessment of pro-inflammatory, pro-proliferative and genotoxic potential. • In later tiers (if applicable): *in vivo* translocation, *in vivo* inflammation and/or mesothelial cell proliferation.
NFs larger than 5 nm (D5NM): NF will not translocate across skin. *Scientific rationale:* If there is no translocation across intact skin in case of dermal exposure, systemic exposure via skin will not occur.	*Regulatory:* Waiving of endpoints related to systemic exposure.	• Size of the NF in relevant media • Translocation studies across skin (*in vitro*, *ex vivo*).
NFs which are incorporated into a solid matrix (SNEP): NF will be released as free NF depending on the use/aging process & matrix. *Scientific rationale:* The probability and form of release is mainly determined by the type of matrix, dispersed state of the NF in the matrix and use or aging process.	*Precautionary approaches or safe-by-design:* Control-banding (Level 1), minimize exposure or adjustment of NEP. *Targeted testing:* Testing to assess concerns.	• Incorporation of NF into the matrix of the NEP (g/g content, disperse state) • Resilience of matrix under relevant conditions • Forms of release from NEP under relevant conditions

4.4 Framework Level 1c: Generate basic hypothesis

If no hypothesis with clear implications is applicable, a basic hypothesis is generated considering the Level 1a information and the intention of the user of the grouping framework, i.e. what is the specific

purpose of grouping different NFs. To facilitate the user, a series of potential Level 1c hypotheses have been elaborated in Table 3. Also potential consequences of such grouping are presented. GRACIOUS D4.2 provides a more complete list of hypotheses along with a template that facilitates generation of a new hypothesis if required.

Table 3: Non-exhaustive list of potential Level 1c hypotheses for grouping. For each hypothesis, the potential consequences of the fact that the material fits in the group and suggested relevant IATA(s) are given. GRACIOUS D4.2 provides a more complete list of hypotheses.

Group description and hypothesis	**Potential implications**	**Relevant testing (in IATA where appropriate)**
NFs with low dissolution rate and chemical composition of low toxicity: NF will accumulate in humans and the environment and may lead to increase of the likelihood for long term toxicity after chronic exposure. *Scientific rationale:* These NFs will show low solubility particle behaviour and toxicity.	*Targeted testing:* Testing to address accumulation and long term toxicity can be performed to reduce concerns. *Regulatory:* Read-across to other poorly soluble passive NFs may be possible (in subsequent Level).	• Dissolution rate in water and relevant media. • Deposition in alveoli by MPPD modelling (in case of exposure via inhalation). • Reactivity.
NFs with low dissolution rate and specific toxicity: NF will accumulate in humans and the environment and may increase the likelihood for long term toxicity after chronic exposure as well as specific toxicity related to the active nature of the NF. *Scientific rationale:* These NFs will show particle behaviour and toxicity as well as specific toxicity due to active nature.	*Targeted testing:* Testing to address active nature, accumulation and long term toxicity can be performed to reduce concerns. *Regulatory:* Read-across to other similarly poorly soluble active NFs may be possible (in subsequent Level).	• Dissolution rate in water and relevant media. (including ion release of impurities) • Transformation in relevant media • Deposition in alveoli by MPPD modelling (in case of exposure via inhalation). • Mobility in soils • Dispersion stability in aquatic phase • Reactivity.
Moderately dissolving NFs: NF will partly transform in the molecular or ionic form and partly be taken up as NFs leading to fate, kinetic and toxicity similar to the ionic or molecular form and similar to poorly soluble nanoparticles. *Scientific rationale:* Toxicity due to both ion shedding and particle characteristics is possible. Effects due to ion/molecule shedding at specific sites (after distribution in body or environment) should be considered.	*Targeted testing:* Testing to address/reduce concerns related to effects of ions/molecules and poorly soluble nanoparticles. *Regulatory:* Read-across to NFs that shed similar ions/molecules may be possible (in subsequent Level).	• Dissolution rate in water and relevant media. • Transformation in relevant media

Group description and hypothesis	Potential implications	Relevant testing (in IATA where appropriate)
Biopersistent NFs that are neither charged nor sterically stabilized whilst released to the aquatic environment: NF will show quick sedimentation in environment making aquatic toxicity less relevant. *Scientific rationale:* Negligible concentrations of NFs in the aquatic environment.	*Targeted testing:* Focus on sediment dwelling organisms. *Regulatory:* Potential waiving of testing of aquatic species.	• Hetero-agglomeration, attachment efficiency assays. • Zeta potential. • Sedimentation rate (dispersion stability).
NFs with a common core which show rapid dissolution of a coating/shell: NF will show similar fate, kinetics and toxicity compared to similar non-coated NFs. *Scientific rationale:* Exposure is essentially to the core material.	*Regulatory:* Read-across to the common NF (core). Precondition: the coating/shell does not impact fate or toxicokinetic behaviour and does not cause any effect itself.	• Dissolution rate in water and relevant media. • Solute IATA of shell

The basic hypothesis triggers a tailored IATA, which should allow the Level 1c hypothesis to be accepted, rejected, or to provide information for further refinement. Several options for testing are suggested in Table 2 and Table 3. These will be part of IATAs that will be further detailed and elaborated throughout the duration of the GRACIOUS project.

4.4.1 'What they are'-IATA

Physicochemical characterisation of NFs and its relevance to risk is further advanced than other IATAs as it can exploit to a larger extent existing knowledge. A starting point for the IATA on physicochemical properties ('What they are'-IATA) is therefore already developed in the present GRACIOUS draft framework. The "intended use" and the "anticipated relevant exposure route(s) to humans and/or relevant environmental compartment(s)" allow deriving "relevant media" for testing extrinsic properties. Initially, the 'What they are'-IATA of GRACIOUS considers the following extrinsic properties:

- Density (differentiating tap powder density and skeletal density)
- Surface hydrophobicity
- Surface charge (in water or in relevant media, or both),
- Dissolution rate in relevant media (see methods in section 6.1)
- Dispersibility (including agglomeration and dispersion stability) in relevant media
- Dustiness
- Biological reactivity (for the final framework, we will chose which abiotic method correlates best and will adapt the name of the property accordingly. As a result, one could restrict the scope to "ROS creation", or specify by methodology)

ECHA also proposes photoreactivity as a property for grouping (ECHA, 2017b), but this is not experimentally addressed in GRACIOUS. Surface chemistry is (partly) a system dependent parameter (extrinsic parameter) as for example the zeta potential and presence of hydroxyl groups depends on the external conditions. The conditions to report on the surface chemistry may need to be described and preferably standardized. The ECHA best practice on NF registration does not actually require a measurement of surface chemistry. Disclosing the identity of surface modifiers is sufficient (ECHA,

2017c). GRACIOUS will perform measurements and consider if such data is useful for the final 'What they are'-IATA.

Size distribution and specific surface area are related properties. However, when the calculation of the specific surface area based on size distribution does not match with the measured specific surface area, this provides valuable information. The particles may not be spherical, there may be pores/spaces or either of the two has not been measured accurately. Therefore, it is recommended to determine both size distribution and specific surface area.

These listed properties, including the ones described under basic information (section 4.2.1), are recommended in the ECHA approach on grouping and read-across for NFs but are not all mandatory under REACH (ECHA, 2017b). The OECD sponsorship program documents contained some more intrinsic and less extrinsic properties, but were not exclusively focused on grouping purposes. The list is overall in line with the DF4nanoGrouping approach (Arts et al., 2015) which however does not use all the above to come to grouping decisions. For instance, surface charge and hydrophobicity are not used by DF4nanoGrouping, which instead uses the "functional assay" strategy of measuring agglomeration and reactivity directly. In task 3.2, JRC has crosschecked the physicochemical properties requested in the legal text of REACH (including the revised Annexes;EU, 2018) and recommended in ECHA documents, and confirmed the appropriateness of the 13 physicochemical properties listed above.

Additional properties need to be taken into consideration to link the 'what they are'-IATA with the information on life-cycle. These were not included in the ECHA selection of properties, which did not include properties related to emission, release and exposure. The expansion of the DF4nanoGrouping framework from occupational to also consumer and environmental grouping purposes in the nanoGRAVUR framework (2015 – 2018) uses additional extrinsic properties as represented in Table 4.

Table 4: Overview of physicochemical information requirements in the ECHA (ECHA, 2017b) and DF4nanoGrouping (Arts et al., 2015) frameworks and the more comprehensive 2018 nanoGRAVUR framework. Properties related to "what they are" and "what is the NEP" in the first rows (indicated in blue), followed by properties related to "where they go: release/exposure" in orange, properties related to "where they go: in relevant media" in purple, and at the bottom lines properties related to "what they do" in brown.

Properties	ECHA guidance 2017 [2]	DF4nanoGrouping 2015 [2]	NanoGRAVUR [2] (O) Occupation (E) Environment (C) Consumer
Constituent particle shape	Proposed	Criterion	Criterion O,C,E
Constituent particle dimension	Proposed		Criterion O,C,E
Composition GHS [1] (including impurities)	Proposed	Criterion	Criterion O,C,E
Specific surface area (BET/VSSA)	Proposed	Supplementary	
Surface Chemistry (descriptive)	Proposed	Supplementary	
Surface Charge (zeta-potential)	Proposed	Supplementary	
Hydrophobicity	Proposed	Supplementary	

Properties	ECHA guidance 2017 [2]	DF4nanoGrouping 2015 [2]	NanoGRAVUR [2] (O) Occupation (E) Environment (C) Consumer
Rigidity (for fibres)	Proposed		Proposed O
NEP classes & intended use scenarios			Criterion (NEP) O,C
Specific NEP: g/g content of NM			Criterion (NEP) O
Specific NEP: dispersion state of NM			Criterion (NEP) O,C
Dustiness	Proposed	Qualifier	Criterion O
Critical shapes upon exposure			Criterion (NEP) O,C
Agglomeration of NM upon NEP application			Criterion (NEP) O,C
Resilience of NEP Matrix			Criterion (NEP) O,C
Dispersability (dispersion stability: TG318)	Proposed	Criterion	Criterion O,C,E
Solubility in water (screening test)	Proposed	Criterion	Criterion O,C,E
Dissolution rate in relevant media	Proposed	Criterion	Criterion O,C,E
Ion releasing			Criterion E
Transformation "change of what they are"			Criterion E
Mobility (in soils)			Criterion E
Mobility (systemic) by alternative method			Criterion C
Affinity (heteroagglomeration)			Proposed E
Reactivity (abiotic)	Proposed	Criterion	Criterion O,C,E
Reactivity (*in vitro*)	?	Criterion	Criterion O,C
Reactivity (photo-)	Proposed		

1 GHS: classification of substance and impurities as by the Globally Harmonized System (GHS).
2 Proposed: Property is not mandatory, but proposed for decision-making
Criterion: Property with quantitative cut-off for decision-making
Supplementary: Property without use in decision-making
Qualifier: Required to select appropriate conditions in further testing

The additional properties were considered to be especially useful for the environmental and value-chain perspectives: 6 additional properties may enable grouping hypotheses based on the NEP and lifecycle (top right, blue + orange); another 3 additional properties may enable the UBA grouping framework based on environmental fate and hazard (Hund-Rinke et al., 2018), with the following hypotheses:

- Based on “ion-releasing” together with “composition (incl. impurities)” the hypothesis/IATA related to dissolution can be triggered to determine adverse effects of dissolved ions, which may eventually dominate especially the ecotoxicity hazard.
- By “transformation (change of what they are)”, the IATA related to newly formed particulate species is triggered that adds to or replaces the pristine NFs. Mitrano et al. stressed that both an assimilation of different NFs to the same transformation product (and thus a hypothesis for grouping) or diversification of one NF into several transformation products may occur (Mitrano et al., 2015).
- The form of release (which relates to a hypothesis of grouping of different NEPs) can be modelled based on the “specific NEP: g/g content of NF”, “specific NEP: disperse state of NF”, “resilience of NEP matrix”. This will be supported by the description of intended use.
- The similarity of “mobility (in soils)” can substantiate the grouping of NFs for environmental fate.

Finally, information on physico-chemical properties, human toxicity and/or ecotoxicity from the non-NF may be relevant input to targeted testing, but are not in themselves grouping criteria.

Considering that the DF4nanoGrouping and the ECHA frameworks did not elaborate environmental hypotheses or emission/release/exposure hypotheses, it may be necessary to consider such additional properties for GRACIOUS. GRACIOUS will explore the validity of these descriptors, which may (or may not) be selected to incorporate in the final ‘What they are’-IATA.

Table 4 also indicates that the DF4nanoGrouping and nanoGRAVUR frameworks attributed low predictive relevance to properties such as specific surface area or zeta potential, and do not use them to come to grouping decisions. This may or may not be revised in the final GRACIOUS framework.

4.5 Assess information

After Level 1c a new assessment will need to be done to evaluate the substantiation of the grouping hypothesis so far. In many cases this will show that Level 1c groupings will not be specific enough to address the needs of the user, and further refinement in Level 2 is necessary.

4.6 Framework Level 2: Generate refined hypothesis

Level 2 of the framework can be used for refinement of the grouping hypothesis as described in Level 1, if the similarity is deemed insufficiently substantiated for the purpose. Further discussion to support the discussion when a grouping is “sufficiently substantiated” is needed.

With the available information from Level 1 and knowledge on potential source materials[4], it is expected that the generation of a hypothesis resulting in read-across that can be used in a regulatory dossier, is more likely in Level 2 than in Level 1 of the framework. When such regulatory application is intended, the user should assess for which endpoints further information is needed, as required by the applicable regulation, which may be addressed by read-across. The read-across justification should be provided per endpoint, which may differ in hypothesis and source materials (see Figure 7). The hypothesis can involve a comparison of fate/toxicokinetic behaviour (“where they go”) related to the endpoint (e.g. will the target site be reached in a similar way) between the source material and the ‘test’ NF (also referred

[4] A source material is a material for which a known property or hazard can be used to estimate the same property or hazard for a target chemical to fill a data gap for that target material (OECD, 2014).

to a target material), in combination with a comparison of hazard also related to the endpoint (e.g. will the same mechanism be affected). In the IATA corresponding to Level 2 of the framework, this comparison would be based on *in vitro* and *in silico* methods. Hence, these *in vitro* and *in silico* methods should allow for a correct ranking, in view of the *in vivo* situation, of the fate/toxicokinetic behaviour and hazard of the materials investigated.

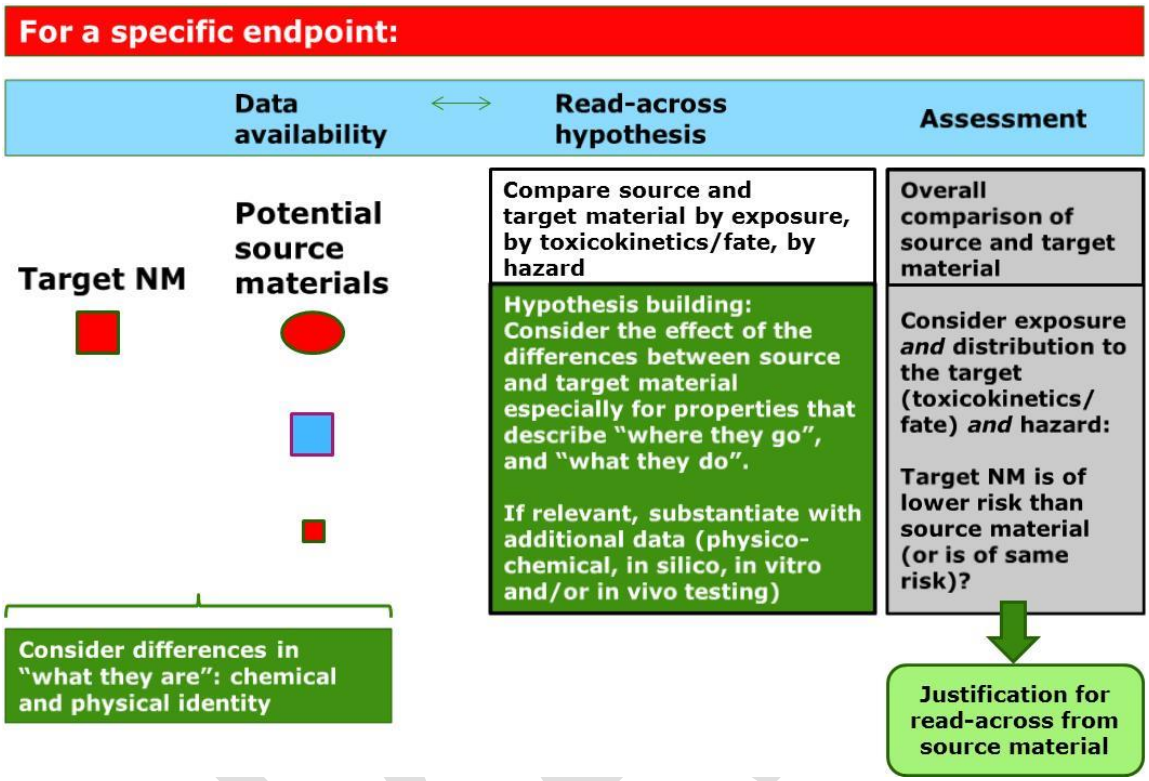

Figure 7: Approaches on developing a read-across justification for one or more NFs for a specific endpoint (Figure adapted from Oomen et al., 2015, with permission). Hypothesis development for read-across justification and identification of potential source materials are related processes. The justification can be substantiated by arguing that a very similar or smaller amount of the target material reaches the target site, and the target material is equally or less hazardous than the source material. This justification can be based on available knowledge on relationships between physicochemical properties and exposure, toxicokinetics/fate and hazard and may be complemented with physicochemical data, in silico, in vitro or if needed in vivo data.

When QSARs on NFs that are accepted for regulatory decision making (see section 6.5.1) are available, these may also be used to fill in data gaps for specific endpoints.

In an occupational setting, grouping according to type of activity (e.g. bagging) may be used to estimate the potential life-cycle transformations of the NF, i.e. as primary particle, aggregated/agglomerated, attached or bonded to other substances, as well as an indication of the level of exposure. The amount of NF being handled, the exposure duration and the presence of exposure controls can be used to develop a more refined hypothesis on the level of exposure. The tonnage produced over a year could be used to group the level of release to the environment. For consumer exposure from products, the product category and application will yield information on the exposure route and population group exposed. For construction materials that are aged by weathering leading to environmental release, the type of matrix and years of use are key factors. Such grouping factors will help to clarify where NFs will go, the properties of the NF released (single particles, embedded in a matrix, agglomerates) and an indication on the release or exposure level.

Table 5. Non-exhaustive list of potential Level 2 refinements of hypotheses for grouping. For each hypothesis, the potential consequences of material fitting in the group is given along with suggested relevant IATA(s).

Group description and hypothesis	**Potential implications**	**Relevant testing (in IATA where appropriate)**
Further refinement of Level 1c hypothesis		
Read-across: in general (see also Figure 7)		
Target material (= NF entering framework) is of lower risk than source material	*Regulatory:* Read-across from data of another NF, endpoint specific. E.g., combine argument on 'where they go' (i.e. same or smaller amount of NF reaches target site than source material) and argument on 'what they do' (i.e. hazard of NF is same or less than source material). Data explaining the mechanism* are valuable to support that the same hazard endpoints are applicable.	• Dissolution in lung lining/gastrointestinal tract fluid and lysosomal fluid. • Deposition in alveoli by MPPD model. • *In vitro* reactivity, cytotoxicity and immunotoxicity assays.
Read-across: example		
NFs with low dissolution rate and potency for induction of mesothelioma: NF (= target material) will accumulate to a smaller extent in human lungs and translocate to a smaller extent in the pleural cavity than long fibre amosite asbestos (or another HARN). In addition, the NF is less potent in inducing mesothelioma than source material (asbestos or another HARN).	*Regulatory:* Read-across from data on asbestos or another HARN with data on (early stage of) mesothelioma for endpoint carcinogenicity.	• Dissolution in lung lining/gastrointestinal tract fluid and lysosomal fluid. • Deposition in alveoli by MPPD model. • Translocation to pleural cavity. • Biomarker studies related to mode-of-action of mesothelioma (inflammation, granuloma formation etc).

* Here a link to AOP could be made

4.7 Level 3: Further refine hypothesis in view of purpose/context (in vivo)

It is anticipated that the Level 3 hypothesis is similar to the Level 2 hypothesis, but may be more refined as the Level 2 information is not sufficiently conclusive to address the needs of the user. The topic of the Level 3 IATA may be the same as the Level 2 IATA, but can comprise *in vivo* testing and detailed exposure assessment such as personal exposure estimation. Exposure assessment in Level 3 relies increasingly on actual data relevant to the NF in question than on the estimation via grouping.

4.8 Grouping sufficiently substantiated for purpose?

After gathering of the information as indicated in the relevant IATA, the Framework will guide the user on how to determine whether the NF is indeed part of the hypothesized group. This should also include an assessment of whether the grouping is sufficiently substantiated for the purpose (precautionary, safe-by-design, regulatory, including waiving or read-cross). As a start, Chapter 5 presents aspects to be addressed when assessing if a grouping is “sufficiently substantiated”.

If a grouping is sufficiently substantiated, one can use the proposed grouping as intended.

If the weight of evidence is not enough for grouping, either the required information can be generated for the individual NF, or an alternative grouping hypothesis can be generated.

5 Quality assessment to assess if a hypothesis is sufficiently justified

In this chapter aspects are addressed that are important when evaluating if a hypothesis is sufficiently justified. Obviously, the justification would be more stringent for regulatory purposes as compared to targeted testing, precautionary approaches and SbD. The following section is directed towards the justification for grouping and read-across for regulatory purposes. First, the quality assessment of the available information is considered related to relevance, reliability and adequacy (section 5.1). Secondly, the elements for scientific justification for grouping and read-across of substances are discussed in general, and what changes when grouping and read-across is applied to NFs (section 5.2). Section 5.3 elaborates on criteria to conclude whether a scientific justification is acceptable for substances in general and what changes when grouping and read-across is applied to NFs.

5.1 Quality assessment of the available information under REACH: an overview of legal requirements and recommendations by ECHA

ECHA (2011) requires that the information gathered in the context of Annexes VI-XI of REACH (by means of direct (animal) testing or application of alternative test methods including grouping and read-across) is evaluated in terms of completeness and quality. Quality refers to the relevance, reliability and adequacy of the available data (ECHA, 2011).

The relevance of, for example, a test method describes the relationship between the test and the effect in the target species and whether the method is meaningful and useful for a particular purpose (OECD, 2005). In a shorter definition, Klimisch et al. (1997) state that data are relevant when the “test describes the effect correctly”. According to ECHA, this occurs when the test substance is representative of the registered substance, the test species, route of exposure and dose/concentration are appropriate, and the critical parameters that influence the endpoint under investigation are considered (ECHA, 2011).

Data are adequate when they are useful for the purpose (ECHA, 2011). In the context of REACH, the purpose can be compliance with the information requirements triggered by tonnage in Annexes VII-X, hazard classification including PBT/vPvB assessment, and risk assessment (for example, DNEL/PNEC derivation required in the Chemical Safety Assessment under REACH).

The reliability of a test method is defined by OECD as the extent of reproducibility of the results within and among laboratories over time (OECD, 2005). In order to evaluate the reliability of (eco)toxicological test results, ECHA recommends following the systematic approach developed by Klimisch et al. (1997). Accordingly, (eco)toxicological data are reliable when the studies are conducted according to generally valid and/or internationally accepted test guidelines (for example, OECD, ISO, EU Test Methods Regulation) and in compliance with the principles of Good Laboratory Practice (GLP). Data that are not generated according to an international or national standard but result from a scientifically sound and well documented method (including a clear description of the scope of investigation, test substance and related impurities, test organism, test conditions, applied dose/concentration, use of controls) can also be considered to be reliable. ECHA (2011) underlines that less reliable and unreliable data should not be automatically excluded from further consideration by experts as that data may be used as supporting information in certain situations.

In the case of *in vitro* data, REACH requires that the methods are “suitable”, which means “sufficiently well-developed according to internationally agreed test development criteria” such as the pre-validation criteria defined by ECVAM (Curren et al., 1995; Hartung et al., 2004) or the validation criteria recommended by OECD (2005).

In the case of (Q)SAR data, REACH requires that the "scientific validity" of the model is established and the model used only for substances that fall in its applicability domain. OECD (2007a) provides the criteria to consider to assess whether a (Q)SAR model can be accepted for regulatory purposes.

In the case of data obtained from application of a grouping and read-across approach, although REACH does not make any explicit reference to the need to validate grouping when used to fill data gaps in a dossier, the registrant is required to provide a robust "scientific justification" and demonstrate that the approach is adequate for the regulatory purpose (ECHA, 2011). REACH also requires that "adequate and reliable documentation" is provided (EC, 2006). In this context, Schultz et al. (2015) identified two main sources of uncertainty in a read-across prediction that needs to be accommodated and documented to facilitate regulatory acceptance: 1) the uncertainty associated with the justification of similarity between the source and target substances; and 2) the uncertainty associated with the application of a particular read-across exercise.

5.2 Elements to be documented in a scientific justification for grouping and read-across

In the guidance on grouping of chemicals, ECHA provides the registrant with the steps to follow to develop and verify a grouping hypothesis (ECHA, 2008, 2017d). ECHA also gives clear indications on how the information should be reported in a transparent and systematic way. The main elements to be documented are:

- The **hypothesis**, which includes a description of the similarities defining the chemicals as a group. To this end, it is fundamental that the substance identity (e.g. chemical composition, impurities and crystalline structure) of each group member is well known and documented, otherwise similarities cannot be assessed.
- The **applicability domain** of the group, which includes a description of the inclusion and exclusion rules that provide the ranges of values within which reliable estimations can be made for the group members.
- The **endpoint(s)** covered by the hypothesis.
- The group **members**, including their structure, chemical composition, and impurities.
- The **data matrix** (endpoint(s) vs members). The available data on all physicochemical, toxicological and ecotoxicological properties needs to be gathered for each group member and the data quality evaluated (against relevance, reliability and adequacy). The available data should be organised in a matrix which helps highlight the data gaps and identify both the target chemicals and the possible source chemicals for each endpoint.
- The group **justification**, which is an explanation of how the available experimental data for the members of the group verify the robustness of the whole group. The data should give an indication of the trend(s) in properties across the group members for each endpoint and also show that features which are not in common to all members do not affect the predicted property.
- The **conclusions** for each endpoint and uncertainties that need to be addressed.

If the scientific justification underpinning the grouping approach is robust enough and the available information on the members is sufficient, then read-across can be applied to fill the data gaps for the endpoint(s). If not, testing may be needed to either generate further information supporting the scientific foundations of the grouping approach and justifying read-across or generate the missing data for the target chemicals (in the latter case, grouping and read-across are thus abandoned).

5.2.1 What changes when grouping and read-across is applied to NFs

Similarity based on a wider spectrum of physicochemical properties

When grouping and read-across are applied to different NFs of the same registered substance (instead of different substances) the same elements as described above need to be documented (section 5.2).

A deviation in the elements to be documented is expected for the description of the hypothesis and the group members. This deviation is related to the fact that the concept of similarity underpinning grouping and read-across for NFs does not only rely on information on structure and chemical composition (incl. impurities) but considers a broader range of physicochemical properties (ECHA, 2017b). As a consequence, the identity of the source and target NF(s) falling in the same group needs to be defined and reported in line with the minimum set of information identified by ECHA, which, in addition to chemical composition, impurities and crystalline structure, includes size, shape and surface chemistry (ECHA, 2017c).

5.3 Criteria to conclude whether a scientific justification for grouping and read-across is acceptable

The Read-Across Assessment Framework (RAAF) developed by ECHA provides principles for examining the scientific aspects of a read-across case and assessing whether such a case is compliant with REACH provisions (ECHA, 2017d). It was developed as an internal tool and is intended to be used by experts in ECHA in their dossier evaluation activities (ECHA, 2017d). The RAAF covers read-across predictions of (eco)toxicological effects and environmental fate.

According to ECHA (2017d), the application of the RAAF results in “a structured assessment, which recognises the strengths of the read-across and identifies possible shortcomings in documentation, scientific reasoning and/or supporting evidence. The outcome of the assessment is a conclusion on whether the read-across is scientifically acceptable or not”.

There are two pre-conditions for a read-across case to be assessed and accepted:

- **Substance identity of the registered substance**. ECHA (2017d) states that “unambiguous substance identity, for both the target and the source substance, is a prerequisite for read-across assessment”. Substance identity comprises chemical composition, including any other constituent such as impurities, and structural information.
- **Sufficient documentation**. The main elements are listed in section 5.2 (ECHA, 2008).

In the RAAF document (ECHA, 2017d), six read-across “scenarios” are described, which are based on the most frequently applied read-across hypothesis in REACH registration dossiers. The scenarios result from the combination of three aspects:

- The grouping and read-across **approach**;
- The read-across **hypothesis** choice, which is either “(bio)transformation to common compound(s)” or “different compound(s) that have the same type of effect(s)”; and
- Whether quantitative **variations** in the predictions are observed according to a regular pattern.

Each scenario is characterised by “assessment elements” (AEs), which address different scientific aspects deemed crucial to judge the adequacy and scientific robustness of that scenario. Each AE consists of a number of questions. Each AE starts with the question on whether the scientific aspect of the AE has been addressed. If the answer is yes, then the adequacy and scientific robustness of the supporting evidence needs to be assessed. A conclusion is derived by choosing one of a predefined set of “assessment options” (AOs) accompanied by a justification (ranging from acceptable with high, medium or just sufficient confidence to not acceptable). The outcome of the assessment is based on the conclusions derived for all the AEs.

The supporting evidence may range from theoretical considerations to results from experimental studies. The supporting evidence needs to be “sufficient” but no rules are provided for the type of

supporting evidence due to the diversity of properties and possible explanations (ECHA, 2017d). The following indications are given:

- Toxicokinetic data and quantitative mechanistic toxicological data are valuable supporting evidence for read-across predictions of toxicological properties.
- For read-across predictions of environmental fate and ecotoxicological properties, data on interrelated endpoints (e.g. degradation, bioaccumulation) constitute valuable supporting evidence.
- *In vitro* and *in silico* studies may increase the robustness of the scenario but are usually not sufficient as standalone information.
- A data matrix is useful to outline consistency of information within a given scenario and exclude contradictions.
- Available studies for the target chemical are of specific importance. However, the hypothesis must be endpoint-specific and information on other properties is not sufficient to justify a read-across case for the one under investigation.

The six scenarios have some common AEs that must be assessed. They concern the following scientific aspects:

- The identity and characterisation of both source and target substances
- Structural similarities and differences within the group
- The link of structural similarities and differences with the proposed prediction
- Consistency of properties in the data matrix
- Reliability and adequacy of the source data (see section 5.1)
- Bias that influences the prediction

Bias that influences the prediction mainly refers to the selection of the source substance(s). Documentation must be provided on how the source substance(s) has (have) been chosen and what other substances were considered and why they were discarded (ECHA, 2017d).

Other AEs are specific to the hypothesis option and the type of property addressed (human health effects or environmental fate and effects).

5.3.1 What changes when grouping and read-across is applied to NFs

Worth et al. (2017a) applied the ECHA RAAF (ECHA, 2017d) to identify and summarise the different sources of uncertainty associated with two read-across case studies: nano-TiO_2 and MWCNTs. This exercise was also aimed at evaluating the applicability of the RAAF to NFs and proposing specific adaptations. The RAAF was considered in general applicable to NFs and Scenario 6 (category approach, common compound(s), no variation in properties) was chosen for both case-studies. The general conclusions were that: (1) the RAAF criterion for structural similarity should be extended to nanoforms by considering nanospecific properties regarding identification, behaviour and reactivity (although for soluble NFs the similarity in chemical structure may be applied); (2) when dealing with the compound an organism is exposed to, not only the parent/(bio)transformed compound has to be taken into consideration, but also the NM as such, or its coating/impurities. In general, the analysis also underlines that the variability in the available data on NM characterisation and the variability in the applied toxicity protocols hampers linking the NM characteristics to the effects (Worth et al., 2017a; Lamon et al., 2018).

Both target and source NFs must be clearly identified

As pointed out by (Worth et al., 2017a), in the case of a read-across case applied to the NFs of a registered substance, the pre-condition on substance identity needs to be extended to each NF. Chemical composition (incl. impurities) and structural information are not sufficient. ECHA recently suggested a minimum set of information which, in addition to chemical composition, impurities and crystalline structure, includes size, shape and surface chemistry (ECHA, 2017c). This set of information

should be available for each source and target NF to enable the assessment of similarities and differences within the group.

The read-across hypothesis may need to be extended

The two RAAF options for a read-across hypothesis, "(bio)transformation to common compound(s)" and "different compound(s) that have the same type of effect(s)", are in principle applicable to the case of a read-across between (nano)forms. For example, this hypothesis was chosen for both TiO_2 and CNTs (Worth et al., 2017a). However, the concept of "compound" as used in the two RAAF hypotheses may need to be extended to account for the case of (bio)transformation to a common (nano)form and of different (nano)forms of the same substance showing the same effect (Worth et al., 2017a).

A preliminary attempt has been made to compare the four "grouping hypotheses with clear implications" illustrated in Table 2 (section 4.3) with the two RAAF options to verify whether the GRACIOUS hypotheses are covered:

- The "Quickly dissolving NFs" hypothesis is based on the assumption that the NF(s) quickly dissolve into the ionic or molecular form and have therefore the same fate, kinetic and toxicity profile as the ionic or molecular form. This hypothesis seems to be covered by the "(bio)transformation to common (nano)form(s)" option.
- The "Respirable Biopersistent Rigid High Aspect Ratio NFs" hypothesis is based on the assumption that the NFs sharing these properties are similar enough to show the same toxicokinetic and toxicological behaviour (translocation to the pleural membrane and frustrated phagocytosis by macrophages with potential of causing mesothelioma). This hypothesis seems to be covered by the "different (nano)form(s) that have the same type of effect(s)" option.
- The "NFs larger than 5 nm" hypothesis is based on the assumption that the NFs sharing the property of having size larger than 5 nm show the same toxicokinetic behaviour (no translocation across skin, no systemic exposure via the skin). This hypothesis seems to be covered by the "different (nano)form(s) that have the same type of effect(s)" option.
- The "Nanoforms incorporated into a solid matrix" hypothesis assumes that NFs incorporated into a solid matrix are released over time in similar forms depending on the intended use, the associated aging process and the nature of the matrix. The hypothesis might be covered by the "(bio)transformation to common (nano)form(s)" option, or by waiving of information requirements due to absence of release of NFs and thus exposure for e.g. consumers (to be further discussed).

Evaluation of reliability needs nanospecific criteria

ECHA recommends that each study result used in a read-across case (either to justify read-across or to predict the missing data for the target substance) is evaluated in terms of relevance, reliability and adequacy. For chemicals, reliability is defined by the OECD (2005) and under REACH is generally addressed by applying the Klimisch criteria (Klimisch et al., 1997) as implemented in the ToxRTool (Schneider et al., 2009).

In the case of data obtained from (eco)toxicological studies with NFs, reliability cannot be evaluated based only on study design and related documentation. The data needs to also be accompanied by a complete and adequate physicochemical characterisation of the material(s) used in the test (as pristine material(s) and in the test media). This information is of utmost importance to compare studies and identify the parameters that might influence toxicity (Card and Magnuson, 2010).

To this end, several evaluation methods of data quality have been published in the literature. In GRACIOUS, the following evaluation schemes are considered:

- For human health risk assessment:
  - Two-step process by Card and Magnuson (2010);
  - DaNa Literature Criteria Checklist (DaNa, 2016); and
  - GUIDEnano quality assessment approach (Fernandez-Cruz et al., 2018).
- For environmental risk assessment:

- NanoCRED evaluation method (Hartmann et al., 2017); and
- GUIDEnano quality assessment approach (Fernandez-Cruz et al., 2018).

In brief, concerning the evaluation methods for toxicological data, the two methods developed by DaNa and GUIDEnano follow a list of criteria to verify the reliability of the study. Even if criteria may be phrased and grouped differently, nearly the same aspects are covered, which are the physicochemical characterisation of the material and the study design (including sample preparation, test organism, test parameters, result documentation). The following differences have been identified:

- A couple of aspects that are assessed by DaNa seem not to be explicitly addressed by GUIDEnano: the overload/non-overload conditions in relation to the test dose and the compliance with a standard (for instance, OECD Test Guidelines, SOPs).
- The list of physicochemical properties of NFs considered by GUIDEnano (size, surface area, shape, surface charge, surface reactivity, and cluster formation - in addition to composition, impurities and surface chemistry which is basic information in substance identity) is similar to the one provided by DaNa (chemical composition incl. impurities, particle size, specific surface area, surface chemistry and shape).
- GUIDEnano includes a scoring system, which is an evolution of the two-step process proposed by Card and Magnuson (2010).

Concerning the evaluation methods for ecotoxicological data, GUIDEnano criteria cover or overlap with many of the NanoCRED criteria, which are however more thorough and detailed. The following aspects seem not to be explicitly considered in GUIDEnano:

- Compliance with a standard (e.g. OECD, ISO)
- GLP conditions
- The appropriateness of:
  - The test organisms, incl. source, acclimatisation, pre-exposure
  - The experimental system for the test material and the test organism
  - The range of dispersant/stabiliser/solvent used
  - The spacing of exposure concentrations
  - The range of biomass loading of organisms in test system
- Sufficient number of replicates and organisms per replicate (controls and test conditions)
- Sufficient data available for checking the calculation of endpoints

The GUIDEnano approach appears therefore to be a powerful tool for data quality evaluation of both toxicological and ecotoxicological studies performed with NFs. However, some aspects that are not considered in the GUIDEnano approach but are covered in other schemes, for example DaNa and NanoCRED could be included. A decision on how to evaluate the reliability of data on NFs will be made during the development of the GRACIOUS Framework. In this respect, it should be noted that both the GUIDEnano approach and DaNa criteria are being further developed in two on-going projects, which are caLIBRAte and NanoGRAVUR, respectively. Future modifications will be taken into account in the GRACIOUS Framework.

The aspects addressed in this Chapter will be used to further elaborate when a hypothesis within the GRACIOUS framework is sufficiently substantiated.

6 Relevant tools and methods for incorporation into IATAs

The aim of this section is to summarise the tools considered necessary in the development of the GRACIOUS framework. For the purpose of this chapter, *tools* include:

- standard protocols on intrinsic and extrinsic properties, on release and exposure
- models (*in silico*, release, fate and exposure, and physiologically-based toxicokinetic models)
- *in vitro* and *in vivo* tests protocols, and
- databases including existing inventories of standard protocols, models, data to build models and data to validate *in vitro* protocols.

The tools selected for the draft framework and their sources are shortly summarised below. The protocols and methods for physicochemical properties (section 6.1) are more advanced as compared to other areas as these can exploit to a larger extent existing knowledge.

6.1 Experimental protocols for intrinsic and extrinsic physicochemical properties

There are numerous sources of information relevant to physicochemical characterisation, including regulatory guidance (ECHA, 2017b, c), reviews (especially those from ProSafe on methods: Steinhäuser and Sayre, 2017; and on properties used in frameworks: Oomen et al., 2018) and project documents (Jantunen et al., 2017; Wohlleben et al., 2018). These manuscripts are the basis for the basic physicochemical information (see section 4.2.1) and 'what they are'-IATA (section 4.4.1).

Importantly, the ECHA guidance does not specify methods, but refers to guidance R7.1 for "advice on some of the parameters", and refers to ECETOC (DF4nanoGrouping) for "supplementary information table that includes available analytical methods for parameters relevant for read-across and grouping of nanomaterials" (ECHA, 2017b). In R7.1 (ECHA, 2017a), many alternative methods are provided for each property, whereas DF4nanoGrouping selects a specific method per property. We note that the US-EPA recommends, to differentiate discrete forms "using the same test medium and method […] as even minor changes […] can results in large differences in the measured results" (US-EPA, 2017).

During the GRACIOUS project we may explore several alternative methods to identify the method and metric with highest relevance for grouping and read-across purposes, but we anticipate that the final 'What they are'-IATA may follow the approach of the EPA with respect to recommending a single method to assess similarity between materials.

The following sources of information are perceived as essential for GRACIOUS physicochemical *methods*, because they focus on grouping and read-across with a selection on methods:

- DF4nanoGrouping, Table SI_2 (intrinsic) and SI_4 (extrinsic) in (Arts et al., 2015), which are specifically mentioned in the ECHA grouping guidance.
- ProSafe review on methods, Table 1 in (Steinhäuser and Sayre, 2017) that serve grouping purposes and provide up to four alternative methods for each property.
- ECHA guidance R7.1 (ECHA, 2017a) as a supporting reference, because it is specifically mentioned in the ECHA grouping guidance. However, itdoes not mention methods for most of the extrinsic properties that are ranked high in most grouping frameworks (as shown by Table 2 in Oomen et al., 2018).

There are numerous alternative sources of physicochemical methods for different purposes, as reviewed extensively in the above key sources. GRACIOUS is aware of important parallel developments, including OECD TGs. Recently Rasmussen et al. (2018) assessed the performance of

physicochemical methods in the OECD sponsorship program and generated a rating of 'suitable' or 'suitable with restrictions' for each. Methods relevant to GRACIOUS, with their rating have been summarised in table 6 (below).

In previous sections of this document, Table 4 (section 4.1.1) summarized the selection of properties in the ECHA and DF4nanoGrouping frameworks. GRACIOUS has merged the ProSafe and DF4nanoGrouping tables on physicochemical methods, updated by the environmental and consumer perspectives in nanoGRAVUR (Wohlleben et al., 2018) in order to generate a *draft* selection of experimental protocols summarised in Table 6 . The nanoGRAVUR project (Wohlleben et al., 2018) filled several method gaps identified by ProSafe (Steinhäuser and Sayre, 2017) and improved the methods originally proposed by DF4nanoGrouping (Arts et al., 2015). This is most notably for "solubility". Early in the 'What they are'-IATA quickly soluble materials may be identified (see above hypotheses), e.g. by using the Health Canada static method (Avramescu et al., 2017), which is very similar to the "screening method" in the OECD draft TG on solubility. At a later stage, dynamic dissolution testing may be required (Bove et al., 2017), such as the flow-cell geometry selected by WHO/IARC for fibre biodissolution ranking (IARC, 2002), which then also requires the dissolution rate metric in ng/cm²/h as recommended by(Oberdörster and Kuhlbusch, 2018).

Table 6 will be revised throughout the GRACIOUS project to make the final 'What they are'-IATA. The Technology Readiness Level (TRL) of the methods is estimated for orientation. The TRL criteria requires an interlaboratory comparison and/or standardisation to achieve high TRL (above 7). We refer to the full reasoning for the specific selection in GRACIOUS D3.1, where we will also check if the NANoREG toolbox contains any methods that correlate better for grouping purposes, and backup methods if the main selected method should not be applicable to a specific substance (e.g. purely organic NF). Additionally GRACIOUS tasks will check for which properties a NANoREG database template exists.

The importance of representative test materials for grouping was already stressed in the seminal concept by NIOSH (Kuempel et al., 2012). For the majority of properties, no certified reference materials or reference materials currently exist.

Table 6: The GRACIOUS draft selection of experimental protocols for the physicochemical properties as listed by ECHA, DF4nanoGrouping, or by both (Table 4). Different hypothesis may require information on different properties.

Property	**Method**	**More input expected from ongoing projects**	**Descriptors [metric]**	**Representative test materials**	**TRL of method**
Constituent particle shape (triggers HARN hypothesis)	SEM or TEM (NanoDefine methodology, consistent with ECHA nanoforms)	NANoREG on family of TiO_2 (nano)forms	Aspect ratio [unitless]	NanoDefine IRMM-repository and interlaboratory validations used: BaSO4 IRMM387 (=NM220), BaSO4 IRMM381, CaCO3 IRMM384, TiO2 IRMM388 . HARN: NM402, NM400	High

Property	Method	More input expected from ongoing projects	Descriptors [metric]	Representative test materials	TRL of method
Constituent particle size distribution	SEM or TEM (NanoDefine methodology, consistent with ECHA nanoforms) (alternative technique for size of colloids: centrifugal AC, consistent with NanoDefine)		Median of minimum external size [nm]	NanoDefine IRMM-repository and interlaboratory validations used: BaSO4 IRMM387 (=NM220), BaSO4 IRMM381, CaCO3 IRMM384, TiO2 IRMM388 . HARN: NM402, NM400	High
Composition incl. impurities and additives (cf ECHA footnote to CLP)	Identify composition by XRF (or ICPMS, XRD) (applicable for inorganic materials) HPLC (for organics)	ACENano	Impurity > 1% (consistent DF4nanoGrouping and ECHA footnote to CLP)	Not required. Cutoffs given by CLP and Swiss reporting scheme for NFs	High
Chemical nature of the surface (surface chemistry)	Tentatively: XPS, TGA-MS/IR, LC-MS (or combinations)	ACEnano	Informative for GRACIOUS IATA and methods. Probably not in itself a criterion of similarity of (nano)forms, instead several surface-induced biological interactions are measured directly		Medium
Specific surface area	BET, VSSA by N2 adsorption, (ISO, 2010; Hackley and Stefaniak, 2013)		Informative for GRACIOUS IATA and methods. Probably not in itself a criterion of similarity of (nano)forms, instead several surface-induced biological interactions are measured directly		High
Surface charge	Zeta-potential with pH titration (generic) Charge density (for silica)		Informative for GRACIOUS IATA and methods. Probably not in itself a criterion of similarity of (nano)forms, instead several surface-induced biological interactions are measured directly		High

Property	Method	More input expected from ongoing projects	Descriptors [metric]	Representative test materials	TRL of method
Surface hydrophobicity	Potentially: sessile drop, water contact angle (Xiao and Wiesner, 2012; Nowak et al., 2013)	ACEnano	Informative for GRACIOUS IATA and methods. Probably not in itself a criterion of similarity of (nano)forms, instead several surface-induced biological interactions are measured directly		Low
Density	He-pycnometry		informative for GRACIOUS IATA, probably not in itself a criterion of similarity of (nano)forms		high
Physico-chemical hazards (non-NF), human toxicity (non-NF), ecotoxicity (non-NF)	Non-NF GHS CLP		H-phrases: informative for GRACIOUS IATA, probably not in itself a criterion of similarity of (nano)forms	Not required	High
Rigidity (for HARN IATA)	No valid method established yet		Modulus of elasticity [MPa] (for MWCNT: diameter [nm])	NM400 (non-rigid)/ NM401 (rigid)	Low
Solubility (quickly soluble)	OECD TG draft: in 5 mM NaHCO3, pH7 at 10 mg/L, 24h	OECD	% dissolved	CuO (OECD): soluble (given in TG draft)	High
Solubility: Ion-releasing (triggers the solute IATA)	OECD TG draft: incubate 100 mg/L in relevant medium, measure ions	OECD	dissolved ions [mg/l]	CuO (OECD): ion releasing with >0.1 mg/L Cu^{2+}	High / medium
Dissolution in relevant media	Flow-through dissolution + ICPMS as requested by Oberdörster and Kuhlbusch (2018) and implemented by Koltermann-Jülly et al. (Submitted) and Wiemann et al. (2018)	PATROLS, ACEnano	Pulmonary: k [ng/cm²/h] (Oberdörster and Kuhlbusch, 2018)	BaSO4 NM220, CeO2 NM212, ZnO NM110 or 111	High / medium
Transformation "changes of what they are"	Flow-through dissolution: TEM, optional SAD, XPS (Koltermann-Jülly et al., Submitted)	PATROLS, ACEnano	Qualitative or NanoDefiner image analysis		Medium / low

Property	Method	More input expected from ongoing projects	Descriptors [metric]	Representative test materials	TRL of method
Dispersability (approximated by Homo-agglomeration)	Human perspective (DF4nanoGrouping) : Agglomeration in serum-cont. medium + DLS or AUC Env. perspective: TG318 in relevant medium (instead of 3*3 Ca*NOM media = 54 measurements)	ACEnano	Follow up to read-across similarity by DLS/VCM, AUC.	TiO2 NM105, Ag NM300 (OECD, 2017c)	High / medium
Dispersability (approximated by Affinity and thus hetero-agglomeration)	Tbd. Possibility after Geitner et al. (2016)? to be explored by T4.4	ACEnano, nanoFATE	Attachment efficiency (α)		Low
Biological Reactivity	ESR cell free in water (ecotoxicity), ESR cell-free +FRAS (on human serum) (humantoxicity)	ACEnano, PATROLS ISO TS18827	ESR: relative to negative representative test material FRAS: relative to LoD and positive representative test material	BaSO4 (neg), Mn2O3 (pos)	High / medium

Persistent method gaps with low TRL, where we cannot endorse ProSafe proposals (Steinhäuser and Sayre, 2017), are:

- Hetero-agglomeration: development is needed in GRACIOUS, supported by ongoing projects such as NANOFASE or ACEnano (Hendren et al., 2015; Geitner et al., 2016; Geitner et al., 2017)
- Rigidity of fibres: BAuA Forschungsprojekt F 2365 (curvature analysis + oscillatory measurement) explores options, but still needs to achieve validation and standardization.
- Hydrophobicity: GRACIOUS preferred contact angle measurement (Xiao and Wiesner, 2012; Gao and Lowry, 2018) not sorption of probe molecule due to low reproducibility in earlier attempts, despite reports of equivalent rankings (Xiao and Wiesner, 2012; Gao and Lowry, 2018)
- Redox potential and energy band gap both are intended as proxies of reactive damage. The strategy of the 'What they are'- IATA is to substantiate the assumed predictive value of proxies by direct measurement. Instead of modelling reactive damage based on band energies, the IATA requires direct determination of biological oxidative damage (sBOD from ESR, FRAS or others), supported by GRACIOUS, ACEnano and others
- ISO photoreactivity (Methylene Blue) cannot be recommended due to low reproducibility and affinity issues; the OECD-listed (OECD, 2016a) alternative Rhodamine B is more reproducible, but still insensitive. For both, numerous contextual parameters are needed to describe the protocol.

Beyond the comparison to ProSafe, an experimental metric is not established for comparison of surface chemistry that would go beyond the descriptive "chemical nature of the surface" as required for NF identification (ECHA, 2017c).

Hypotheses based on release and exposure, such as release of NFs from NEPS in which the NF is incorporated into a solid matrix, may require characterisation of additional physicochemical properties, as listed in Table 7.

Table 7: The draft selection of experimental protocols for the properties that are relevant for hypotheses based on intended use, release and exposure: Dustiness was proposed by ECHA, but only addresses powders. The form and rate of release after incorporation into a solid matrix may be assessed by these properties in a 'What they are'-IATA.

Property	Method	More input expected from ongoing projects	Descriptors, [metric]	Representative test materials	TRL of method
Dustiness	CEN update to EN 15051 Methods (RD, CDD) and alternative methods with similar strain intensity (e.g. SRD, SHA, SDD)	CEN dustinano	dustiness coefficient dependent on mass and number [mg/kg, #/kg] factor of emission in number metric	For particles: BaSO4_b from CEN-Project. (high dustiness index) ; For fibres: NM400/NM401	High / medium
Agglomeration of NF upon application of NEP	For fiber powder handling: perform dustiness, analyse the CEN-required filter sample by SEM For spray consumer / occupational: perform intended use, sample aerosol onto filter, analyse by SEM	CEN dustinano	fibres: quantity of primary particle (fibres) in agglomerates in relation to amount of single fibres within the dust (%)	fibres: NM400, NM401	low
Specific application in nano-enabled product (NEP): State of dispersion of NF	Assignment to three fixed categories, which determine the disperse system as well as the type of embedding and agglomeration	ACEnano ??	Disperse system - composites - suspensions - powder Embedding into a dispersed system - complete embedding - partly embedding - attachment - isolated Agglomeration in dispersed system - Agglomerated - individualized	Not required	Medium
Specific application in NEP: content (g/g) of NF in NEP	Acid digestion + ICPMS (for inorganic NF)		Mass-% NF in NEP		High

Property	Method	More input expected from ongoing projects	Descriptors, [metric]	Representative test materials	TRL of method
Resilience of NEP matrix (as introduced in ISO TC229 PG29)	For mechanical stress: tensile elongation (ISO) For chemical stress: matrix lifetime / solubility	nanoGRAVUR, nanoFATE	tensile strength [MPa] or elongation at break [%]	Mechanical stress release: PA = low, Epoxy = moderate, cement = high	Medium
Critical dimensions upon exposure	CEN update to EN 15051 Methods (RD, CDD) + SEM analysis of shape	nanoGRAVUR	Amount of WHO objects from total number (%)	NM400 0.4% NM401 20.4%	Medium / low

6.2 Databases

A table itemising recent Nano-Environmental Health & Safety databases of potential significance to the GRACIOUS framework has been generated (available on a personal basis via GRACIOUS). It is organised by:

- Database Title – its name, or equivalent project title
- A brief Description of the resource
- A Reference to literature or website.

The list includes relevant sources available from approximately the previous five years of NanoEHS project and related activity. It was initially derived partially from information from the EU NanoSafety Cluster (NSC) Workgroup F (Data Management, formerly Databases) Survey of NANO-EHS Databases (Mustad et al., 2014), and from ProSafe (Ritchie et al., 2016).

This material was updated and supplemented with more recent information and expert knowledge of FP7, H2020 and wider project developments, and was also informed through the efforts to compile the "EU US Roadmap Nanoinformatics 2030" (Haase and Klaessig, 2017) in the previous 12 months, in order to compile a contemporary picture of the database landscape. Where relevant entries are classified to indicate whether they are study data containing databases (DB) or predominately Nano-EHS Knowledge Bases (KB) or portals. For the former an indication is given of their data availability status (at around 28/02/18) in terms of: existing accessible datasets (subject to appropriate permissions or Data Sharing Agreement (DSA)); not yet available, or potential data source, (subject to permissions, DSA, and/or upload/conversion to the eNanoMapper Database platform.

6.3 Tools relevant for release estimation and exposure assessment

Possible sources of information on available tools for release estimation, environmental fate and exposure assessment and on occupational and consumer exposure are:

- Environmental exposure models
  - NanoReg Toolbox (Jantunen et al., 2017)
  - Models for environmental fate are reviewed in Nanocomput chapter 3 (Worth et al., 2017a) and an Inventory is available in the JRC ScienceHub as supporting material file S2 (.xlsx file) (Worth et al., 2017b)

- caLIBRAte deliverable D3.2 “List with suitable existing environmental hazard, exposure and risk assessment models at the different stage gates defined.” (not – yet – publicly available)
- ProSafe task Force result: mass flow models and environmental fate models have been developed to calculate predicted environmental concentrations of NMs. A comprehensive review is available by Nowack (2017)
- MARINA result: Frameworks and tools for risk assessment of manufactured nanomaterials (Hristozov et al., 2016)

- Occupational and consumer exposure models
  - NANoREG Toolbox (Jantunen et al., 2017)
  - caLIBRAte deliverable D2.2 “Review of current hazard, exposure and (integrated) health risk assessment models considering their input requirements and applicability at the Cooper innovation stage-gates defined.”, specifically table 2 (not – yet – publicly available)
  - ProSafe task Force result: a review on NMs exposure assessment for workers, consumers and of the public via the environment is performed in Kuhlbusch et al. (2018)
  - Liguori et al. (2016) provide a review on control banding tools for occupational exposure assessment of nanomaterials
  - Tools for evaluation of exposure and hazard of NMs contained in consumer products: NanoRiskCat (Hansen et al., 2014), Licara Nanoscan (https://www.empa.ch/web/s506/licara), ConsExpo Nano tool (https://www.consexponano.nl/),
  - MARINA result: Frameworks and tools for risk assessment of manufactured nanomaterials (Hristozov et al., 2016)

6.4 *In vitro* models

Possible sources of information on *in vitro* methods and SOPs are:

- ProSafe task Force released a comprehensive review on *in vitro* methods applied to NMs (Drasler et al., 2017)
- NanoReg Toolbox (Jantunen et al., 2017)
- ECVAM DataBase service on Alternative Methods to Animal experimentation (DB-ALM) is available in https://ecvam-dbalm.jrc.ec.europa.eu/methods-and-protocols. At present, it contains one *in vitro* method applicable to NFs (DB-ALM Method Summary n° 185: Assessment of mitochondrial health and cell viability with HTS/HCA in HepaRG cells exposed to nanomaterials).
- OECD guidelines for the testing of chemicals: https://www.oecd-ilibrary.org/environment/oecd-guidelines-for-the-testing-of-chemicals-section-4-health-effects_20745788
- ISO/ TC 229 Nanotechnologies: https://www.iso.org/committee/381983/x/catalogue/
- SOPs on *in vitro* methods are released by NanoValid: http://www.nanovalid.eu/index.php/sops-standard-operating-procedures
- NANoREG Result Repository: Deliverables and SOPs WP5
- Nanocomput chapter 1 contains information on OECD *in vitro* tests and their applicability to NMs (Worth et al., 2017a)
- Hristozov et al. (2016) provided an overview of protocols developed across EU and US research projects

- FP7 projects: SUN, ENPRA, NanoGenoTox, Marina

6.5 *In silico* models

In silico methods include several techniques that involve the application of computational tools to obtain an outcome. These methods include data mining techniques to extract patterns and knowledge from large datasets and models built on this knowledge, such models being able to predict the outcome of an experimental test.

Data mining can be supervised or unsupervised. In unsupervised methods, no target variable is identified and they are applied for instance to identify features that can be useful for categorization, and gain insight into the nature or structure of the data. An example of an unsupervised method is clustering. On the other hand, supervised methods identify a rule or a model to relate an object to a class or category that is already identified; an example of supervised learning are decision tree models.

QSARs are a specific type of *in silico* method. These methods are based on the assumption that the activity of the substances is related to its structure.

In this section physiologically based toxicokinetic/dynamic models (PBK) are also taken into consideration, as they are computer models representing the kinetics (the fate of a substance in the human body) or the dynamics (the effect a chemical has on a cell or organ) in the human body.

6.5.1 QSARs and QSPRs

QSARs are based on the assumption that the activity of a substance is related to its structure. QSPRs apply the same concept to predict physicochemical properties. QSARs are accepted in regulatory decision making when the model(s) is validated according to the OECD Guidance Document on the Validation of (Quantitative) Structure Activity Relationship (QSAR) models (OECD, 2007a). According to this Guidance, a QSAR is considered reliable and applicable if it responds to five validation principles: a QSAR should be built for a defined endpoint, it should consist of an unambiguous algorithm, have a defined domain of applicability and appropriate measures of goodness-of-fit, robustness and predictivity, and a mechanistic interpretation (OECD, 2004). QSARs can be documented following the QSAR Model Reporting Format (QMRF), an internationally harmonised template for summarising and reporting key information on QSAR models, including the results of any validation studies (https://qsardb.jrc.ec.europa.eu/qmrf/).

QSARs are considered together with grouping approaches as possible alternative methods in chemicals regulatory assessment. Although the identification of groups of chemicals (or NMs) and the development of QSARs are underpinned by the same principles of chemical similarity, there is no formal process for “validating” a chemical group, which has to be justified on a case-by-case basis.

QSARs and QSPRs have been developed and applied to NMs in recent years (Chen et al., 2017; Worth et al., 2017a). A detailed list of models available in the literature is provided in the inventory from GRACIOUS Task 1.1. An inventory of QSARs and QSPRs where information is extracted following an updated version of the QMRF, thus reporting the available models by endpoint, and exploiting the size of the dataset supporting model development, is available through the JRC ScienceHub as supporting material file S1 and a description of the state of the art on QSARs and QSPRs is given in Worth et al. (2017a) and in Burello (2017) and Chen et al. (2017). A comprehensive review covering grouping approaches and QSARs/QSPRs applied to NMs is in preparation (Basei et al., in prep).

6.5.2 Physiologically based toxicokinetic models

Physiologically based toxicokinetic models (PBK) describe chemical fate in the human body and are useful in determining internal effective target organ concentrations. Information on NFs human kinetics

are deemed important to support a grouping hypothesis, and hence the application of PBKs is relevant for grouping.

Worth et al. (2017a) developed an inventory on PBK models on NFs, including both toxicokinetics and toxicodynamic models, and dosimetry models. Toxicokinetics study the fate of a substance (including NFs) in the body, whereas toxicodynamics concern what a substance does to the body once it in contacts an organ. These dosimetry models predict the fate and the internal dose of NFs in a defined *in vitro* or *in vivo* system.

An inventory is available in the JRC ScienceHub as supporting material file S2, and explicitly reports for each model the NF-dependent and independent parameters, the specifics on the NFs to which the models are applied, and model assumptions (Worth et al., 2017b).

6.5.3 Tools to assess similarity

As reported in section 2.3, several grouping approaches have been developed by application of computational methods to investigate similarity in a set of NFs in order to read-across hazard endpoints. Substantiation of a group is in principle the same as showing the similarity between NFs. Similar to grouping, similarity assessment often goes beyond comparison of a set of physicochemical properties. The hypothesis adds a scientific rationale to grouping.

Within the GUIDEnano project, a methodology was developed to compare the similarity between a NF that has been tested in toxicity studies and the NF for which risk needs to be evaluated, for the purpose of extrapolating toxicity data between the two materials. GUIDEnano developed a series of pragmatic and systematic rules for assessing NF similarity (Park et al., 2018).

GRACIOUS aims at designing a *blueprint* describing both the aspects (algorithms, decision trees/tables, rules) suited as a base for implementation and embedding (or parts of) the GRACIOUS grouping and read across framework into existing and future software-based risk assessment (RA) tools.

Other possible sources:

- Reviews from Tantra et al. (2015)
- ECVAM: https://qsardb.jrc.ec.europa.eu/qmrf
- NanoReg toolbox (Jantunen et al., 2017)

6.6 *In vivo* tests

OECD collects, develops and updates guidelines for the testing of chemicals also for *in vivo* toxicity testing (https://www.oecd-ilibrary.org/environment/oecd-guidelines-for-the-testing-of-chemicals-section-4-health-effects_20745788). Some OECD test guidelines are considered suitable for application to NFs whereas others need adaptation (Rasmussen et al., 2016). Lately, some OECD test guidelines have been updated for applicability to NFs, like Test Guidelines 412 and 413 on subacute and subchronic inhalation toxicity studies that include considerations for application of the test to NMs (OECD, 2017a, b). In addition, an update of OECD Guidance Document 39 on inhalation toxicity testing has been endorsed in April 2018 (publication expected ca. September 20. The relevance of exposure-dose-response *in vivo* testing of NFs, and the need of studying biokinetics for identifying secondary targets is discussed in Oberdörster and Kuhlbusch (2018).

In ecotoxicology the need for adaptation of OECD test guidelines to address NFs has been identified (Rasmussen et al., 2016) and the OECD WPMN has initiated a guidance document on aquatic toxicity testing of nanomaterials for TG 305 on bioaccumulation in fish, which will be available in the near future (Hjorth et al., 2017). An in-depth discussion on how to obtain more reliable ecotoxicity tests for NFs is presented in (Hjorth et al., 2017).

Other sources:

- NANoREG Result Repository: Deliverables and SOPs WP4
- NanoValid SOPs: http://www.nanovalid.eu/index.php/sops-standard-operating-procedures

7 Subsequent steps for further development of the framework

As indicated before, the draft GRACIOUS framework will be discussed with stakeholders and adapted according to feedback. This document will also be used to further define the detail of the work to be done to generate the GRACIOUS Framework. During the course of the GRACIOUS project the framework will be tested and refined using case studies.

Several actions for further developing the GRACIOUS framework have already been identified. They include:

- Evaluation of the scientific validity of the hypotheses, including assessment of the applicability domain of the hypotheses. This evaluation can be performed using existing knowledge (e.g. literature) or by generating new data. It should be prioritised which hypotheses are evaluated. Alignment between the different work packages will be needed as hypotheses often comprise elements of 'what they are', 'life-cycle', 'where they go' and 'what they do'.
- Development of the IATA's in relation to hypotheses, including assessment of the predictive value of the different tools, methods and protocols.
- Determination of quality criteria, based on existing approaches, for quality assessment of data from studies with nanomaterials, and evaluation of the data needed to substantiate the hypothesis (i.e. data curation).
- Determination of quality criteria for the different tools, methods and protocols used in the IATAs and for the tools, methods and protocols needed to generate the basic information (Level 1a). For application of *in vitro* studies in read-across justification, it is relevant to evaluate the ability of *in vitro* and *in silico* methods to correctly rank hazard as well as fate and toxicokinetics behaviour as compared to the *in vivo* situation.
- Further elaboration to assess when a hypothesis is sufficiently substantiated, using Chapter 5 as starting point.
- Determination of the case studies to be used to test the framework, and stakeholders to assess usage of the framework.
- Adverse outcome pathways may be useful in justifying grouping for read-across by providing mechanistic evidence of the effect related to identified relevant physicochemical properties; this aspect can be investigated and implemented in the GRACIOUS framework.

  It should be noted that hypothesis substantiation is in principle the same as an assessment of similarity of NFs that often goes beyond the comparison of a set of physicochemical properties relevant to the grouping hypothesis. The hypothesis adds a scientific rationale to the similarity assessment, and thereby is leading in the identification of a relevant set of properties to justify the grouping/similarity and the identification of relevant information to be obtained and assessed in a structured approach (i.e. the IATAs).

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Appendix A – The proposed definitions for the GRACIOUS key terms and their regulatory references.

Consistent use of terminology is important in any field of science and technology to ensure common understanding of concepts and tools among the involved experts and stakeholders. For emerging or rapidly evolving fields, it often happens that existing concepts are used, but have acquired further nuances giving them a different meaning in a certain discipline, or brand new terms are quickly introduced both in scientific and legislative languages. This also applies to the field of environmental, health and risk assessment of NMs and may become an issue in multidisciplinary research projects with a regulatory outlook such as GRACIOUS.

In GRACIOUS seven terms have been identified as key, namely nanomaterial, nanoform, grouping, read-across, classification, safe-by-design, and representative test material/benchmark material. Section 2.1 shortly describes how these terms have been defined starting from the harmonised definitions developed in NANoREG (Gottardo et al., 2017) and NanoReg2 (Hernandez and Noorlander, 2016). Table 1 provides short definitions for each of the seven GRACIOUS key terms. More comprehensive information, including their use in the EU regulatory context, can be found in the Table below.

KEY TERM	PROPOSED DEFINITION
NANOMATERIAL (NM)	According to the definition published by the European Commission (EC) in the Recommendation 2011/696/EU (EU, 2011a), a nanomaterial is: **"A natural, incidental or manufactured material containing particles, in an unbound state or as an aggregate or as an agglomerate and where, for 50 % or more of the particles in the number size distribution, one or more external dimensions is in the size range 1 nm - 100 nm.** In specific cases and where warranted by concerns for the environment, health, safety or competitiveness the number size distribution threshold of 50 % may be replaced by a threshold between 1 and 50 %. **By derogation from the above, fullerenes, graphene flakes and single wall carbon nanotubes with one or more external dimensions below 1 nm should be considered as nanomaterials."** The definition in the EC Recommendation 2011/696/EU is not legally binding and was developed as a reference definition to promote the harmonised use of this term across different areas of legislation in the EU (Rauscher et al., 2017). It was then used as a basis for the legal definition included in the Biocidal Products Regulation (EU, 2012) and is currently used in the revised Annexes of REACH (EU, 2018). The EC Recommendation 2011/696/EU is now under review. If a revised definition will be published, this section will be updated accordingly. Since 2009, legally-binding sector-specific definitions of the term nanomaterial, which partly deviate from the EC Recommendation 2011/696/EU, have been included in some EU legal acts. For example in the Cosmetic Products Regulation (EC, 2009) nanomaterial means: "an insoluble or biopersistant and intentionally manufactured material with one or more external dimensions, or an internal structure, on the scale from 1 to 100 nm" In the Food Information for Consumers Regulation (EU, 2011c) and Novel Food Regulation (EC, 2015) engineered nanomaterial means:

KEY TERM	PROPOSED DEFINITION
	"any intentionally produced material that has one or more dimensions of the order of 100 nm or less or that is composed of discrete functional parts, either internally or at the surface, many of which have one or more dimensions of the order of 100 nm or less, including structures, agglomerates or aggregates, which may have a size above the order of 100 nm but retain properties that are characteristic of the nanoscale. Properties that are characteristic of the nanoscale include: (i) those related to the large specific surface area of the materials considered; and/or (ii) specific physico-chemical properties that are different from those of the non-nanoform of the same material." The EC intends to amend the sector-specific definitions by harmonising them with the Recommendation 2011/696/EU as soon as the review process is accomplished (Rauscher et al., 2017).
NANOFORM (NF)	At European level, the REACH Committee endorsed the revised REACH Annexes in April 2018 (EU, 2018), after which it will be scrutinised by the European Parliament and Council for three months before it will be published. This revision of the REACH Annexes (EU, 2018) includes the following definition: "On the basis of the Commission Recommendation of 18 October 2011 on the definition of nanomaterial, a nanoform is **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. For this purpose, 'particle' means a minute piece of matter with defined physical boundaries; 'agglomerate' means a collection of weakly bound particles or aggregates where the resulting external surface area is similar to the sum of the surface areas of the individual components and 'aggregate' means a particle comprising of strongly bound or fused particles. A nanoform shall be characterised in accordance with section 2.4 below. A substance may have one or more different nanoforms, based on differences in the parameters in points 2.4.2 to 2.4.5." Section 2.4 states the following parameters in 2.4.2 to 2.4.5. (EU, 2018): "2.4.2. Number based particle size distribution with indication of the number fraction of constituent particles in the size range within 1 nm – 100 nm. 2.4.3. Description of surface functionalisation or treatment and identification of each agent including IUPAC name and CAS or EC number. 2.4.4. Shape, aspect ratio and other morphological characterisation: crystallinity, information on assembly structure including e.g. shell like structures or hollow structures, if appropriate 2.4.5. Surface area (specific surface area by volume, specific surface area by mass or both)" This is similar to the way term was already defined and used by the European Chemicals Agency (ECHA) in the best practice on how to a prepare REACH registration dossiers for a substance that also exists in nanoform(s) (ECHA, 2017b). ECHA establishes that, in the context of REACH, a nanoform is **a form of a substance that meets the requirements of the European Commission (EC)**

KEY TERM	PROPOSED DEFINITION
	Recommendation 2011/696/EU on the definition of the term nanomaterial ((EU, 2011a) and has a specific shape and surface chemistry (ECHA, 2017b). This means that, in the case of a REACH dossier covering different forms of the same substance, registrants are advised to consider particle size distribution (which is the parameter used to verify whether the form meets the EC definition of nanomaterial or not), shape and surface chemistry as the minimum criteria to identify and characterise the nanoforms (ECHA, 2017b). This definition is also considered in the ECHA appendix for nanomaterials to the guidance on grouping of chemicals (ECHA, 2017a). Both of these ECHA documents will be updated in line with the definition now included in the revised REACH Annexes. It is important to underline that the official guidance published by ECHA provides industry with recommendations that are not legally binding but aimed to facilitate the implementation of REACH provisions. The term nanoform is also used in the legal text of the Regulation on Plastic Food Contact Materials (EU, 2011b) but not defined.
GROUPING	At international level, the Organisation for Economic Co-operation and Development (OECD) defines grouping as **the general approach for considering more than one chemical at the same time** (OECD, 2014). Grouping is undertaken to predict unknown properties of some members of the group based on the known properties of other members of the group. The way in which grouping is undertaken depends on the purpose of the prediction, which may be: commercial decision-making, screening and priority-setting of chemicals for further evaluation, hazard identification for risk assessment and classification and labelling, filling information requirements in different regulatory schemes (OECD, 2014). According to OECD (2014), the rationale underpinning grouping may be based on: - Common functional group(s); - Common constituents or chemical classes, similar carbon range numbers; - A common mode or mechanism of action or adverse outcome pathway; - The likelihood of common precursors and/or breakdown products via physical or biological processes that result in structurally similar chemicals; - An incremental and constant change across the category. Grouping may include formation of a chemical category or identification of (a) chemical analogue(s) (OECD, 2014). The terms category approach and analogue approach are therefore used to describe techniques for grouping of chemicals, whilst the term read-across is reserved for a technique of data gaps filling in either approach (OECD, 2014). At European level, Annex XI to REACH addresses grouping and read-across between different substances and establishes that structural similarity is a prerequisite for any grouping and read-across approach aimed to fulfil the standard information requirements. Annex XI to REACH was recently revised to include specific provisions for nanoforms and extend the applicability of the concept of grouping and read-across to different nanoforms of the same substance (EU, 2018). In this transition period, the European Chemicals Agency (ECHA) published an appendix for nanomaterials to the guidance on grouping of chemicals (ECHA, 2017a), which aims at providing registrants with recommendations on how to apply grouping and read-across to nanoforms of the same substance. ECHA clarifies that the principles used to justify sharing of hazard data between nanoforms of the same

KEY TERM	PROPOSED DEFINITION
	substance are similar to those used for grouping and read-across between different substances and refers to the terminology and rationale established at OECD level (OECD, 2014) and reflected in the parent guidance (ECHA, 2008). ECHA also explains that the assessment of similarity between different nanoforms of the same substance starts from considerations on the chemical composition (including impurities, additives and crystalline structure) and considers a wide range of physicochemical properties such as size, surface area, shape, surface chemistry, solubility (including dissolution rate), and hydrophobicity among others (ECHA, 2017a).
READ-ACROSS	The Organisation for Economic Co-operation and Development (OECD) defines read-across as **a technique to fill in data gaps where the test information concerning a certain endpoint for one chemical, referred to as source chemical, is used to predicted the test information concerning the same endpoint for another chemical, referred to as target chemical, which is considered to be similar based on a scientific justification** (OECD, 2014). Theoretically, read-across can be applied to test information concerning any type of endpoint, including physicochemical properties, environmental fate, human health effects, and ecotoxicological effects (OECD, 2014). For any of them, read-across can be performed in a qualitative or quantitative manner (OECD, 2014). The aim of read-across for any endpoint is to provide a prediction that is (more or less) equivalent to the omitted (animal) study and hence acceptable for regulatory purposes (Schultz et al., 2015). At European level, Annex XI to REACH addresses grouping and read-across between different substances and establishes that structural similarity is a prerequisite for any grouping and read-across approach aimed to fulfil the standard information requirements. Annex XI to REACH was recently revised to include specific provisions for nanoforms and extend the applicability of the concept of grouping and read-across to different nanoforms of the same substance (EU, 2018). In this transition period, the European Chemicals Agency (ECHA) published an appendix for nanomaterials to the guidance on grouping of chemicals, which aims at providing registrants with recommendations on how to apply grouping and read-across to nanoforms of the same substance (ECHA, 2017a). ECHA clarifies that the principles used to justify sharing of hazard data between nanoforms of the same substance are similar to those used for grouping and read-across between different substances and refers to the terminology and rationale established at OECD level (OECD, 2014) and reflected in the parent guidance (ECHA, 2008). ECHA also explains that the assessment of similarity between different nanoforms of the same substance starts from considerations on the chemical composition (including impurities, additives and crystalline structure), and considers a wide range of physicochemical properties such as size, surface area, shape, surface chemistry, solubility (including dissolution rate), and hydrophobicity among others (ECHA, 2017a). ECHA underlines that, as for chemicals, read-across between nanoforms is endpoint-specific (ECHA, 2017a).
CLASSIFICATION	At European level, the European Chemicals Agency (ECHA) defines the hazard classification of a substance or a mixture as **the assignment of a standardised description to its physical, health or environmental hazard** (ECHA, 2015). Examples of classification include sensitizer or carcinogen. The classification reflects the type and severity of the hazards of a substance or mixture (and should

KEY TERM	PROPOSED DEFINITION
	not be confused with risk where a given hazard is linked to the actual exposure of humans or the environment to the substance or mixture) (ECHA, 2015). Determining whether a substance or a mixture has properties that lead to a classification is the scope of the Classification Labelling Packaging (CLP) Regulation (EC, 2008). It obliges manufacturers and importers to classify the hazard of substances and mixtures before placing them onto the European market (ECHA, 2015). Relevant and available information on all hazardous properties of a substance or mixture needs to be gathered and rigorously assessed in order to decide whether the substance or mixture should be classified. In case of data gaps, testing may be considered. The assignation to a hazard class is based on a direct comparison between the available information on a specific endpoint with the established classification criteria (ECHA, 2015).
SAFE-BY-DESIGN	According to NANoREG (Gottardo et al., 2017) and NanoReg2 (Hernandez and Noorlander, 2016), the Safe-by-Design (SbD) concept **aims at reducing risks of a NM or nano-enabled product for human health and the environment, and associated uncertainties, starting from an early stage of the innovation process.** This concept requires measures which enable the consideration of safety aspects in the design process of a NF or a NEP, with the objective of eliminating or minimising the risk of adverse effects during the life cycle. Within the SbD concept, the functionality of a NF or nanomaterial-containing product and its safety are therefore considered in an integrated way. Such a concept maximises the use of resources and expedites the development of new nanomaterials and nanomaterial-containing products that are safer by design.
REPRESENTATIVE TEST MATERIAL/ BENCHMARK MATERIAL	The term benchmark material is often used to compare the properties and behaviour of a test material with that of another material (the benchmark material) that serves as a point of reference for the assessment of the applicability of existing methods to new kinds of materials, modification of existing methods, development of new methods, validation of methods and quality control of routinely used methods (Roebben et al., 2013). To this end, benchmark materials have specific requirements with respect to the homogeneity and stability of their properties (Roebben et al., 2013). As the term benchmark material is not very precise it should not be used in the context of the GRACIOUS project. It is proposed to use instead the term representative test material, for which a precise definition, which has gained widespread acceptance, was established in the literature (Roebben et al., 2013): **"A representative test material (RTM) is a material from a single batch, which is sufficiently homogeneous and stable with respect to one or more specified properties, and which implicitly is assumed to be fit for its intended use in the development of test methods which target properties other than the properties for which homogeneity and stability have been demonstrated."** All materials used in GRACIOUS as benchmark or reference materials are in fact representative test materials. It is also proposed that, if the use of the term benchmark material cannot be avoided, it should be regarded as having the identical meaning of representative test material within the context of GRACIOUS.

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Appendix B – Addressing NM and/or NF in the EU legislation

EU legislation addressing chemicals either broadly covers almost all chemicals (horizontal legislation, for example REACH; EC, 2006) or covers a specific use of chemicals (sector-specific legislation, for example the Cosmetic Products Regulation; EC, 2009c; and Novel Food Regulation; EC, 2015).

Currently, many of the pieces of legislation addressing specific uses of chemicals explicitly cover nanomaterials (see terminology in section 2.1) by means of dedicated definitions, information requirements and safety assessment provisions. Examples are: Novel Foods Regulation (section B.3), Regulation on Plastic Food Contact Materials (section B.4), and Cosmetic Products Regulation (section B.7). The horizontal legislation such as REACH (section B.1) and Classification Labelling Packaging (CLP) Regulation (section B.2) do not yet specifically mention nanomaterials in the legal text but nanomaterials are considered to be implicitly covered by the legal definition of the term substance, which is identical for both REACH and CLP. To ensure clarity that REACH, and thereby also CLP, addresses nanomaterials, the REACH Annexes were recently revised by the European Commission (EC) to include a legal definition of the term nanoform (see terminology in section 2.1) and specific provisions (EU, 2018).

The way that the most relevant regulations address application of grouping and read-across concepts to nanomaterials (see terminology in section 2.1) is briefly described below. In sector-specific legislation, a separate safety assessment based on data generated from direct testing of the nanomaterial is the preferred option. At the same time, read-across from a non-nanoform or a nanoform of the same substance or from a different nanomaterial is generally not excluded but needs to rely on a robust scientific justification for each endpoint to be accepted. To this end, dedicated guidance would be beneficial. In the context of REACH, guidance on how to identify nanoforms and share data between nanoforms of the same substance within a registration dossier has recently been provided by ECHA (ECHA, 2017a, b). The European Food Safety Authority (EFSA) is also updating the guidance describing what the information requirements are to enable the risk assessment of a nanotechnology application in food and feed products (EFSA Scientific Committee, 2011). A draft version was released for public consultation in January 2018 (EFSA Scientific Committee, 2018) and contains a section dedicated to grouping and read-across.

More information on this subject can be found in the next paragraphs and in (Mech et al., 2018).

B.1. REACH

The legal text of REACH (EC, 2006) does not explicitly refer to nanomaterials. However, since the definition of substance covers substances in any size, shape or physical state, all provisions of the legal text of REACH apply in principle to nanomaterials to the same extent as they apply to chemicals in general (EC, 2008a).

Annex XI of REACH allows the use of grouping and read-across between different substances as an adaptation to the standard testing regime. This means that the information (of physicochemical, toxicological or ecotoxicological nature) to be submitted by industry in the registration dossier of a substance can be obtained by means of read-across to similar substances instead of direct testing for a certain endpoint, as long as a robust scientific justification is provided.

REACH Annexes, including Annex XI, were recently revised to include specific provisions for nanoforms and extend the applicability of the concept of grouping and read-across to different nanoforms of the same substance (EU, 2018).

ECHA has published an appendix for nanomaterials to the guidance on grouping of chemicals under REACH, which describes a strategy for read-across between (nano)forms (including non-nanoforms) of the same substance (ECHA, 2017a). ECHA clarifies that the principles used to justify sharing of hazard data between nanoforms of the same substance are similar to those used for grouping and read-across between different substances and refers to the terminology and rationale established at OECD level (OECD, 2014) and reflected in the parent guidance (ECHA, 2008). It also notes that, when applying grouping and read-across to different forms (including nanoforms and non-nanoforms) of the same substance, similarity claims should not be limited to the considerations on structure and chemical composition (including impurities, additives and crystallinity) but should include other physicochemical properties such as size, surface area, shape and surface chemistry (ECHA, 2017a). These properties can indeed affect exposure, toxicokinetics, fate and (eco)toxicological behaviour of each nanoform and are considered to be baseline information by ECHA (2017a). ECHA also refers to size (as particle size distribution), shape and surface chemistry as the minimum criteria to use to distinguish different nanoforms of the same substance in substance identification (ECHA, 2017b). When developing a grouping hypothesis for the purpose of fulfilling REACH information requirements, additional physicochemical properties may influence the hazard of the different nanoforms, and these are: solubility (including dissolution rate), hydrophobicity, zeta potential, dispersibility, dustiness, biological reactivity and photoreactivity (ECHA, 2017a). The properties that are used to demonstrate similarity in a read-across case can vary depending on the investigated (nano)forms and also change over the life cycle (Mech et al., 2018). Physicochemical similarity may not be sufficient to justify a group or read-across and other information, e.g. the toxicokinetic profile of a nanoform, may be needed (ECHA, 2017a).

ECHA also underlines that, as for chemicals in general, read-across read-across between (nano)forms is endpoint-specific. Moreover, it is not the conclusion of a study but the result of a study with a source (nano)form, which is used to predict the result for the target nanoform within the defined group (ECHA, 2017a). In the conclusion of a study, which is a comprehensive interpretation of the result of a study based on expert judgment, the result itself (e.g. the numerical value) is evaluated along with other available information about the test material, including the associated uncertainty. This context may therefore change when the same result is used to predict the property of the target nanoform in a read-across case.

B.2. Classification Labelling Packaging Regulation

The Classification Labelling Packaging (CLP) Regulation (EC, 2008b) does not explicitly address nanomaterials. However, the CLP definition of a substance is identical to the definition in REACH (section B.1) and thus nanomaterials are considered to be covered by CLP. Individual nanoforms of the same substance may therefore be classified differently depending on their specific hazard profile (see terminology in section 2.1).

CLP requires that all available information, including information generated according to Annex XI to REACH, is gathered to classify the physical, health or environmental hazard of any substance or mixture. Grouping and read-across between substances are therefore applicable for classification purposes and REACH provisions apply (Mech et al., 2018).

B.3. Legislation on Food and Novel Foods

The Novel Foods Regulation (EC, 2015) covers the authorisation and use of novel foods, which are defined as foods not consumed to any significant degree in the EU prior to 15 May 1997, including newly developed, innovative food, and food produced using new technologies and production processes. According to this Regulation, food containing or consisting of engineered nanomaterials is also considered to be novel food and requires a case-by-case pre-market authorisation on the basis of a scientific risk assessment performed by the scientific panels of the European Food Safety Authority

(EFSA). The Novel Food Regulation requires an explanation of the "scientific appropriateness" of the test methods used to meet the information needs in the case of a nanomaterial, including any adaptation or adjustment applied to such methods (EC, 2015).

The Food Additives Regulation (EC, 2008e) requires that a substance modified through "the use of nanotechnology" is registered on a list of approved food additives as a new substance or by changing the specifications of an existing entry, before that substance can be placed onto the market. In the Food Enzymes Regulation (EC, 2008d), an already authorised food enzyme, which is produced with significantly different methods or starting materials or shows a change in particle size, needs to be submitted for evaluation by EFSA panels. The Flavourings Regulation (EC, 2008c) and Food Supplements Directive (EC, 2002) do not refer to nanomaterials or nanotechnology in their legal text. However, food supplements containing or consisting of nanomaterials are considered to be novel foods and thus undergo a case-by-case pre-market authorisation.

Grouping and read-across is never mentioned in the legislation on food briefly introduced above. In addition, no dedicated guidance exists. For this reason, these approaches are not commonly used by EFSA in the safety assessment of novel foods (Mech et al., 2018). Read-across has however been applied for some food additives and flavouring agents, for example in the case of 4-methylbenzophenone (EFSA, 2009).

In the case, for example, of a novel food containing engineered nanomaterials or a food additive modified through the use of nanotechnology, EFSA refers to the "Guidance on the risk assessment of the application of nanoscience and nanotechnologies in the food and feed chain" (EFSA Scientific Committee, 2011), which describes the information requirements to enable a nanospecific assessment. This guidance is currently being reviewed and updated. In the draft version that was released for public consultation in January 2018, EFSA states that "in principle toxicological data from a nanomaterial may be used for safety assessment of another variant of the same nanomaterial, if it can be shown that there are close similarities in their physicochemical properties and toxicokinetic behaviour. Justification that a source (nano)material exhibits toxicokinetics behaviour that is more 'worst case' than the target nanomaterial would also be possible" (EFSA Scientific Committee, 2018). To this end, adequate physicochemical characterisation of the source and target nanomaterial is essential for establishing their similarities. The EFSA guidance explicitly refers to the step-wise strategy for grouping and read-across between (nano)forms recommended by ECHA (ECHA, 2017a). However, EFSA concludes that there is "considerable uncertainty" on the value of read-across for risk assessment of nanomaterials and "it is likely that experimental data for read-across substantiation would be needed in a majority of cases" (EFSA Scientific Committee, 2018).

At the time of this document, grouping and read-across have not been used by EFSA panels for the assessment of engineered nanomaterials in food and novel foods (Mech et al., 2018).

B.4. Legislation on Food Contact Materials

The Regulation on Plastic Food Contact Materials (FCMs) (EU, 2011b) refers to "engineered nanoparticles" and "substances in nanoform" in the recitals. It furthermore specifies that authorisations based on the risk assessment of the conventional particle size of a substance do not cover engineered nanoparticles. Accordingly, nanoforms are only allowed if they are authorised based on a case-by-case safety assessment performed by EFSA panels and entered onto a positive list (accompanied, if needed, by specific safety based restrictions and migration limits). The Regulation on Active and Intelligent FCMs (EC, 2009a) contains similar requirements for nanoforms.

Neither regulations exclude using alternative methods for evaluating human health hazards of substances, and to date read-across has been applied to certain endpoints for a few non-nanoforms (Mech et al., 2018). In the case of nanomaterials, EFSA states that relevant information obtained by means of read-across from the non-nanoform or other nanomaterials may be used under specific

assumptions (EFSA Scientific Committee, 2011; EFSA CEF Panel, 2016). To date, however, all nanomaterials have been evaluated separately on a case-by-case basis as limited information is available on how their specific properties affect the release from FCMs or the toxicokinetic and toxicological profiles (EFSA CEF Panel, 2016).

B.5. Biocidal Products Regulation

The Biocidal Products Regulation (BPR) (EU, 2012) requires a case-by-case risk assessment for all active substances and for all products. Active substances are assessed at EU level and, if approved, entered onto a positive list for a limited time period. Products containing approved active substances are authorised at Member State level or at EU level via ECHA.

BPR was the first legal act defining nanomaterials using as a basis the EC Recommendation 2011/696/EU (EU, 2011a). The Regulation states that the approval of an active substance does not cover nanomaterials unless explicitly mentioned (EU, 2012). Moreover, where nanomaterials are used in a biocidal product, the risk to human health, animal health and the environment needs to be assessed separately (EU, 2012).

When test methods for identifying hazards are applied to nanomaterials or to products containing them, the “scientific appropriateness” for nanomaterials of the test methods and, as relevant, the technical adaptations or adjustments made in response to the nanospecific properties must be explained (EU, 2012).

Annex IV of BPR establishes the rules for adaptation of data requirements, including those for grouping and read-across. Since BPR and REACH provisions for grouping and read-across are almost identical, the considerations for REACH (section B.1) seem to apply to BPR (Mech et al., 2018).

To date, two nanoforms of silicon dioxide have been approved after individual evaluation based on data generated by means of direct testing, not read-across (Mech et al., 2018).

B.6. Plant Protection Products Regulation

The Plant Protection Products Regulation (PPPR) (EC, 2009b) does not explicitly mention nanomaterials in the legal text. PPPR requires a case-by-case risk assessment for all active substances and for all products. Active substances are assessed by EFSA panels and, if approved, entered into a positive list valid at EU level for a limited time period. Products containing approved active substances are authorised at Member State level.

PPPR does not exclude using alternative methods. For products, toxicity data for the required endpoints are in practice often extrapolated from similar products based on information on the active substance(s) and co-formulants, to reduce the workload and animal testing (Kah et al., 2013). However, grouping and read-across should not replace the risk assessment based on measured data in the evaluation of active substances as residues in food. Prior to product authorisation this scenario should be assessed using the data generated from toxicological tests that are reported in the dossiers (EC, 2009b).

B.7. Cosmetic Products Regulation

The Cosmetic Products Regulation (CPR) (EC, 2009c) prohibits the marketing of products containing ingredients or combinations of ingredients that have been subject to animal testing (unless obtained before July 2013 or generated for other legislation). The risk evaluation of cosmetic ingredients is performed by the European Commission's Scientific Committee on Consumer Safety (SCCS), which must consider all available scientific information, including that generated by alternative methods

replacing animal testing (for example, *in silico* data, chemical categories, grouping, read-across, *in vitro* and *in vivo* data). If needed, the SCCS may request the applicant to submit further information.

CPR explicitly addresses nanomaterials and provides a legal definition that a nanomaterial is "an insoluble or bio-persistent and intentionally manufactured material with one or more external dimensions, or an internal structure, on the scale from 1 to 100 nm" (EC, 2009c).

Any intended use of nanomaterials in cosmetic products must be notified to the European Commission at least six months prior to placing them on the market (except where they had already been placed on the market before July 2013). In case the use of a nanomaterial causes concern, the European Commission shall request a scientific opinion from the SCCS, which should use the full information made available by the applicant to derive a conclusion. The SCCS recognises that the physicochemical properties, biokinetic behaviour and biological effects of nanoforms of substances may differ from those of the non-nanoforms and emphasises that data is required from tests carried out considering the nanoscale properties (SCCS, 2012). Moreover, the nanomaterial must be characterised at different stages: as raw material, in the formulation, and during the toxicological test (SCCS, 2012).

In principle, grouping and read-across are applicable under CPR if the underlying data is sufficiently robust. In the case of nanomaterials, the SCCS concluded that "unless there is a close similarity between different nanomaterials, it is advisable to include a complete set of supporting data on each nanomaterial" (SCCS, 2013). In case of close similarity, the scientific justification that enables read-across should not only rely on the chemical composition of the core nanomaterial but also on the physicochemical and morphological features of the surface coating or other modifications (SCCS, 2013). Moreover, the SCCS clarifies that safety of a nanomaterial cannot be assumed on the safety of the non-nanoform without specific evidence that supports such a conclusion (SCCS, 2013).

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