

# An integrated approach to testing and assessment (IATA) to support grouping and read-across of nanomaterials in aquatic systems

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## ABSTRACT

Even small changes in physicochemical properties of nanoforms (NFs), can drive differences in their environmental fate and hazard. The large number of new materials being developed means it will not be feasible to test and characterise the fate, behaviour and (eco)toxicity of each individual NF. This is further amplified by transformations of NFs over their lifecycle, changing the processes governing their risk. A common complexity arises from dissolution, where the combined toxicity of the exposure arises from both the solutes and any remaining particles contribution to the overall toxicity of the exposure. For efficient and effective risk assessment, it is the most relevant form of the NF for a given exposure that should be targeted for testing and assessment. In aquatic systems, functional fate processes (including dissolution, dispersion stability and chemical and biological transformations) determine the NF's exposure relevant form. Whilst transformations in the environment alter the initial properties of an NF, different NFs may follow a shared functional fate pathway and ultimately present a similar fate and hazard profile in the environment. Therefore, these processes may be used to scientifically justify grouping NFs and read-across for specific endpoints from data rich NF(s) to verified members of the group that have not been tested yet. Integrated Approaches to Testing and Assessment (IATA) have been used in other regulatory contexts to support the collection and integration of relevant existing information as well as the targeted generation of new data to support grouping and read-across. Here, a new IATA is presented consisting of decision nodes focused on dissolution, dispersion stability, chemical transformations and the relative contribution to toxicity of the particle and dissolved component of the overall exposure. The IATA focuses on the fate of NFs in aquatic systems outside of the body, but it can be considered a template for future assessment of *in vivo* kinetics, which will require further development. Guidance on tiered testing approaches and thresholds for grouping within each decision node are critically discussed. Worked examples for ecotoxicity of metal oxide NFs in aqueous systems (in microbial communities isolated from soils and for lettuce plants in hydroponic systems) demonstrate successful identification of the exposure relevant form of the NF in these case studies and allows for different grouping of NFs through application of the IATA.

## 1. Introduction

Manipulation of materials at the nanoscale has resulted in the development of a wealth of different nanoforms (NFs). The large number of new materials being developed means it will not be feasible to test and characterise the physicochemical properties, fate, behaviour and (eco)

toxicity of each individual NF to support risk assessment. Constraints in time and resources limit the possibilities of such testing. A similar issue has also been encountered in conventional chemical risk assessment, where the development of groups of related chemicals taxes the ability to conduct full risk assessment for all class members. For chemicals, grouping and read-across methods are well established to deal with this

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issue of scale. Grouping and read-across makes maximum use of available data by predicting key properties and behaviours for those members that have not yet been specifically tested. This is encouraged by the European REACH regulation [10], based on Guidance by the Organisation for Economic Co-operation and Development [40]. The potential value of grouping and read-across to support streamlined risk assessment has also been identified for NFs by the European Chemical Agency [12]. However, approaches are only beginning to be developed. Grouping in risk assessment requires that any predictive assessment is based on a sound hypothesis that links physicochemical properties with the fate and hazard characteristics. Scientific justification of the link is essential and generally requires knowledge on the underlying mechanisms that lead to the observed environmental behaviour or hazard response.

To facilitate the use of grouping and read-across of NFs in a regulatory setting and to support innovation, the GRACIOUS Framework provides a prototype grouping approach, representing an effective structure to collect and generate the necessary data to identify similarities and differences for different NFs [20,54]. The GRACIOUS Framework approach is built upon the development of a hypothesis, collecting only those data needed to support the hypothesis that links NF properties to fate and hazard characteristics relevant to risk assessment [31]. For a set of pre-defined hypotheses, integrated approaches for testing and assessment (IATAs) are needed to support the efficient and effective collection of data that enables supporting or rejecting each of these hypotheses. Example IATAs have been developed with human nanotoxicology, including for inhalation [30], oral [9] and dermal [8] routes of exposure. For environmental risk assessment, one of the key compartments considered is surface water. Aquatic based test systems are used for freshwater and marine species, as well as for sediment dwelling organisms and, in some cases, for terrestrial meiofaunal and microbial species, where exposure is predominantly via the soil solution (e.g. nematodes, soil bacteria). An IATA which can identify the exposure relevant form and group NFs according to their fate and hazard in aquatic systems may be applied in all these cases.

The structure of an IATA aims to gather information to either falsify or accept the hypotheses through a series of decision nodes. Based on an assessment of the information gathered, it is possible to use the IATA to identify which group a NF belongs to for risk assessment [31]. Risk assessment groups are defined around identifying the exposure relevant form that drives the hazard of the NF in, for example, aquatic systems, given the functional fate of the NF [53]. Functional fate properties that are key in determining the exposure and thus toxicity of NFs in aqueous systems are the NF's stability in dispersion, chemical transformations and the role of toxic solutes dissolving from the NF [56].

We report here the structure of a general IATA that guides users to group NFs on the basis of the following mechanistic insight: "*Following aqueous exposure, dissolution rate and attachment efficiency (derived from dispersion stability) determine whether lethal and sub-lethal toxicity to representative species in aqueous environments is driven by the fate and toxicity characteristics of either the NF particles, or the solutes, or by both particles and solutes*".

Each decision node in the IATA is taken in turn and the detailed rationale, evidence in support of the decision node, and guidance on testing strategies to answer each decision node are presented. Finally, a worked example is presented that demonstrates the practical implementation of the IATA. Limitations in data availability mean that the worked example focuses on hydroponic exposures using lettuce plants and on microbial communities isolated from soils but exposed via water. However, an added benefit to this is that we demonstrate the applicability of the IATA for any aqueous system in which NFs are delivered in the water phase to the biological target, extending its application beyond the more obvious freshwater or marine environments.

## 2. Structure and definitions of components of the aqueous IATA

An effective IATA should enable the efficient and effective collection of data to support a decision and accept or reject a hypothesis. The structure of the aquatic IATA presented here is that of **decision trees**, comprising of a series of questions (**decision nodes**) which identify the most relevant information needed to reach a grouping conclusion with an underlying scientific justification. The user may have one or more **candidate NF(s)** in mind, for which identification of the exposure relevant form and grouping in aquatic systems is needed to answer regulatory decisions.

Each decision node can be answered through a binary choice of "yes" or "no" for the candidate NF(s). There are four decision nodes in the aquatic IATA. Three consider aspects of the environmental fate of the NF, to establish the **relevant exposure form** and in which environment and/or species to test hazard, for example in a pelagic or sediment dwelling species. These three decision nodes are based on the dissolution, dispersion stability and chemical transformations of the NF. The final decision node is focused on the **hazard of the exposure relevant form** of the NF. This assesses the contribution of solutes, particles, and a combination of the two to the overall toxicity of the NF exposure.

Answering the decision nodes generally relies on existing data, but where data gaps exist, the decision nodes guide the acquisition of new data via experiments. When available in silico approaches to predict fate or hazard from intrinsic properties may be used. All of this necessarily includes a degree of expert judgement, for example, in the choice of information sources and their weighting.

For each decision, information gathering is structured in a tiered approach, with up to three tiers. These are referred to as **Tiered Testing Strategies**. Higher tier testing generally requires more complicated, costly and time-consuming tests. As an example: for hazard testing, tier 1 might encompass an acute hazard assessment using standard species (e.g. *Daphnia* as used in the Organisation for Economic Co-operation and Development (OECD) Test Guideline 202 (OECD, 2004), tier 2 a longer-term chronic hazard study (e.g. *Daphnia* OECD TG 211, 2012), while tier 3 might include mesocosm or field studies. Reaching a conclusion for a decision node will not always require progressing through all tiers. Lower tier tests may already provide sufficiently conclusive results, depending on the purpose of the grouping exercise [31]. Implementing tiers reduces the overall need for testing by allowing for a reliance on simpler tests to draw conclusions where appropriate.

It is desirable to link decision nodes to clear **thresholds** that establish groups of NFs based on existing knowledge or the interplay between the NF property tested and the implications for fate and hazard. However, thresholds can be difficult to quantify and justify due to data variability or a lack of data. Where thresholds cannot be established, decision nodes require alternative methodologies to reach a conclusion. **Similarity assessment** between the candidate NF(s) and/or a source material to justify similar fate, behaviour or toxicity is one such method for decision making. Methods for pairwise assessment of similarity on individual properties have been reviewed and tested in the context of grouping NFs for hazard assessment [24]. These approaches are applicable to decision nodes in the current IATA.

Once all data gaps have been filled for the candidate NF(s) the final grouping conclusion can be any of the following:

- All candidate NFs can be grouped into a single group (i.e. they are sufficiently similar to each other to be viewed as a whole without splitting);
- Some candidate NFs can be grouped according to the original hypothesis, but others cannot. The ones that cannot may be moved to a different grouping hypothesis;
- The hypothesis may be refined to better describe the group [9]
- None of the candidate NFs can be grouped with any other NF.

### 3. An IATA for hazard assessment of nanomaterials in aquatic systems

The aquatic IATA delivers six grouping outcomes, summarised in Table 1, that may be supported or rejected through a series of decision nodes considering:

1. dissolution;
2. dispersion stability;
3. chemical transformations and;
4. toxicity ratio of particles and solutes of the candidate NF(s)

The IATA gathers the information required to best identify the exposure relevant form of the candidate NF(s) in the aquatic environment and in which environmental compartment to test.

Where the exposure relevant form is identified, the IATA also identifies the most appropriate hazard testing strategy to be used that will best support the possibility for read-across to existing source material(s). For example, a NF that dissolves quickly in the aquatic environment is unlikely to reach the target organism in the particulate form. Therefore, the IATA identifies that the toxicity is driven by the solutes from this specific NF. Those NFs would be grouped under hypothesis Aquatic-Q – quickly dissolving NFs (Table 1). As an example, this grouping outcome may be used as evidence to justify read-across to existing hazard information for the solutes of the main constituent elements of the material.

The structure of the aquatic IATA is presented in Fig. 1. Each decision node will be taken in turn and discussed in detail. The context and support for thresholds defining groups (where evidenced) will be provided. The suggested tiered testing strategy to fill data gaps for each decision node will be presented, alongside an explanation on how to escalate between tiers. It should be noted that the tiered testing

**Table 1**

Overview of the different sub-hypotheses in the Aquatic Environment IATA. The hypothesis codes refer to the compartment of exposure (Aquatic) and the summary of the grouping conclusion (Q = quick dissolution; SS = slow dissolution, stable dispersion; SP = slow dissolution, partial stable dispersion; T<sub>s</sub> = toxicity driven by the solute; T<sub>NF</sub> = toxicity driven by the NF; T<sub>NF+s</sub> = toxicity driven by both the NF and the solutes).

Aquatic-Q	NFs with a quick dissolution rate in environmentally relevant aquatic media: Following aqueous exposure lethal and sub-lethal toxicity to representative aquatic species is driven by the fate and toxicity characteristics of the solutes.
Aquatic-SS	NFs with a very slow dissolution rate and a stable dispersion in environmentally relevant aquatic media: Following aqueous exposure lethal and sub-lethal toxicity to representative aquatic species is driven by the fate and toxicity characteristics of the NFs in aqueous environment.
Aquatic-SP	NFs with a very slow dissolution rate and a partial stable dispersion in environmentally relevant aquatic media: Following aqueous exposure lethal and sub-lethal toxicity to representative aquatic species is driven by the fate and toxicity characteristics of the NFs remaining in aqueous environments.
Aquatic-T <sub>s</sub>	NFs that partially dissolve in a (partial) stable dispersion in environmentally relevant aquatic media: Following aqueous exposure lethal and sub-lethal toxicity to representative aquatic species is driven by the fate and toxicity characteristics of both NF particles and solutes in aqueous environments (a high toxicity ratio solute: NF allows read-across to similar solutes).
Aquatic-T <sub>NF</sub>	NFs that partially dissolve in a (partial) stable dispersion in environmentally relevant aquatic media: Following aqueous exposure lethal and sub-lethal toxicity to representative aquatic species is driven by the fate and toxicity characteristics of both NF particles and solutes in aqueous environments (a low toxicity ratio solute: NF allows read-across to similar NFs).
Aquatic-T <sub>NF+s</sub>	NFs that partially dissolve in a (partial) stable dispersion in environmentally relevant aquatic media: Following aqueous exposure lethal and sub-lethal toxicity to representative aquatic species is driven by the fate and toxicity characteristics of both NF particles and solutes in aqueous environments (an intermediate toxicity ratio solute: NF limits possibilities for read-across).

strategies are not prescriptive, in that alternative test methods may be used. They simply provide a guide to current best practice to generate the data required by each decision node to reach a grouping decision. A gap analysis will be summarised for each decision node to highlight current limitations. In addition, a worked example for two aquatic systems will be described: microbial communities isolated from soils and exposed in liquid media, and lettuce plants in hydroponic exposures.

### 4. Decision Node 1: Assess dissolution in the relevant aquatic medium

#### 4.1. Context for the decision node

Dissolution is a functional fate property of an NF. It is an extrinsic property that may be expressed as a rate, a rate constant, a half-time, or as a proportion of the overall NF exposure. The rate of dissolution is dependent on the chemistry of the surrounding media and, if not considering equilibrium conditions, the extent of dissolution as an absolute value is also a function of time.

Dissolution is an important transformation of NFs and is identified as a critical determinant of exposure in the environment [56]. It is positioned as the first decision node in the aquatic IATA as identification of quickly dissolving NFs can already negate the requirement for further testing of the particulate form, thus reducing the burden of testing already at this early stage. As indicated in the OECD Guidance Document 318 [35], the aim is often to determine dissolution rates for nanomaterials under certain environmental conditions. Testing all (relevant) freshwater types and considering all contingencies (e.g., seasonal changes) is impossible for all possible NFs of a substance and a more universal and simpler procedure is needed. Standard methods for testing the dissolution of NFs in a range of environmental and biological media are currently under development, including an OECD dissolution assay adaptation of OECD Guidance Document 29 “Transformation/Dissolution of Metals and Metal Compounds in Aqueous Media” [47] and discussion of the relevance of dissolution for the biodegradability of nanomaterials [38]. In addition, ISO 19057 provides descriptions of procedures to assess the dissolution of NFs in test media typically used in *in vitro* testing [21]. These procedures are briefly described in OECD GD 318 [35] and act as the basis around which the tiered testing strategy for dissolution is proposed.

#### 4.2. Grouping outcomes for the decision node

Transformation of the NF into constituent solutes through dissolution over a relevant period can identify whether the solute alone needs considering in the risk assessment of the NF, or whether properties of the particle must also be considered. A distinction needs to be made between quick dissolution (Aquatic-Q), partial dissolution (Aquatic-T<sub>s</sub>, -T<sub>NF</sub> and -T<sub>NF+s</sub>) and very slow dissolution (Aquatic-SS and -SP). A universal threshold of dissolution rate to classify these three groups is inappropriate, for example, to define “quick dissolution”, it must be considered a function of the relevant residence time in the system. This necessitates some expert judgement in the IATA, but the following principles can be used to identify the three groups of NFs according to their dissolution:

1. **Quick dissolution:** Following aqueous exposure, lethal and sub-lethal toxicity to representative aquatic species is driven by the fate and toxicity characteristics of the solutes (Aquatic-Q).
2. **Very slow dissolution:** Following aqueous exposure lethal and sub-lethal toxicity to representative aquatic species is driven by the fate and toxicity characteristics of the NF in the aqueous environment (Aquatic-SS and SP)
3. **Partial dissolution:** Biota are exposed to a combination of particles and their solutes. The contribution of particles versus solutes to the overall suspension toxicity can be further tested to assist in read-

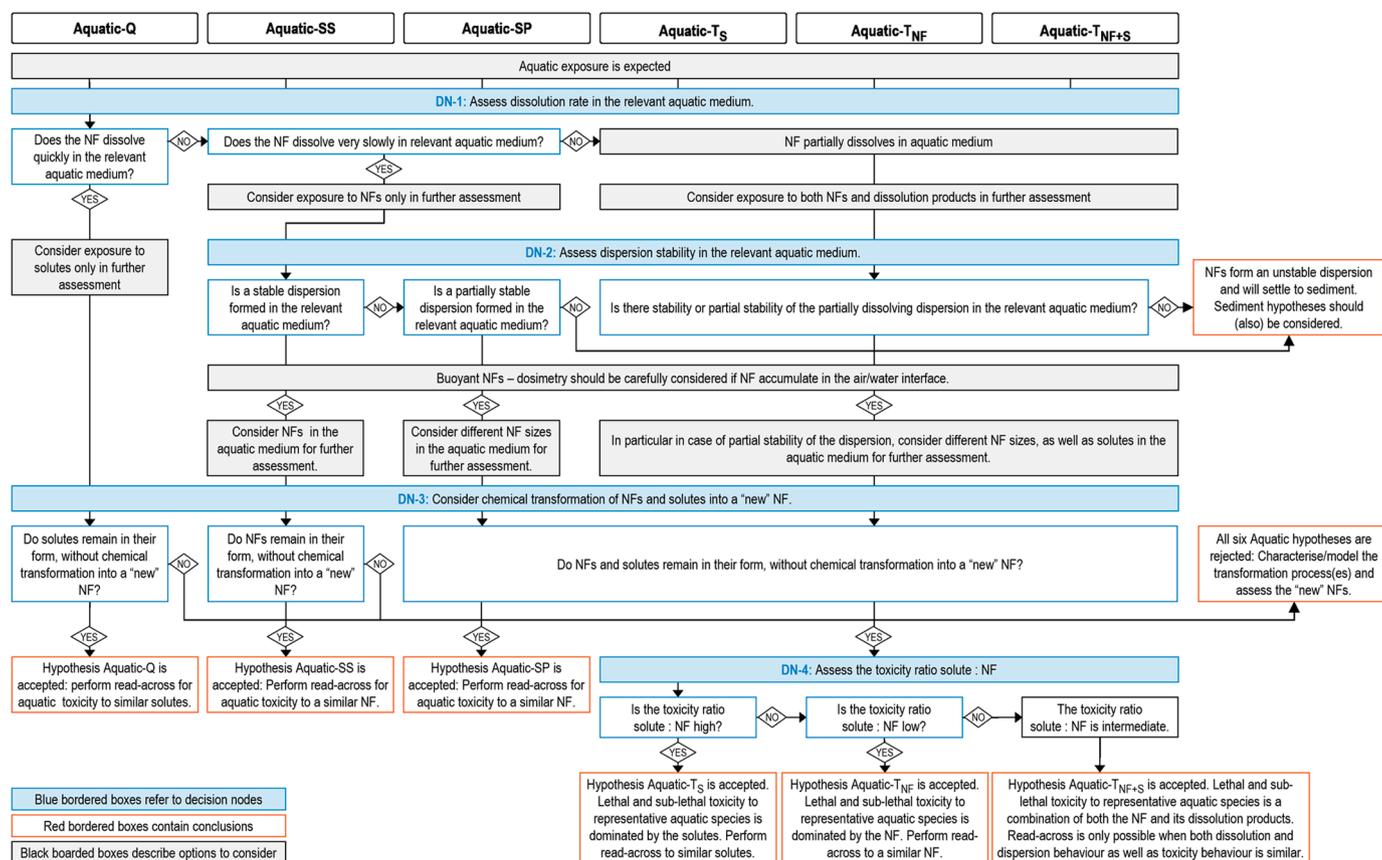


Fig. 1. An IATA for hazard assessment and grouping of nanomaterials in aqueous systems.

across to either the solute or to structurally similar source NFs (Aquatic- $T_s$ , - $T_{NF}$  and - $T_{NF+S}$ ).

#### 4.3. Tiered testing strategy

The tiered testing strategy comprises of three levels of testing (Table 2): a screening batch test of a single time point (Tier 1), an extended batch/dynamic dissolution test to derive dissolution rates in simple media (Tier 2) and an extended dissolution test in specific ecotoxicological media (Tier 3). Tiers 1 and 2 are most suited to identify groups of NFs based on thresholds for quick, partial and very slow dissolution. Tier 3 is particularly suited to similarity assessment between NFs for a specific hazard endpoint (i.e. testing in specific ecotoxicological media). The IATA structure does not preclude performing higher tier tests immediately if they are more suited to the purpose of the grouping.

Table 2

Tiered testing strategy for Decision Node 1 on dissolution.

Tier	Screening batch (24 h) dissolution test [35]
1	10 mg·L <sup>-1</sup> NF in 5 mM sodium bicarbonate buffer (pH 5, 7, 9) 1 time point for measurement at 24 h
2	Extended batch (48 h) [35], or continuous flow (12 h) [21] dissolution test in 5 mM sodium bicarbonate buffer (pH 5, 7, 9) Recommended 8 (batch test) or hourly (continuous flow test) time points for measurement
3	Extended batch (48 h) [35] or continuous flow (12 h) [21] dissolution test in relevant medium, e.g. user defined test medium to be used for toxicity test, specific surface water(s) Recommended 8 (batch test) or hourly (continuous flow test) time points for measurement

#### 4.3.1. Tier 1 screening batch dissolution test

As indicated in OECD GD 29 “The intent of the screening test, performed at a single loading, is to identify those compounds which undergo either dissolution or rapid transformation such that their ecotoxicity potential is indistinguishable from soluble forms”. Therefore, quickly dissolving NFs (Aquatic-Q) may be identified in a first tier through simple screening using a batch dissolution test based on the methodology from OECD GD 29 [47], with adaptations for nano-specific considerations detailed in OECD GD 318 [35]. To capture some of the environmental variability while keeping a simple procedure, we suggest to use a simple 5 mM sodium bicarbonate buffered medium that is adjusted to a range of different pH values (e.g. 5, 7, and 9), as a starting point in Tier 1.

#### 4.3.2. Tier 2 extended batch or continuous flow dissolution test

For those NFs which are not identified as quickly dissolving in the Tier 1 test, the user must consider whether there is sufficient evidence to decide that the NF is considered very slowly dissolving or partially dissolving (OECD GD 318). If there is insufficient evidence, the user escalates to Tier 2.

Based on the results from the Tier 1 screening batch dissolution test, batch or continuous flow tests may be suitable. Current experiences indicate materials with a solubility between 1 and 10 mg·L<sup>-1</sup> may be better suited to testing with the continuous flow system [21], whilst materials with a solubility < 1 mg·L<sup>-1</sup> may be better tested using the extended batch dissolution test [35], due to analytical limits of detection associated with these two methods. Measurements at intervals across the test duration in Tier 2 allows calculation of the dissolution rate expressed as a function of surface area of the NF, using the Noyes-Whitney equation [35].

#### 4.3.3. Tier 3 extended dissolution testing in relevant medium

Escalation to Tier 3 requires expert judgement and is only relevant if dissolution rates in specific media are required by the user. For Tier 3, the same considerations apply for choosing between batch and continuous flow systems as for Tier 2 testing. The choice of relevant environmental or biological media and the relevant test duration must be driven by the specific purpose of the grouping. More details on how to select relevant test durations and starting concentrations can be found in OECD GD 318.

#### 4.3.4. General considerations – duration and starting concentrations

The tiered described approach may need adjustments once a new OECD Test Guideline on dissolution rate of nanomaterials in aquatic environment will be published. Development of such a TG is currently ongoing (Test Guideline Programme Project 3.10, [33] and expected to be published in 2025.

Depending on the NF under investigation and the objective of the testing, appropriate starting concentrations and test durations need to be chosen. For example, if the solubility of the NF is to be measured in Tier 1, a starting concentration above the solubility limit is necessary, so a range of concentrations may be tested (e.g. OECD GD 29, [47]). Alternatively, in Tier 2, when calculating a rate of dissolution, if the starting concentration is too high, the ion concentration in the media may increase to such an extent that the dissolution rate would appear to be reduced [35]. As a default, we recommend an assay duration of 24 h for Tier 1. In the majority of ecotoxicity tests, static conditions are maintained for 24 h for acute studies or in semi-static test designs the test media is replenished at 24-hour intervals. This is also in line with the duration of the screening test described in OECD GD 29. For higher tier testing other time scales may become relevant and should be justified by the user of the IATA, informed by the purpose of grouping or conditions of the test to which read-across is desired for example.

#### 4.4. Thresholds for grouping and supporting evidence

For each grouping outcome of Decision Node 1, we describe existing thresholds and the evidence to support categorisation. The category thresholds identify the exposure relevant forms for testing in ecotoxicity tests. These are first summarised in Table 3, followed by supporting evidence and more details. Where the threshold is dependent on the purpose of the grouping, we describe how a decision for inclusion or exclusion from the group can be evidenced and justified. Calculations for the concentration of solutes and particles over time and calculations of mass-based rates of dissolution and half-times are described in the Supporting Information 1. Note that the different metrics used to describe dissolution have advantages and disadvantages. Proportional dissolution is only useful if strictly constrained to specific conditions of duration and starting concentration of material. Mass based dissolution rate is not constrained by starting concentrations or duration in the same way as the proportional dissolution. Half-times are useful in the context of known residence times or relevant durations.

**Table 3**

Summary of the proposed thresholds to identify quick, very slow and partial dissolution of NFs. Proposed thresholds for different metrics for dissolution are described and where relevant the associated test conditions required for interpretation of the threshold.

Grouping outcome	Threshold specific to different metrics used to describe dissolution		
	Proportional dissolution (%)	Mass based rate (s <sup>-1</sup> )	Half-time (hours)
Quick dissolution	> 90% dissolved in 24 h. Tier 1 batch, single timepoint, starting concentration 10 mgL <sup>-1</sup> .	≥ 2.7·10 <sup>-5</sup>	≤ 7.2 h, assuming (pseudo-) first order kinetics
Very slow dissolution	< LOD after 24 h. Tier 1 batch, single timepoint, starting concentration 10 mgL <sup>-1</sup> .	< 2.7·10 <sup>-5</sup> , ≥ 3.8·10 <sup>-7</sup>	≥ 507 h, 21 days
Partial dissolution	> LOD but < 90% dissolved in 24 h. Tier 1 batch, single timepoint, starting concentration 10 mgL <sup>-1</sup> .	< 3.8·10 <sup>-7</sup>	≥ 7.2 h and < 507 h

#### 4.4.1. Quick dissolution

Quick dissolution is defined as the critical dissolution rate above which the exposure may be considered analogous to the fate of the solute of the same chemical composition. This categorisation needs to be made in the context of the relevant residence time in the aquatic environment, or the residence time related to a particular aquatic hazard test. Therefore, the exact threshold for “quick dissolution” should consider:

1. the relevant starting concentration (informed by expected exposure)
2. relevant residence time for the exposure (informed by the expected exposure or relevant test system conditions)
3. the relative proportion of the NF suspension remaining in the particulate form considered to be insignificant in contributing to hazard

The first two points require judgement on the part of the user using the IATA for their purposes. However, on the third point, some guidance for metal nanomaterials can be established based on existing evidence.

Notter et al. [32] demonstrated that for silver, copper oxide and zinc oxide nanomaterials, NFs were in most cases less toxic on a mass concentration basis than the corresponding dissolved metal. Very few of the studies included within their meta-analysis found a ratio of EC50 values for dissolved/nano that was larger than 2 (Ag: 1.1 %; Cu: 0 %; Zn: 2.8 %), with the majority generally lower than 10. Only a single case out of a total of 453 evaluated showed particles to be > 100-fold more toxic than the relevant solutes.

Using these ratios, we provisionally propose that demonstrating 90% dissolution from a starting concentration of 10 mg L<sup>-1</sup> within 24 h (i.e. t<sub>1/2</sub> = 7.2 hr, assuming first order kinetics, dissolution rate of 2.7·10<sup>-5</sup> s<sup>-1</sup>) in Tier 1 under all three conditions of pH would support read-across to known toxicity data for solutes of the same chemical composition as the candidate NF. This cut-off is related to the achievable measurement accuracy (distinguishing between solutes and very small particles is challenging) and ensures that if the particles are no more than twice as toxic as the solute on a mass basis, at least 80% of the toxicity can be attributed to the solute. The user of the IATA should however consider whether the assumption underlying this threshold is justified on a case-by-case basis for the material under investigation.

#### 4.4.2. Very slow dissolution

Absence of detectable levels of solutes by the analytical approach in the Tier 1 batch screening test can be considered indicative of very slow dissolution [35]. The OECD GD 318 provides further guidance on what is sufficient evidence to conclude that the NF does not dissolve or is very slowly dissolving at this stage. Escalation to Tier 2 extended tests could confirm very slow dissolution, to further strengthen the weight of evidence as part of a grouping decision.

To provide evidence of very slow dissolution, the user may need to identify the exposure relevant form for testing under a particular release or exposure scenario. In this case, it is no longer sufficient to demonstrate very slow dissolution based on the Tier 1 batch test, but rather dissolution rates should be calculated from Tier 2 or 3 tests, considered in the context of time scales relevant to the exposure scenario. Relevant

residence times in the aquatic system are a function of the type of release (continuous versus single event/ pulse discharges) and the dispersion stability of the NF. Expert judgement or modelling is required to define the relevant residence time for a particular exposure scenario. The cut-off rate or half-time below which dissolution is considered very slow should be such that under the given scenario, the rate of dissolution of the NF would result in negligible contribution of the solutes to the overall fate of the particles.

Taking the examples of Cu and Zn containing nanoforms which we used to demonstrate thresholds for quick dissolution, we can estimate the half-time threshold required for very slowly dissolving forms of these nanomaterials, for example if coated to passivate the surface and reduce dissolution. Sensitivity analysis of the SimpleBox4Nano environmental fate and exposure model [28] identifies a lower threshold transformation rate of  $3.8 \cdot 10^{-7} \text{ s}^{-1}$ , above which the predicted aquatic environmental concentration of the particle form of the NF becomes sensitive to dissolution [29]. This critical transformation rate is dependent on the substance and size of the NF and would be between 2 and 20 ng/cm<sup>2</sup>/h for Cu-NFs and between 1.6 and 16 ng/cm<sup>2</sup>/h for Zn-NFs between 10 and 100 nm in diameter that are spherical in nature. Calculations assume that there is no saturation of the suspension with regards to ions. This equates to slowly dissolving NFs having a half-time of 507 h, or 21 days (Table 3, calculations in Supporting Information 1).

Above this critical rate, the predicted concentration of particles in the aquatic environment decreases with increasing dissolution rate of the NF, because the NF dissolves and the smallest particles are completely transformed to their constituent solutes. This may be considered a threshold half-life for very slow dissolution under residence times relevant for aquatic environments, when identifying the exposure relevant form as being the particles themselves.

#### 4.4.3. Partially dissolving

It follows that partially dissolving NFs lie between the two thresholds proposed for quickly and very slowly dissolving NFs. For NFs grouped as partially dissolving, it may be possible to identify a regular pattern such as scaling of effect concentrations to the concentration of dissolved solutes in the NF suspension. Such cases could call for a category approach to read-across as described in the Read-Across Assessment Framework, [13]. The user can consider whether property-function relationships can be established for NFs grouped as “partially dissolving” as part of a read-across justification. For partially dissolving NFs, establishing the relative contribution of the solutes versus the particles to the overall suspension toxicity of the material can allow the user to identify appropriate source materials based on whether the particle or the solute is the main driver of toxicity in the exposure. This is discussed further in the toxicity ratio decision node (DN-4).

#### 4.5. Limitations and outlook

Adaptation of the OECD GD 29 [47] for nano specific amendments is ongoing at the time of publication (OECD Test Guideline Programme Project 3.10) with the aim to develop a Test Guideline.

It should be noted that once the Test Guideline for the Determination of Solubility and Dissolution Rate in Environmental Aquatic Media is finalised, specific test requirements in this guideline should be used to complement the tiered testing strategy outlined above where new guidance supersedes current approaches. In particular, specifics around starting concentrations, durations and escalation between tiers may be updated in light of the outcomes of this new test guideline and should be referred to. All tiered testing strategies in the IATAs are not exclusively prescriptive, but rather indicate current best practices to deliver the data needed in each decision node reach a conclusion on grouping. Whichever method is chosen, the same protocol should be utilised for all NFs and substances within the proposed group in order to allow an appropriate similarity assessment. Use of different protocols, even for the same method, can lead to variance that prevents regulatory decision

making, although it could be sufficient for safe(r)-by-design purposes.

The tiered testing strategy described, whilst more established for metal NFs, may also be applied to non-metal NFs in principle. However, options for analytical methods for non-metal NFs are more limited and would need to consider their ability to distinguish between solutes of, for example, carbon-based NFs from natural organic matter, particularly in higher tier tests in more complex environmental media.

## 5. Decision Node 2: Assess dispersion stability in the relevant aquatic medium

### 5.1. Context for the decision node

Dispersion stability is another key parameter which determines the relevant form and in which environment to test in aquatic systems. Dispersion stability is positioned second in the aquatic IATA as it need not be assessed for NFs already classified as quickly dissolving (Aquatic-Q). The OECD TG 318 provides guidance on testing the dispersion stability of NFs in a simulated environmental media [39]. This test guideline consists of both a screening and an extended test for dispersion stability. The concentration of particles in the supernatant is monitored after a fixed period of time, during which the dispersion may agglomerate and settle out of suspension. Ensuring the tiered testing strategy for this decision node of the IATA is complementary to this standardised test guideline will increase the acceptance of data derived, allowing for data generated to fulfil other testing requirements, and over time, for comparison to other NFs.

In aquatic environments such as surface waters heteroaggregation with natural suspended particulate matter (SPM) is the dominant mechanism by which nanoparticles settle from surface waters and enter sediments [50]. SPM features floc-like structures comprising minerals and organic components, and this complex composition means there is heterogeneity in surface properties of SPM that are important in governing the physicochemical interactions between nanoparticles and SPM. To simulate heteroaggregation processes in natural waters, recent advances have been made in generating reproducible and stable artificial SPM [57]. This is an important adaptation to the existing guidance as now artificial SPM may be used in place of natural organic matter, allowing for more consistent and representative assessment of heteroaggregation in a laboratory setting than previously possible.

Critical attachment efficiencies have been estimated for which the predicted environmental concentration (PEC) of freely dispersed nanoparticles becomes sensitive to their stability against aggregation [29]. This can form the basis for thresholds for stable and unstable NF dispersions. A stable NF dispersion finds the dispersed particles to be the relevant exposure form, whilst instability can trigger consideration of sediment toxicity testing. Stability of NF dispersions has also been proposed as a screen for bioaccumulation potential in fish, meaning data from this decision node may be useful for other frameworks [16,17].

### 5.2. Grouping outcomes for the decision node

The aim of this decision node is to identify the relevant form of exposure and what species to test as critical receptors based on the stability of the NF dispersion in aquatic systems. Three grouping outcomes are possible from this decision node: stable, unstable or partially stable NFs.

**1. Stable dispersion:** Stable dispersions with very slow dissolution (Aquatic-SS). The free particles are considered the exposure relevant form for hazard assessment. Read-across to hazard endpoints for existing source materials or between members of the group may not be justified on the basis of this grouping alone. Physicochemical properties of the candidate NF(s) should also be considered for similarity assessment against potential source materials. For example, different surface chemistries may result in different toxicity

profiles between NFs with the same core chemistry (see DN-4 gap analysis later in the article).

- Unstable dispersion:** These NFs are not grouped under the hypotheses addressed by the aquatic IATA as NF do not remain in the pelagic environment. Assessment of grouping hypotheses that address hazards in sediment environments is flagged as of concern for further assessment.
- Partially stable dispersions:** This intermediate class of stability considers both free NFs but also heteroaggregates/agglomerates of varying sizes environmentally relevant forms. Partially stable dispersions can consist either of a slowly aggregating and sedimenting population of particles, or where two distinct populations of particles exist in the exposure situation, e.g. an unstable fraction which settle quickly and a more stable fraction that either remain in dispersion or sediment out of dispersion at a rate that is not considered sufficient to be classed as unstable [35]. Understanding the mode of action will allow further consideration of aquatic hypotheses and/or address hazards in sediment environments.

### 5.3. Tiered testing strategy

Complementary to OECD TG 318 [39] the tiered testing strategy comprises of three tiers of testing (Table 4): a screening batch test of a single time point (Tier 1), an extended batch test with hourly measurement intervals to understand the dynamics of partially stable dispersions (Tier 2) and several options for higher tier testing specific to the purpose of grouping (Tier 3). Inclusion of NOM or SPM in Tier 1 and 2 is based on the purpose of assessment, whether to evaluate homoaggregation or heteroaggregation respectively. Tier 3 tests are recommended where grouping is desired to be predictive of particular exposure scenarios (for example, in a specific medium associated with a test, or in a wastewater matrix if release to wastewater treatment works is a relevant exposure scenario).

Complete guidance on performing and interpreting the screening and extended dispersion stability tests in Tier 1 and Tier 2 of the testing strategy can be found in the test guideline [39]. The selection of media conditions are based on generating a matrix that covers the majority of conditions normally found in the environment, for example the pH range is based on OECD TG 111 [46]. Practical guidance for escalation between tiers and accepting grouping outcomes based on OECD TG 318 is also available in the GRACIOUS Framework guidance document [20]. The inclusion of a reproducible artificial suspended particulate matter (SPM) further increases the realism of the assessment as compared to just natural organic matter (NOM), allowing for heteroaggregation as well as homoaggregation to be investigated in a comparable manner [57].

Briefly, stable (Aquatic-SS) and unstable dispersions of NFs can be identified in Tier 1. By escalation to Tier 2, extended dispersion stability testing of NFs can inform on the nature of the dispersion of partially stable NFs. Tier 3 is considered for scenarios where similarity assessment may be desirable between NFs under a specific test condition or

**Table 4**  
Tiered testing strategy for Decision Node 2 on dispersion stability.

Tier 1	<b>Screening batch (6 h) dispersion stability test [39]</b> Matrix of 9 conditions of varying ionic strength (0, 1, 10 Ca(NO <sub>3</sub> ) <sub>2</sub> ) and pH (4, 7, 9) + NOM/SPM. Dispersion measured at 0 h and 6 h.
Tier 2	<b>Extended batch (6 h) dispersion stability test [39].</b> Matrix of 9 conditions of varying ionic strength (0, 1, 10 Ca(NO <sub>3</sub> ) <sub>2</sub> ) and pH (4, 7, 9) + NOM/SPM. Dispersion measured at hourly intervals over 6 h.
Tier 3	<b>Dependent on the purpose of grouping. Options include:</b> Extended batch (6 h) dispersion stability test in specific environmentally relevant medium/media [39] Nanomaterial removal in wastewater [34]

environmental exposure. For example, if relevant dispersion stability data exists for a potential source material in a particular aquatic medium used for ecotoxicological testing, comparative data for the candidate NF may be useful to justify similar behaviour and so contribute evidence to a read-across justification to the hazard data in the same test for the source material.

### 5.4. Thresholds for grouping and supporting evidence

The OECD TG 318 describes in detail interpretation of the Tier 1 and Tier 2 tests for dispersion stability [39], which is further elaborated in the accompanying GD 318 [35]. Thresholds for stable, unstable and partially stable NF dispersions are reported here, in addition to other data sources for which thresholds are established. Where the threshold is dependent on the purpose of the grouping, we describe how a decision for inclusion or exclusion from the group can be evidenced and justified. Table 5 summarises the thresholds that have been established to identify three grouping outcomes on the basis of Tier 1 and Tier 2 tests for dispersion stability and on attachment efficiency, two different metrics for describing dispersion stability.

#### 5.4.1. Stable dispersion

A stable dispersion is confirmed when the candidate NF(s) achieve the requirements for “high dispersion stability” described in the OECD TG 318 [39]. NFs which show high dispersion stability of  $\geq 90\%$  remaining in suspension in all conditions of ionic strength and pH in Tier 1 need not progress to Tier 2 testing. It is assumed that this cut-off is sufficiently conservative to be indicative of a stable dispersion being formed in aquatic systems under most conditions that can be expected in freshwaters (e.g. OECD TG 111, [46]). If attachment efficiencies are available for the candidate NF(s) this information can also be used to conclude on the stability of dispersions. Very slowly or partially dissolving NFs with attachment efficiencies below the critical value of  $1.1 \cdot 10^{-4}$  are considered stable in aquatic systems [29]. One special case is for buoyant or extremely hydrophobic NFs. Such materials would also appear stable if they accumulate at the air/water interface. In these cases, implications for dosimetry and adaptations to test design for model aquatic species should be carefully considered. This specific case is discussed in more detail below.

#### 5.4.2. Unstable dispersion

This group can be identified in Tier 1 following the same guidance as

**Table 5**  
Summary of the proposed thresholds to identify stable, unstable and partial stability dispersions of NFs. Proposed thresholds for different metrics for dispersion stability are described and where relevant associated test conditions required for interpretation of the threshold.

Grouping outcome	Threshold specific to different metrics used to describe dispersion stability	
	Proportional stability (%)	Attachment efficiency
Stable dispersion	> 90 % remaining stable under all media conditions (Tier 1 test, 6-hour timepoint)	< $1.1 \cdot 10^{-4}$
Unstable dispersion	< 10 remaining in dispersion under all media conditions (Tier 1 test, 6-hour timepoint)	No specific threshold.
Partial dispersion stability	Between 10 % and 90 % stable in at least one media condition, Tier 1 test. Conditional stability. Tier 2 test can further distinguish between mechanisms underlying partial stability	> $1.1 \cdot 10^{-4}$ . Category approach to read-across within this region could form the basis of a prediction of environmental concentrations based on attachment efficiency

outlined in OECD TG 318 [39]. NFs which show low dispersion stability  $\leq 10\%$  remaining in suspension after 6 h across all conditions of ionic strength and pH are considered to be unstable. No further testing in Tier 2 is required as the assumption is that these NFs will rapidly settle out of suspension in most freshwater environments. This does not conclude a specific grouping under the aquatic IATA, rather it exits the IATA, prompting the user to consider sediment exposures as being most relevant for the risk assessment of the candidate NF(s). Such cases may trigger, for example, hazard testing with sediment dwelling species. A provisional IATA for sediments is presented in Supporting Information 2, but is not substantiated to the same extent as the IATA for aqueous systems. This should be considered a resource available as a starting point for further substantiation or expansion into a full IATA for the sediment environment. If continuous release of the NF is expected or identified for a particular environment during the exposure assessment of the NF, it is still useful for the user to continue the grouping exercise in the aquatic environment as well as consider the sediment compartment. Guidance on how to adapt existing OECD test guidelines for ecotoxicity testing for unstable dispersions (e.g. through static renewal or flow-through test designs to maintain exposure to the NF) can be found in OECD Guidance Document 317 on aquatic and sediment testing of nanomaterials [36].

#### 5.4.3. Partial dispersion stability

NFs which cannot be confirmed as stable or unstable according to the thresholds suggested in the OECD TG 318 are considered to have partial dispersion stability. The extended test in Tier 2 allows for qualitative identification of the aggregation/agglomeration state of partially stable NF dispersions. Monitoring the dispersion over time allows for differentiation between NFs which agglomerate/aggregate slowly resulting in continuous settlement and those for which distinct populations of stable free particles and unstable aggregated populations emerge, full details of which can be found in the guidance document [39].

If the purpose of grouping is for hazard assessment, then NFs grouped as based on both partial dispersion stability and partial dissolution characteristics, continue to further assessment based on the ratio of toxicity between the particles and solutes in the overall dispersion. The free concentration in dispersion of partially stable NFs decreases with increasing attachment efficiency above a critical value  $1.1 \cdot 10^{-4}$  [29]. A category approach to read-across may be employed to predict the concentration of free NF in the aquatic environment within this group.

#### 5.5. Limitations and outlook

The assumption when grouping NFs according to their dispersion stability is that in aqueous exposures, similar stability would indicate similar bioavailability between members of the group. A definition for the bioavailable fraction of stable NFs in aqueous systems is not agreed. For conventional chemicals or metal compounds, the bioavailable fraction is often operationally defined as the fraction that can pass through, rather arbitrarily, a  $< 0.45 \mu\text{m}$  filter [15]. Assuming collisions between natural colloids and NFs occur more frequently than attachment to coarse suspended particles  $> 0.45 \mu\text{m}$  [51], most aggregates/agglomerates of partially stable NFs are also likely to be  $< 0.45 \mu\text{m}$ , so this definition could also apply to NFs. However, our understanding of how different physicochemical properties other than size affect bioavailability of NFs is still incomplete, particularly considering species specific differences. A benefit to the use of the OECD TG318 test structure in the early tiers of testing is that data generated this way may be used for multiple purposes. Whilst thresholds are proposed here to group NFs according to maintenance of an aquatic phase exposure of particles, other interpretations of the test could be considered. For example partially stable or unstable dispersions might act as a chemistry trigger for bioaccumulation testing in fish, where emphasis of the scheme is on dietary bioaccumulation and biomagnification risks [17]. In this way, the IATA is an efficient strategy in that data collected and generated can

be used to address multiple endpoints, where thresholds and interpretation can be set depending on the purpose of the assessment.

There are still metrological challenges in quantifying dispersion stability and attachment efficiency in particular. The tiered testing strategy presented focuses on proportional stability of NFs in dispersion, however, thresholds for attachment efficiency could also be used to define the stable and partially stable groups. Calculation of these attachment values is possible but there are difficulties in proposing an agreed standard for calculating such parameters [56]. Detailed discussion on this topic is beyond the scope of this article, but recent developments have been summarised (e.g., [49]). Attachment efficiencies, predicted or measured, would also be valid information sources to contribute to concluding on this decision node.

Whether measuring stability or attachment efficiency, different assessment methods for hydrophobic materials will be required, as the design of ecotoxicity tests and calculating the delivered dose would be very different for these NFs which accumulate in the air/water interface, than for those dispersed in the aquatic phase. Concentration at the air/water interface presents a challenge for traditional calculation of effect concentrations, even though such case can provide ecologically relevant exposures. For example, some species (e.g. *Lemna spp.* used in OECD TG 221) which float at the surface may be exposed by this route. Guidance is given in OECD GD 317 on dispersion approaches to avoid these conditions (e.g., through application of dispersants) but notes that this should only be done if relevant to the goal of testing [36]. Paradigms for highly hydrophobic or buoyant materials such as nanoplastics with a density  $< 1 \text{ g cm}^{-3}$  concentrating at the air/water interface it appears are missing, but could be relevant to the exposure profile for some NFs with similar properties.

## 6. Decision node 3: Chemical and biological transformations

### 6.1. Context for the decision node

Whilst dissolution is a transformation covered already in the IATA, other abiotic or biotic transformation of the surface or core of the NF, or indeed the formation of new NFs from precipitating solutes, dissolved from the original “as manufactured” particles may generate new exposure relevant forms for assessment. Clearly such transformations are highly dependent on the chemical identity of the NF. Rather than including all possibilities in the IATA, the user is guided towards identifying relevant transformation pathways to define a new NF and proceed with the basic information of the new NF as the target nanoform.

The hypothesis that materials can turn into structurally similar transformation products with similar mechanisms and degrees of toxicity is already acknowledged for the grouping of substances [14]. The main transformations of concern are dissolution, redox/photo-reactions, sulphidation, phosphorylation and interaction of surrounding compounds with the NF [48,56]. For organic nanomaterials and/or surface treatments, biodegradation can also be a significant factor determining persistence and form. Relevant mechanisms of degradation and the available test methods are summarised to date in ECHA’s recent study on (bio)degradation, persistence and safe by design of nanomaterials [11]. Decisions made on the nature of probable transformations for this node can be informed by modelling or predicted chemical speciation change. At this point a fully threshold based tiered testing strategy cannot yet be defined due to limited routine and quantitative methodologies to address this decision node [27].

### 6.2. Grouping outcomes for the decision node

Identification of the chemical transformation of a NF into a “new NF” is currently described in the IATA as a binary choice. The user may select whether a new NF has become the most exposure relevant form. If so, that transformed form is assessed as the candidate NF within the IATA.

For example, if sulphidation of a silver nanoform is identified as

relevant on entering a sewage treatment plant, then this decision node would allow the user to present a case for silver sulphide NFs to be the exposure relevant chemically transformed form for any risk assessment conducted of the effluent and downstream receiving water. Selection of a silver sulphide nanoparticle for further assessment in the hazard assessment or as a source material for read-across could be justified in this way.

Where possible, direct testing of the original NF and a surrogate that represents the likely transformed exposure relevant NF is preferable, to demonstrate that final assessment should be of the transformed NF. However, use of existing evidence or modelled or predicted data should be encouraged to also be included as part of this justification and to answer subsequent decision nodes in the IATA.

If no conclusion can be made as to whether chemical transformations would lead to a more relevant exposure form for which the hazard profile would differ from the original particle (either more or less hazardous), assessment of the original NF should be continued.

### 6.3. Tiered testing strategy

Whilst a tiered testing strategy to consider all chemical and biological transformations cannot yet be established, more specific strategies have been proposed, specifically for biological degradation of organic surface treatments, which then result in a new transformed exposure relevant form [6]. From a grouping perspective, NFs coated with an organic surface treatment may lose this surface functionalisation through a variety of mechanisms, resulting in initially distinct NFs (NFs with a similar core, but different surface chemistry) converging in their fate and toxicity profile as the surface treatment is lost or degraded. A tiered testing strategy to assess the durability of organic surface treatments against biodegradation has recently been proposed. This could be employed in this decision node, allowing grouping of NFs with initially distinct surface chemistries on the basis of demonstrated loss of that organic surface treatment in the environment [6]. Information from this tiered strategy could be one tool to identify a new exposure relevant form after transformation.

It is envisaged that the aquatic system IATA is structured in such a way that other strategies to test relevant transformations would in future be able to be integrated to answer this decision node. The user of the IATA must document clearly what the context, relevance, testing strategy and grouping outcomes based on evidence are for any assessment of NF chemical or biological transformation.

### 6.4. Limitations and outlook

Chemical transformations across the life cycle of a product or NF have been suggested as a long term research priority for use in future grouping and risk assessment of NFs [55]. Sulphidation and phosphorylation of NFs may be indirectly investigated by the variation of the dissolution behaviour of a NF, but also by investigating the modification of the NF's properties (e.g. shape, size, composition) by TEM-EDX or surface properties (XPS). For solid transformation, synchrotron-based x-ray diffractions are a methods of choice to investigate the composition of a new NF [26]. Quantification of most transformations require the use of more specialised techniques that are not ideally suited to more routine assessment. Hence where possible, the emphasis should be on development of predictive models for transformation processes should be favoured.

Biological transformations, both in environmental media external to organisms such as the biodegradation by biological exudates discussed, but also within the body are currently poorly understood in the context of how these processes may result in similar or dissimilar hazard profiles for NFs. Some studies are available concerning the kinetics of transformations such as dissolution within select species and for specific materials e.g. in vivo bioimaging of dissolution of silver NFs in the gut of *Daphnia magna* [58]. However, there is insufficient understanding of the

mechanisms of action, key events and eventual adverse outcome triggered by specific distribution or kinetics of nanomaterials within the body of non-mammalian species to include such considerations in an effective way in the IATA as it stands. Developments in adverse outcome pathways for nanomaterials will assist in this and allow extension of the IATA or new IATA to be developed which are concerned with in vivo kinetics and interactions, similar to the recent developments of IATA for specific human health disease outcomes or exposure pathways [3,8,9,30]. Developments in standardised testing to measure and quantify nanomaterials in biological samples will also support inclusion of such decision nodes in future adaptations of the IATA, such as the OECD TGP Project 1.10 Guidance Document on the determination of concentrations of nanoparticles in biological samples for (eco)toxicity studies (details on current status of OECD GD and TG relevant for nanomaterials available in [18]). These methods are well advanced for metallic nanoparticles [25] but methods are weighted towards inorganic NFs, with more progress required particularly for the detection of organic NFs in biological samples [1].

## 7. Decision node 4: Toxicity ratio of solutes versus particles in the overall suspension

### 7.1. Context for the decision node

This decision node is relevant for those NFs which are found to undergo partial dissolution and be at least partially stable. For these materials, grouping may still be possible through identifying whether it is the solute, the particle or a combination of both, that is responsible for the overall toxicity. Testing in this decision node allows the user to come to a decision about what component of the overall exposure best accounts for the observed toxicity. In this way, read-across either to the solutes or particle toxicity can still be performed, even for NFs which are grouped by their fate as partially dissolving, a key advancement of this IATA.

### 7.2. Grouping outcomes for the decision node

Three grouping outcomes are possible:

1. **Aquatic-T<sub>S</sub>**: NFs that partially dissolve in a (partial) stable dispersion in environmentally relevant aquatic media: Following aqueous exposure lethal and sub-lethal toxicity to representative species is driven by the toxicity characteristics of the solutes in aqueous environments (a high toxicity ratio solute: NF allows read-across to similar solutes).
2. **Aquatic-T<sub>NF</sub>**: NFs that partially dissolve in a (partial) stable dispersion in environmentally relevant aquatic media: Following aqueous exposure lethal and sub-lethal toxicity to representative species is driven by the toxicity characteristics of the NF particles in aqueous environments (a low toxicity ratio solute: NF allows read-across to similar NFs).
3. **Aquatic-T<sub>NF+S</sub>**: NFs that partially dissolve in a (partial) stable dispersion in environmentally relevant aquatic media: Following aqueous exposure lethal and sub-lethal toxicity to representative species is driven by the fate and toxicity characteristics of both NF particles and solutes in aqueous environments (an intermediate toxicity ratio solute: NF limits possibilities for read-across).

If the user concludes that it is the particles that are responsible for the observed toxicity (Aquatic-T<sub>NF</sub>), the outcome of grouping in this case is that read-across between NFs within this group is possible. Similarity assessment of relevant physicochemical properties should also be considered to support the read-across to potential source materials. In addition, for partially stable NFs, demonstrating similar stability in DN-2 would further support the read-across by evidencing a similar delivered dose of particles to the target organism.

If the user concludes that the observed effect can be attributed to the solutes, then read-across to soluble forms of the material is justified as a source material. The two critical functional properties of dissolution and dispersion stability that determine the exposure and delivered dose of the NF in aquatic systems have been demonstrated to be similar and so support the read-across justification.

### 7.3. Tiered testing strategy

This proposed tiered testing strategy is constructed around an assumption of increasing data requirements as you progress through the tiers such as longer-term effects data. Each tier uses the same rationale and experimental design to establish the contribution of solutes versus the NF to the overall suspension toxicity, but applies this approach in different test systems (moving from acute toxicity, to chronic population relevant effects, Table 6). Tier 1 requires assessment of the ratio of toxicity between solutes and the NF only in a single relevant acute ecotoxicity test. Tier 2 extends this test to multiple relevant species with an aim to assess the generality of the ratio of solute versus particle toxicity. Tier 3 considers if the solute or NF drives the responses of chronic endpoints in either the most sensitive or the most relevant species for the exposure. The level of testing undertaken is guided by the information requirements needed to be fulfilled and the purpose of using the IATA. Selection of test species and relevant tier of testing can be informed by existing approaches such as the OECD guidance on threshold approach for acute fish testing [43].

For each tier of testing, the same test design is followed. Two dilution series are prepared for exposures, one of the NF and one of a soluble form of the material. Dose response curves are calculated for both the solute only control exposures and the NF exposures, in which the dissolved fraction is monitored over time. The response addition model for mixture toxicity is then used to establish the relative contribution of the solutes versus the particle to the overall mixture toxicity of the suspension. Within the tiers, differences between species may occur and expert judgement may be needed to decide on the most relevant species for use in grouping. A practical guide on how to conduct this assessment and the assumptions that underly it are available [20]. A full example of this test design and the associated calculations is reported in Song et al., [52] and is used as part of the worked example testing the IATA. Full details of the calculations are also provided as a guide in Supporting Information 3.

**Table 6**

Tiered testing strategy for Decision Node 4 on the toxicity ratio of solutes versus particles. Comparison between multiple NFs should assess data from the same test method. Standardised tests should be used where possible (examples provided in the table), but non-standardised approaches and/or new approach methodologies can also be justified. Model species selection can be purpose specific.

Tier	<b>Assessment of single acute model</b>
1	Assessment of the ratio of solute versus particle toxicity in acute toxicity tests in any relevant model, e.g. Daphnia Immobilisation test OECD TG202 [45] or algae OECD TG201 [44], based on comparison of generally used effect levels like EC20, EC50, LC20, LC50. The aim is demonstrating which component of the overall suspension is responsible for the observed acute toxicity in a single relevant model.
Tier	<b>Assessment of acute toxicity in multiple models</b>
2	Extended assessment of the ratio of solute versus particle toxicity in multiple relevant acute toxicity tests, e.g. algae (OECD TG201, 2006), daphnids (OECD TG202, 2004a), fish early- life stage toxicity test [41], based on comparison of generally used effect levels like EC20, EC50, LC20, LC50. The aim is assessing the generality of the ratio of solute versus particle toxicity.
Tier	<b>Chronic assessment focusing on the most sensitive species</b>
3	Extended assessment of the ratio of solute versus particle toxicity in relevant chronic tests, e.g. daphnids reproduction test [42], rainbow trout chronic toxicity on juveniles (OECD TG215, 2013) based on comparison of generally used effect levels like EC20, EC50, LC20, LC50. The aim is assessing the generality of the ratio of solute versus particle toxicity for chronic endpoints

Some important principles should be followed in this tiered testing strategy:

- When comparing toxicity data between NFs, it is always preferable to only compare data derived from the same assay. If this cannot be done, justification for comparison between data for different model species for example must be evidenced in detail.
- Standardised tests are preferred and are listed as examples in the tiered testing table (Table 6). However, the approach should not be limited only to standardised tests if good quality data is available from non-standardised approaches.
- Relevant model species can be selected either based on the expected release pathway or behaviour of the NF in the environment or based on expectations of what may be the most sensitive model species.
- The user may start the assessment at any Tier depending on the purpose of the grouping.
- Escalation between tiers is purpose driven. For example, if a specific release pathway is considered for the NF, testing of the most relevant species in Tier 1 may be sufficient if NFs are grouped as acute toxicity driven by the solutes. The user must present a justification that if solutes drive the acute toxicity, then at longer timescales, the solutes are still responsible for the chronic toxicity of the NF. Such evidence could use existing data for acute and chronic toxicity of soluble forms of the substance.

### 7.4. Limitations and outlook

This decision node establishes whether it is the particle, the solute, or a combination of both being primarily responsible for the toxicity of the overall suspension of the NF. For the Aquatic-T<sub>3</sub> group, read-across to fill data gaps for hazard may be justified to soluble forms of the material. For the two groups for which the particles contribute significantly to the overall toxicity of the suspension (Aquatic-T<sub>NF</sub> and Aquatic-T<sub>NF+S</sub>), similarity assessment of physicochemical properties between the target (s) and source NFs is recommended to justify read-across to fill data gaps for hazard assessment relating to the effects of exposure to the NF. Any such assessment assumes that the properties of the “as manufactured” NF are still relevant for similarity assessment between source and target NFs as part of a read-across justification. For example, that the original size of the particles still largely determines the effect of the NF, even when incorporated into agglomerates. Such assumptions require evaluation across a wider range of NFs for which individual properties are systematically varied to be fully substantiated. Substantiation of rules for similarity assessment of the physicochemical properties of NFs for which the particles contribute most to their observed ecotoxicity in aquatic systems is a major knowledge gap which must be addressed. We summarise the outlook for this knowledge gap here.

Tools and approaches for a quantitative similarity assessment based on individual properties have been proposed [24]. However, there are outstanding gaps in our knowledge that present a challenge to drawing conclusions from these similarity assessment approaches to date. These are:

1. Validation of acceptable limits of similarity on individual physicochemical properties based on in vivo comparison to aquatic ecotoxicity outcomes is outstanding (i.e. the magnitude of change in an ecotoxicological response associated with a defined change in a physicochemical property of the NF)
2. Relevance of individual physicochemical properties of NFs under particular exposures may be not be applicable for all properties and all exposure scenarios [24]
3. Assessment of the achievable accuracy and precision with which we can describe physicochemical properties of the nanoform is possible [5], but the same is not known for responses in model test systems for ecotoxicology

Rules for acceptable limits of similarity have been proposed for individual physicochemical properties in the context of defining sets of NFs in a specific regulatory context [22,23]. However, the context and applicability domain of these rules for grouping NFs into sets is very different from the applicability domain covered by the aquatic IATA. New rules to cover the domain of the aquatic IATA are needed. In principle, acceptable limits of similarity can be substantiated for physicochemical properties of NFs in aquatic systems if they correctly assign NFs to groups for which the hazard profile of these same NFs in vivo is similar. Reactivity, dissolution and morphology have also been identified as relevant for grouping for ecotoxicity of NFs, but thresholds have not been established for acceptable limits of similarity for these properties between NFs of the same chemical composition [19]. However, systematic review with targeted supporting testing may provide an approach to develop such rules.

It is important to note that similarity assessment on the basis of physicochemical properties is applicable only to those NFs which are grouped for concerns over toxicity from the NF in the dispersion, not the solutes (i.e. those grouped as Aquatic-SS, slowly dissolving, stable; Aquatic-SP, slowly dissolving, partially stable; and Aquatic-T<sub>NF</sub>, partially dissolving but NF responsible for the toxicity). It is only within these groups that a read-across prediction from a source material to a similar target NF based on physicochemical properties is possible. Two NFs of the same chemical composition may have considerable differences in physicochemical properties, but if the functional fate of both NFs is to dissolve quickly, differences in properties between the two NFs beyond acceptable limits of similarity should not preclude read-across between the two.

For NFs where a combination of NF and solutes contribute significantly to the toxicity (Aquatic-T<sub>NF+S</sub>), this picture is more complex. In these cases, it may not be sufficient to justify similarity based on the properties of the particles alone, but also on an acceptable limit of similarity in the rate or extent of dissolution. An acceptable limit of similarity for dissolution rate or extent within a relevant exposure has not been established for aquatic ecotoxicity and would require the same rationale to be followed as above for intrinsic properties of the NFs.

## 8. Worked example

This worked demonstrates how following the IATA can identify the exposure relevant form for testing and allow for the contribution of the solutes versus the particles to be quantified. For demonstration, datasets must be available that give the required level of information for each decision node. Given the relatively recent developments in experimental design and modelling to test the toxicity ratio of solutes versus particles, few datasets are available which can fulfil the requirements for each node. However, some studies do require the necessary data. Of these, two publications focusing on zinc oxide (ZnO-NFs) were selected which contained sufficient data to road test the IATA Song et al., [52] and Zhai et al., [59].

Song et al., [52] assessed the toxicity of 25 nm spherical ZnO particles and the role of dissolution on lettuce growth exposed hydroponically in ¼ Hoagland medium. Zhai et al., [59] assessed the impact of five ZnO NFs and submicron particles on microbial activity in liquid soil extracts as determined by means of the BIOLOG culture based bacterial substrate utilisation test system. In the latter studies, average well-colour development in the BIOLOG plate was used as a proxy for functional diversity of the microbial communities exposed to the ZnO-NFs, from which to calculate effect concentrations (EC50). The BIOLOG test could also be used for species representative of both aquatic and sediment environments as in principle isolates from any environments can be used as the starting inoculum community for the test. In this way it has direct application relevance for the aquatic environment as well.

Whilst the worked examples do not contain a classical aquatic environment model, the two publications selected represent test systems

in which delivery of the NFs is through aqueous exposure, and so are within the applicability domain of the IATA. A benefit of this is that we are able to demonstrate application of the IATA beyond model species for the aquatic environment and demonstrate its relevance for any aqueous exposed environmental test system.

Six nanofoms of zinc oxide (ZnO-NFs), distinguished by different sizes and shapes, were evaluated in total within the overall assessment:

- ZnO-25: Spherical, 25 nm ZnO (Lettuce exposure, [52])
- ZnO-18: Spherical, 18 nm ZnO (all subsequent NFs: BIOLOG microbial community, [59])
- ZnO-43: Spherical, 43 nm ZnO
- ZnO-150: Rod shaped, 150 nm length ZnO
- ZnO-200: Cuboid, 200 nm ZnO
- ZnO-900: Cuboid, 900 nm ZnO

Euclidean distances are calculated as part of a similarity assessment to compare NFs responses for each decision node, following Jeliazkova et al., [24]. Dissolution and dispersion stability were measured after 48 and 96 h. Two-dimensional Euclidean distances were calculated to integrate the distances for both 48- and 96-hour timepoints, into a single similarity assessment.

*Equation 1.* The distance,  $d(p,q)$ , is calculated between each pair of NFs, which have two co-ordinates ( $p_1$  and  $p_2$ ,  $q_1$  and  $q_2$ ), one in each dimension of the property being contrasted.

*Equation 1:*

$$d(p,q) = \sqrt{\sum_{i=1}^n (q_i - p_i)^2}$$

We use this approach to qualitatively group NFs as part of a comparative approach. In the worked example, we focus on the ZnO-NFs within these studies. However, additional results for Cu and Pb based NFs are also reported in Supporting Information 3, full discussion of which is made in the original article [59].

Constructing a data matrix on the basis of the IATA decision nodes for these six materials concludes a number of grouping outcomes, including a quickly dissolving NF, (ZnO-25, [52]) where the exposure relevant form of zinc was solutes from the material (Aquatic-Q), and partially dissolving ZnO-NFs (the materials tested in Zhai et al., [59]). Of these partially dissolving NFs, two groups are identified, smaller spherical ZnO < 100 nm for which toxicity is driven by the solutes (Aquatic-T<sub>S</sub>) and non-spheroidal ZnO-NFs > 100 nm for which the particles drive the toxicity (Aquatic-T<sub>NF</sub>).

A schematic summary of the grouping outcomes from the worked examples is provided in Fig. 2.

### 8.1. Data matrix from assessment against the IATA decision nodes

Each decision node is taken in turn and data assessed against the proposed thresholds for different groups. Similarity assessment of each decision node using Euclidean distances also road-tests the thresholds proposed for each Tiered Testing Strategy, supporting the validation of these groups based on the functional fate of NFs against in vivo outcomes for ecotoxicity.

Full methodological details supporting parameter measurements for each worked example may be found in the original publications. Dissolution and dispersion stability were measured in the test media (¼ Hoagland's media in lettuce exposures and soil pore water extracted with 10 mM BIS-TRIS buffer in microbial exposures). Both the proportional extent of dissolution and the dispersion stability were measured after 48 and 96 h. To calculate the ratio of toxicity of solutes versus particles, the soluble salt Zn(NO<sub>3</sub>)<sub>2</sub>·6 H<sub>2</sub>O was used to assess the toxicity of Zn(II). Detailed demonstration of the calculations required for the assessment of the toxicity ratio (time weighted average concentrations, response addition model of mixture toxicity etc.) are reported in

## Worked example A: Aquatic-Q

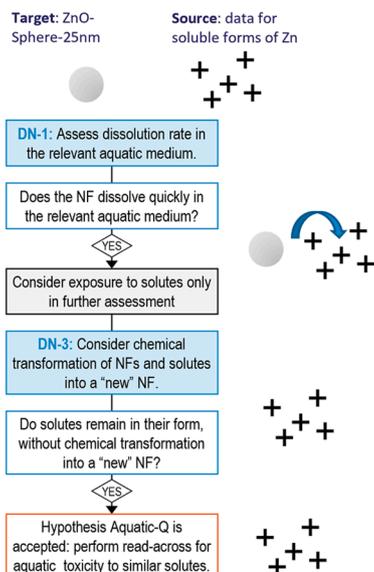
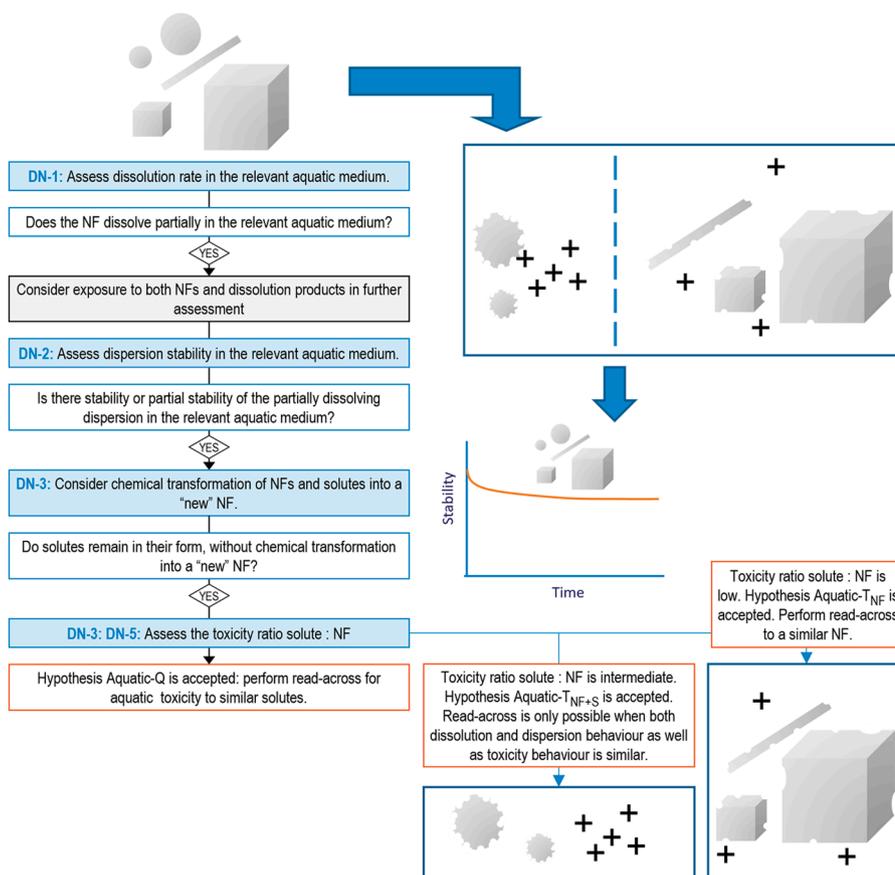
Worked example B: Partial dissolution NFs grouped as solute and particle driven toxicity (Aquatic- $T_{NF+S}$ ) or particle only driven toxicity (Aquatic- $T_{NF}$ )

Fig. 2. Schematic demonstrating the two worked examples, one using data from [52] as an example of NFs being grouped as quickly dissolving NFs in Aquatic-Q (Panel A) whilst the second example using data from [59] demonstrates particles initially grouped as partially dissolving, stable NFs, being split into two groups: Aquatic- $T_S$  where toxicity of the overall suspension is driven by the solutes, supporting read-across to similar soluble forms of the substance and Aquatic- $T_{NF}$ , where toxicity of the overall suspension is driven by the particle fraction, and so read-across may be justified to similar NFs with existing data.

## Supporting Information 3.

The summary data matrix for the ZnO-NFs is represented in Fig. 3. As well as the results for each individual ZnO-NF, a similarity assessment based on the two-dimensional Euclidean distance approach is also represented in the table for each decision node, highlighting the provisional groups that can be identified for each decision node.

## 8.2. Dissolution decision node

ZnO-25 is considered quickly dissolving, Aquatic-Q according to the IATA, with  $> 90\%$  of the  $10 \text{ mg}\cdot\text{L}^{-1}$  starting concentration dissolved after 48 h. This threshold was substantiated with comparison to the in vivo data for lettuce plants exposed to both ZnO-25 and a solute exposure of  $\text{Zn}(\text{NO}_3)_2\cdot 6 \text{ H}_2\text{O}$ , where both treatments resulted in the same  $\text{EC}_{50}$  of  $0.5 \text{ mg}\cdot\text{L}^{-1}$ . This NF is not compared in the subsequent similarity assessments as it falls outside of the applicability range for the assessment, i.e., it is already grouped beyond an existing threshold, so further similarity assessment does not provide additional information.

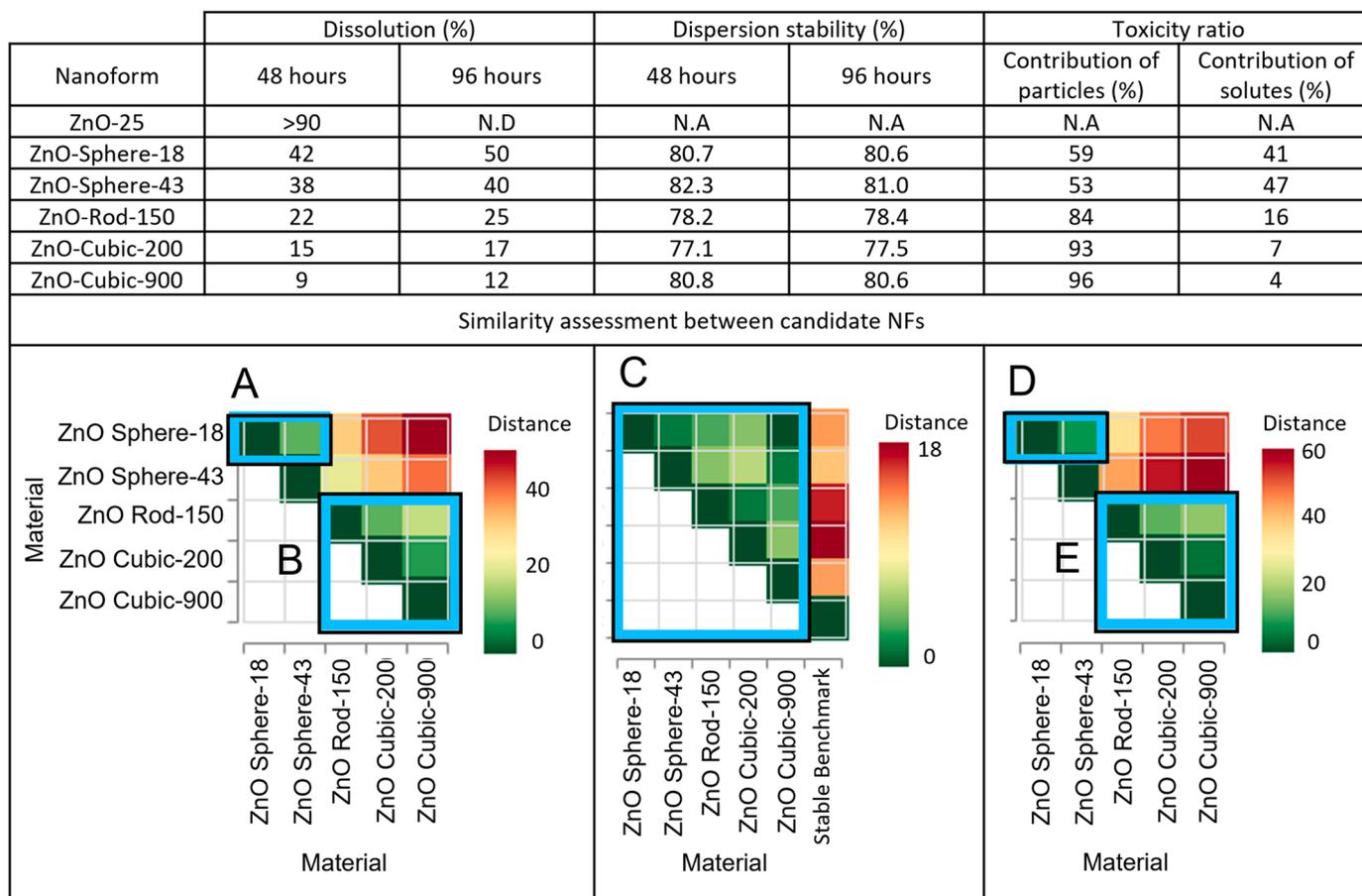
The remaining ZnO-NFs were monitored for dissolution across the test duration (96 h) in the relevant test media for the BIOLOG test, following the rationale of Tier 3 in the tiered test for dissolution. All NFs are considered partially dissolving according to the proposed thresholds for dissolution, with between  $10\%$  and  $90\%$  dissolved across the duration of the exposure. Two distinct provisional groups can be observed, as highlighted in blue boxes A and B (Fig. 3).

## 8.3. Dispersion stability decision node

Dispersion stability was not tested for ZnO-25 in  $\frac{1}{4}$  Hoagland media in the lettuce growth inhibition test as it was already found to be rapidly dissolve, meaning this NF had already been grouped under Aquatic-Q in the IATA and so further assessment was not needed. For the remaining ZnO-NFs, as these particles were partially dissolving, adjustment for this was essential to calculate the proportion of remaining particles which were stable in the dispersion, following the guidance in OECD TG 318. All ZnO-NFs would be considered partially stable in this instance, with between  $80\%$  and  $90\%$  of the material remaining dispersed consistent with a hypothetical benchmark for complete stability i.e.,  $90\%$  (Fig. 3, box C). No partitioning of particles to the air/water interface was reported in the study, with sonication used to initially disperse the NFs in water prior to series dilution in the same test media used for toxicity assessment. Therefore, the results from the dispersion stability assessment are considered valid, without interference from either extremely hydrophobic or buoyant particles.

## 8.4. Chemical transformation decision node

No assessment was made of chemical transformation. By following dissolution in the ecotoxicity test media, solutes from the ZnO-NFs are expected to behave analogously to those of the soluble zinc salt used as a solute control in the same media, so no further assessment of the fate of these solutes is deemed necessary for the purpose of this grouping.



**Fig. 3.** Data matrix for dissolution, dispersion stability and toxicity ratio for the worked example data. N.D refers to no data, whilst N.A refers to “not applicable” in the case of ZnO-25 where quick dissolution is already demonstrated and so further testing not required in the subsequent decision nodes. Within each column representing a decision node, the bottom row of the table includes a similarity assessment for that property, from left to right representing dissolution (two-dimensional Euclidean distances integrating 48 h and 96 h timepoints), dispersion stability (two-dimensional Euclidean distances integrating 48 h and 96 h timepoints) and the toxicity ratio (one-dimensional Euclidean distance using the contribution of solutes for comparison). Green cells represent more similar pairwise comparisons between NFs in the x and y axes, whilst red cells represent more dissimilar pairwise comparisons. Blue boarded boxes overlaying these similarity plots highlight provisional groupings of NFs within each decision node. These are labelled A – E for referencing in the main text.

### 8.5. Toxicity ratio of solutes versus particles decision node

No calculation of the ratio of solutes versus particles was required for the ZnO-25 NF, with > 95 % of the mass dissolving and the  $EC_{50}$  of 0.5  $mgL^{-1}$  calculated was similar to that of the soluble  $Zn(NO_3)_2 \cdot 6 H_2O$  exposure. Thus, this worked example substantiates the hypothesis that underpins Aquatic-Q, that quick dissolution leads to the solutes being the exposure relevant form and so justifying read-across to soluble forms.

The toxicity ratio of solutes versus particles of the five remaining ZnO-NFs appears to identify two provisional groups, similar to the dissolution decision node (Fig. 3, D and E). The two spheroidal particles < 100 nm in diameter with close to 50% of the effect contributed by solutes from the exposure (Aquatic- $T_{NF+S}$ ), and the other rod and cubic ZnO-NFs > 100 nm for which the particles contributed close to or more than 90 % of the observed toxicity of the overall suspension (Aquatic- $T_{NF}$ ).

One of the interests in understanding the relative contribution of solutes versus particles to the overall suspension toxicity, is that the exposure relevant form of the material can play a significant role in toxicokinetics, bioaccumulation and localisation of effects within organisms. Particles may take different pathways into biota than the dissolved solutes. For example transdermal uptake into the sediment dwelling worm *Lumbriculus variegatus* was observed for soluble Ce(III), whilst only dietary accumulation was possible for very slowly dissolving

CeO<sub>2</sub>-NFs [7]. Therefore, one implication for being separately grouped in Aquatic- $T_{NF}$  (as the non-spherical submicron ZnO particles > 100 nm were) is that properties of the NFs or particles must be considered in further justification for read-across within this group for chronic toxicity endpoints or bioaccumulation. It must first be demonstrated that the dispersion stability between NFs in this group is similar, and so expected delivered dose of the NF particles similar, before read-across could be justified.

For NFs grouped as Aquatic- $T_{NF+S}$ , the 18 and 43 nm spherical ZnO-NFs, toxicity is a combination of both the solute and the particle. Read-across between members of this group would require acceptable limits of similarity to be established for the ratio of solute versus particle toxicity. Given that the difference between these NFs was within ~1.15 fold, it might provisionally be considered that plus or minus 15 % of the mean of the provisional group would be an acceptable limit of similarity. This provisional limit would require substantiation across more case studies including NFs of different core compositions.

## 9. Summary and Conclusions

To be operationalised, grouping of NFs for risk assessment needs to use available information to identify the state of the exposure relevant form of the candidate NF(s) in aquatic systems and threshold values that may lead to change in exposure and effect. By making use of data relating to key NF behaviours affecting form and exposure in aqueous

media, this IATA is intended to aid grouping for hazard testing. Beyond regulatory applications, this IATA will be useful for other purposes such as implementation within safe(r)-by-design strategies [4,37]. For example, the IATA could be used to identify which component (e.g. solutes or particles) drives the hazard of the NF dispersion may allow targeting of interventions in the design of the NF, to reduce those aspects of the NF responsible for the observed hazard.

Utilising standardised tests of dissolution and dispersion stability and extending their interpretation to support ecotoxicity testing allows us to establish the toxicity ratio of solutes versus particles, an important advance in allowing grouping of NFs which may not undergo quick dissolution, but for which read-across to the solutes may still be justified. This assessment framework may also be suitable for those multi-component nanomaterials which consist of a (partially) dissolving component, which would extend the applicability domain of the IATA beyond the worked examples for metal oxide nanoparticles described here. The rationale and supporting evidence for each decision node has been taken in turn and gaps identified where further substantiation is needed. Decisions around chemical and biological transformations into a “new” NF are lacking established test methods, predictive models or a framework within which these new NFs can become the focus of grouping. We include this decision node here to demonstrate that the IATA can provide a framework within which new NFs through chemical transformations are identified, reintroduced to the IATA and propagated through the decision tree to allow for more suitable source materials with similar chemistries to be identified. However, more work is required to establish the tiered testing strategies that would allow for this decision node to be implemented within the decision-making process of the IATA.

The IATA is focused on grouping NFs according to their environmental fate prior to internalisation by organisms. An assessment of in vivo kinetics would be beneficial to extend the IATA and widen its application to further grouping according to ecotoxicity. Currently such a strategy is insufficiently developed to include in the IATA design, which led to the focus on fate prior to internalisation. However, this does not preclude its inclusion in the future and developments in this area should be encouraged. To widen its application for read-across between NFs grouped as the particle driving the toxicity of the suspension, further development into low tier assessment of properties relevant for driving the toxicity of the particulate form is also needed. Better understanding of the mechanisms of action, key events and eventual adverse outcome triggered by specific distribution or kinetics of nanomaterials within the body of non-mammalian species are required. Elements such as abiotic or in vitro reactivity testing (e.g. [2]) could prove useful in such an IATA but should be included only if justified following the principles of adverse outcome pathways.

Through the worked examples, we provide first evidence that the proposed thresholds based on modelling and empirical data, can be substantiated through comparison of the groupings delivered by these thresholds and in vivo ecotoxicity data, using the example of metal oxide NFs. As more data becomes available, these thresholds can be further validated or refined through application of the IATA to new examples, in the manner presented for the worked example. Emphasis should be made to generation of data for non-metallic nanoparticles and the extension to multi-component materials as these are significant data gaps. The use of technical standardised guidance for dissolution and dispersion stability encourages the use of this kind of data for multiple purposes and delivers on the aim of the IATA to efficiently collect data to support or reject a hypothesis for grouping.

#### CRediT authorship contribution statement

**Richard K. Cross:** Writing – Original draft, Methodology, Visualization, Formal analysis, Conceptualization, Writing – review & editing. **Dave Spurgeon:** Conceptualization, Writing – review & editing. **Claus Svendsen:** Conceptualization, Supervision. **Elma Lahive:** Supervision,

Writing – review & editing. **Simon Little:** Conceptualization, Writing – original draft. **Frank von der Kammer:** Conceptualization, Writing – original draft. **Frédéric Loosli:** Writing – original draft. **Marianne Matzke:** Conceptualization. **Teresa F. Fernande:** Conceptualization, Writing – original draft. **Vicki Stone:** Conceptualization, Methodology, Writing – review & editing. **Willie J.G.M. Peijnenburg:** Conceptualization, Writing – original draft, Formal analysis. **Eric A.J. Bleeker:** Conceptualization, Writing – original draft, Writing – review & editing.

#### Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

#### Data availability

Data for the worked example is provided in the supporting information as well as all calculations.

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#### Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at [doi:10.1016/j.nantod.2023.102065](https://doi.org/10.1016/j.nantod.2023.102065).

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