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GUIDANCE DOCUMENT FOR THE TESTING OF DISSOLUTION AND DISPERSION STABILITY OF NANOMATERIALS AND THE USE OF THE DATA FOR FURTHER ENVIRONMENTAL TESTING AND ASSESSMENT STRATEGIES

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INTER-ORGANIZATION PROGRAMME FOR THE SOUND MANAGEMENT OF CHEMICALS

A cooperative agreement among **FAO, ILO, UNDP, UNEP, UNIDO, UNITAR, WHO, World Bank and OECD**

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Foreword

Following a number of discussions within the OECD Working Party on Manufactured Nanomaterials (WPMN) (OECD, 2014a), dissolution rate and dispersion stability in the environment where recognised as important parameters in understanding the environmental fate of nanomaterials and nanomaterials (bio)availability. Both parameters are important in environmental risk assessment of nanomaterials. The OECD decides to develop Test Guidelines for these parameters. As a first step, the OECD developed the Test Guideline 318 on dispersion stability of manufactured nanomaterials in simulated environmental media, (OECD TG 318, 2017). Another Test Guideline, under development, will address dissolution.

Nevertheless, it was acknowledged that these parameters are often interlinked and thus the need to develop a document that could provide an overarching guidance. Accordingly, this GD focus on the dissolution and dispersion stability of nanomaterials, and the use of the data for further environmental testing and assessment strategies. It includes an excel spreadsheet as an accompanying document.

This document was led by Germany and benefited from the inputs of the Joint WNT/WPMN Expert Group on Fate and Ecotoxicity of Manufactured Nanomaterials.

1 Introduction

1.   In the OECD Expert Meeting in Berlin 2013 (OECD, 2014a), it was identified that dissolution rate and dispersion stability in the environment are important parameters for nanomaterials, i.e. these parameters are main drivers in environmental fate of nanomaterials and nanomaterials (bio)availability, and as such important in environmental risk assessment of nanomaterials. It was concluded that Test Guidelines (TGs) should be developed for these parameters. As these parameters are often interlinked it was acknowledged that an overarching guidance document (GD) would be beneficial as well.

2.   A Test Guideline (TG 318, OECD 2017) on dispersion stability of manufactured nanomaterials in simulated environmental media is already available since 2017.

3.   A number of projects are being developed simultaneously at OECD to address different environmental parameters. For example, a TG on dissolution rate in environmental media which purpose is to develop and adequately validate a robust method in standardised conditions for dissolution of nanomaterials. Meanwhile, also other relevant methods for dissolution rate testing in water and biological fluids as well as transformation in the environment will be included in upcoming TG and GD for nanomaterials. In view of the current lack of harmonised methods, this GD includes dissolution relevant content based on available information in scientific literature, and GD 29 (OECD 2001) also takes into account the current draft document on dissolution rate in environmental media. In doing so, the GD provides interim guidance on experimental steps and procedures of batch and dynamic flow-through methods and decision support when to use them for nanomaterials (Chapter 2) until new TGs and GDs become available. When these OECD projects are finalised and TGs available, an update of this GD might be needed.

4.   This document provides guidance for the methods to address dissolution rate and dispersion stability for nanomaterials with focus on environmental aqueous media. The definition of nanomaterials as having one dimension between 1 and 100 nm is generally accepted (ISO 2017a, EU 2011). The guidance provided here is relevant for solids in the nanoscale as well as their aggregates and agglomerates and it focuses on their fate and behaviour in aqueous media. In particular it presents the influence of various experimental conditions on the performance and outcomes of the discussed methods. In addition, this GD addresses modifications or additions to the methods and aims to give support for the interpretation of the test results.

5.   Chapter 2 provides guidance for the determination of solubility and dissolution rate based on batch test and flow-through methods as well as on how to evaluate and report the gained test results.

6.   Specific guidance on TG 318 is given in Chapter 3 including further experimental conditions than described in the TG, guidance to account for heteroagglomeration (section 3.3), and deriving attachment coefficient(s) (section 3.4). In order to address the latter issues, the state of the knowledge was included from available scientific literature. Furthermore, guidance is provided on the interpretation and presentation of data addressing the endpoint.

7.   The use of data generated by dissolution testing and testing of dispersion stability using TG 318 for possible further nano-specific fate and effect testing and assessment strategies is presented in Chapter 4. A testing strategy is presented in section 4.2. Furthermore, influence of the two endpoints to each other is discussed, i.e. dispersion stability will influence dissolution rate and vice versa.

8.   In Chapter 5, this GD provides recommendations on the use of output data from dissolution rate and dispersion stability tests to derive input parameters for exposure models.

9.   Chapter 6 provides information on the use of this GD in relation to other OECD TGs and GDs, including the foreseen GD on aquatic and sediment toxicity testing (see section 6.5) and the foreseen GD on the apparent accumulation potential of nanomaterials in fish (see section 6.7). As fate estimations of nanomaterials in soil and sediment are challenging to conduct, the GD also gives advice on screening possibilities for dispersion stability and dissolution rates by varying the environmental conditions to mimic those in soil and sediment (see section 6.8), and this is linked to the foreseen nano-specific GD to support the implementation on the OECD TG 312 for nanomaterials safety.

2 Testing of solubility and dissolution rate

10.   It is important to clearly distinguish between the terms solubility (the ability of a substance to dissolve in a solvent), dissolution (the process under which a substance dissolves over time) and dissolution rate (the amount of dissolved substance versus time). Solubility and dissolution rate of nanomaterials are important to predict their fate and behaviour in the environment and for understanding the changes in their bioavailability, reactivity, fate, and toxicity. Dissolution rates from nanomaterials are particularly important in determining risk/hazard since the rate of release of ions/molecules prior to interaction/complexation with ligands may be more important than equilibrium concentrations.

11.   General methods for the determination of solubility and dissolution rate are available e.g. OECD TG 105 (OECD, 1995), Misra et al. 2012, and ISO 19057 (ISO 2017b). All these methods feature different advantages and disadvantages for nanomaterial testing. So far, no specific OECD TG is available for determination of solubility and dissolution rate for nanomaterials. However, there are two WNT projects underway one addressing *Dissolution Rate of Nanomaterials in Aquatic Environment*, and a second on the *Determination of Solubility and Dissolution Rate of Nanomaterials in Water and Relevant Synthetic Biological Media*, aiming to provide harmonised approaches for testing solubility and dissolution rate of nanomaterials via static batch testing and dynamic flow-through methods, respectively.

12.   In the existing OECD TG 105 (OECD 1995) two methods are described for the determination of solubility of substances, a static batch test and a dynamic test. For determining dissolution (rate) of nanomaterials the same set-up may be used with some modifications. In addition, the existing OECD GD 29 (OECD 2001) for metals and metal compounds may be applicable to some nanomaterials. Scientific basis for the flow-through method can be found in literature e.g. in Koltermann-Juelly et al. 2018.

2.1 Static Batch Test

13.   Currently a draft TG is in preparation on Dissolution Rate of Nanomaterials in Aquatic Environment. This TG is based on OECD GD 29 (OECD 2001, Guidance Document on Transformation/Dissolution of Metals and Metal Compounds in Aqueous Media) while making some nanospecific amendments. Care should be taken regarding the applicability of OECD GD 29 when considering the purpose of testing solubility and dissolution rate of nanomaterials. OECD GD 29 aims to provide supplemental information for aquatic ecotoxicity testing of metal and metal compounds. 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". As such the original purpose of OECD GD 29 is not to provide a harmonised approach to determine solubility or dissolution rate to provide information on environmental fate. Some of the pros and cons of a static batch test as described in GD 29 are discussed in this section.

14.   The testing of nanomaterials' solubility and dissolution rate often goes beyond the scope of GD 29. For example, GD 29 asks for testing the smallest available particle of a metal or metal compounds to achieve maximum concentration of the dissolved ion of an investigated metal. Inversely, for nanomaterial the solubility or dissolution rate of a specific nanomaterial under investigation is of interest, which can differ by e.g. available surface area or surface modification. As another example, the screening test in GD 29 requires test conditions where a metal/metal compound shows highest solubility. For nanomaterials, the

testing of solubility/dissolution rate often relates to the question about the nanomaterial's fate under certain conditions (i.e. not necessarily the highest solubility).

15.   For testing of solubility and dissolution rate the use of the 24 h screening test of GD 29 can still be of interest for these questions and can be in principle applied, depending on the nanomaterial properties. This seems to work at least for silver nanomaterials (Wasmuth et al, 2016). For instance, the screening test can provide solubility limits (maximum amount of a solute that can dissolve in a solvent at a specified temperature) for all nanomaterials and estimation of dissolution rate for sparingly soluble and slowly dissolving nanomaterials. However, for determining dissolution rate, the concentration of dissolved ions needs to be measured over the time with short measuring intervals as long as steady state concentration is not reached. This is of particular importance for such nanomaterials that dissolves very fast, i.e. within a few hours.

16.   GD 29 prescribes a 0.20 µm filtration method for separating dissolved and non-dissolved compounds. This is not appropriate for nanomaterials that have individual particles or aggregates that are usually smaller than this size cut-off, so further separation is needed. ISO 19057 (ISO 2017b) reviews separation techniques that are applicable for nanomaterials related to in vitro biodurability testing.

17.   For the screening test the OECD GD 29 proposes the use of reconstituted standard water with pH range between 6 to 8.5 (i.e. water of known composition, for details on media composition see OECD GD 29). However, in principle and based on the purpose of the testing, the 24 h screening test can be also performed under different media conditions (e.g. ecotoxicity media, natural water, simulated media according to TG 318). Anyway, it is essential to characterise and report test media characteristics as detailed as possible, as media composition influences nanomaterial's dissolution (Misra et al., 2012). Reporting should include at least pH at start, after equilibrium time and the end of testing, ionic strength, if possible the presence and the concentration of polyvalent ions, and the composition and concentration of DOC (Dissolved Organic Matter; e.g. NOM (Natural Organic Matter).

18.   If the nanomaterial fully dissolves during the test duration, the solubility limit of the nanomaterial is equal or higher than the applied starting concentration. Therefore, starting concentration of the investigated nanomaterial has to be high enough to determine the solubility limit. If the solubility limit is not reached within 24h, the test duration can be prolonged in principle. However, this will not change the general statement about the solubility of a nanomaterial (e.g. poorly or highly soluble). When performing the batch test for determination of dissolution rate the following aspects should be considered: The ion concentration in the test media may increase to such an extent that the apparent dissolution rate will reduce. This is caused by a too long observation period (as measuring points will become too far apart and result in a slow increase of the straight that represents the dissolution rate). Here, shorter measurement intervals can provide a corrective. Derivation of dissolution rate is not possible for those nanomaterials that show such a rapid concentration increase of the dissolved fraction during testing which cannot be resolved by measurement.

19.   Speciation calculations can be useful for the investigated nanomaterial to estimate its general tendency of dissolution in the used test media e.g. using the freely available software Phreeqc (The software PHREEQC (ver. 3 from the United States Geological Survey: https://www.usgs.gov/software/phreeqc-version-3/ or VMinteq (https://vminteq.lwr.kth.se/). When dissolution tendency is calculated by such software, the full characteristic of the test media should be used. As the various existing databases which exhibit data on substance properties, specifications, and on media characteristics for calculation have different level of information, it should be verified that the chosen database contains relevant information for the case under consideration. However, not all nanomaterials are covered by existing data base entries.

20.   Three main methods exist in the literature to separate nanomaterials and their aggregates from their dissolved fraction: ultra-centrifugation, dialysis and centrifugal ultrafiltration. Research has shown that filter pore sizes of 0.1 to 0.02 µm could be suitable for separation of some nanomaterials from dissolved

species (Jünemann and Dressman, 2012) by filtration. As retention depends also on the filter material this should be reported together with filter pores size in case filtering was used for nanomaterial separation. To reveal possible artefacts (e.g. nanomaterials sticking to filter membranes), mass balance between the amount of nanomaterial initially introduced, the retained, and passed fraction can be useful.

21.   Ultra-centrifugation is not recommended for several reasons. Firstly, it is difficult to calculate the optimal centrifugal settings (speed and time) to guarantee complete centrifugation of nanomaterials, especially for the case of non-spherical particles and when the rotor has no swing-bucket design but is e.g. a fixed angle rotor. Secondly, theoretical optimal centrifugal times are often long relative to the dissolution rate, especially in the case of relatively small nanomaterials or nanomaterials with low density. For those nanomaterials, proportionally long centrifugation times are needed to be separated them from their dissolved ions. At the same time, they might dissolve faster compared to larger nanomaterials of the same composition. A too slow separation technique thus hampers the determination of relatively fast dissolution rates. Also, often the accurate density of the investigated nanomaterial (e.g. nanomaterials with coating/ligands) is not known and it is difficult to calculate the correct speed settings. Finally, back-diffusion of centrifuged nanomaterials into the centrifuge vial during sampling is likely and may cause artefacts of overestimating dissolution. To minimise sampling of back-diffused nanomaterials it is therefore recommended to sample just below the surface.

22.   Dialysis is also not recommended for separating nanomaterials and their dissolved substances. In this technique, nanomaterials are suspended in a medium within a dialysis bag. Dissolved substances thus need to diffuse through the dialysis membrane into a second compartment where they can be sampled for quantification of the dissolution rate. This process may again be too slow compared to the dissolution process itself (see e.g. Franklin et al., 2007). Furthermore, it is difficult to determine if any sample gets stuck to the tubes. Thus, the sample that finally reaches the dialysis bag might not be the same as introduced.

23.   Centrifugal ultrafiltration is the recommend method for separating nanomaterials and their dissolved substances taking into consideration specific measures (see paragraph 24). Here, a mixture of nanomaterials and their dissolved substances are injected in centrifugal ultrafiltration devices. During centrifugation, nanomaterials and dissolved substances and the test medium are transported towards an ultrafiltration membrane through which nanomaterials cannot pass while their dissolved substances can. The dissolved substances can then be measured in the filtered media.

24.   The pore diameter of ultrafiltration is expressed in terms of molecular weight cut-off (MWCO), i.e. the molecular weight of different molecules in the filtration process (usually dextran or polyethylene glycol) that are retained for 90 % by the membrane (Ren et al., 2006). There is also a pore size distribution, rather than a single pore size. For separation, a MWCO between 3-10 kDa is recommended. This MWCO value corresponds with pore sizes between 2-3nm (Ladner et al., 2012). However, care should be taken as the use of low MWCO filter membranes can lead to a built up of ions in front of the filter. This results into a measurement of a lower dissolution rate. Depending on the properties of the nanomaterials, their ions, and the membrane properties adsorption of the nanomaterial and/or the ions to the filtering membrane can occur. Furthermore, media compounds like NOM can block the membrane. In such a case, filter with MWCO values above 10 kDa may be useful considering that individual nanomaterials can pass the membrane, too.

25.   The centrifugal speed and time required to drive a sufficient amount of aqueous solution containing dissolved species to cross the membrane depends on the MWCO and hydrophobicity of the membrane, as well as the chemistry of the medium. The centrifugal settings should be optimized to achieve a filtrate volume sufficient for subsequent measurement. Prewashing of the filter membrane by centrifuging ultrapure water through the membrane is prerequisite for any filtration step to remove dissolved chemicals that could influence the dissolution process. Modifying or pre-treating the membranes can be used if significant issues are observed, e.g. binding to the membrane (Cornelis et al., 2010; Hedberg et al., 2011).

Prior to use, a centrifugal filtration device should be evaluated concerning the interaction with the investigated nanomaterial. This can help to assess possible loss of ions and to avoid an underestimation of dissolution rate (Kennedy et al., 2010).

26.   In principle the batch test procedure may also be applicable for testing non-metal nanomaterials, but the current analytical possibilities still limit these options. The selection of an analytic method needs to include considerations of e.g. the detection limit of the method in relation to the investigated nanomaterial or the necessary effort to distinguish between the investigated nanomaterial and the media compound (e.g. NOM and carbon-based nanomaterials). Therefore, the decision on an appropriate analytic method currently can only be done case-by-case.

2.2 Dynamic testing of dissolution rates

27.   An OECD project under development on the determination of solubility and dissolution rate of nanomaterials in water and relevant biological media aims to include two different methods: both a static batch test, and dynamic dissolution testing by a flow-through system. The method applied for a flow-through dissolution test is based on the amended Continuous Flow System mentioned in ISO TR 19057 (ISO 2017). Here, simulated media is continuously pumped from a reservoir through a cell containing a nanomaterial samples. After the media has passed the nanomaterial sample the solute concentration in the fluid can be measured. The method was applied in the past to determine the bio-durability for mineral fibre and its applicability to nanomaterials was presented by Koltermann-Juelly (2018) for the dissolution of 24 (nano)forms of 6 substances (Figure 1 below) for various human lung fluids and by Bove et al. (2018) for various gastro fluids.
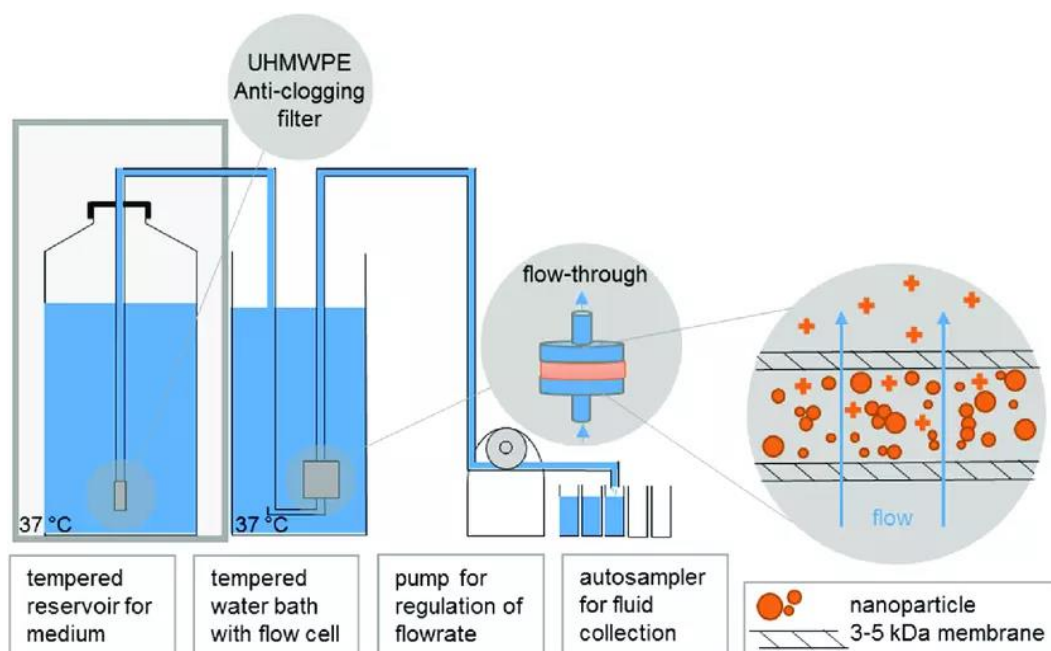
Figure 1. Possible experimental setup for flow-through testing (from Koltermann-Juelly et al. (2018)) (UHMWPE = Ultra High Molecular Weight Polyethylene)

28.   In principle the dynamic method can be adapted to measure dissolution rate in environmental media. However, depending on the purpose test conditions might need to be adapted for differences in environmental compared to biological media. Apart from differences in test media composition (e.g. pH, ionic or organic compounds), considerations for adaptation include applied test concentration, flow rates and test duration. For instance, for the determination of dissolution rate under environmental relevant

conditions considerably different concentrations (e.g. media composition, temperature) and flow rates (first suggestion µg and 1 ml/min) should be used compared to those used for the dissolution testing in biological media (mg and 2 ml/h), thus saturation effects are unlikely to occur.

29.   The dynamic dissolution test should mimic the condition in a natural water, where the nanomaterial is highly diluted and freely diffusing. The dissolution rate of intermediate and highly soluble materials is among other parameters controlled by the thickness of the boundary layer surrounding the nanomaterial and the concentration gradient of dissolved ions in the boundary layer. For those nanomaterials the transport of ions away from the particle surface is a limiting factor. The flow rate in the experiment should ideally mimic those conditions. With 2 ml/h saturation effects have been observed with $BaSO_4$, at 1 mL/min these effects are reduced but not totally prevented. Flow rate and test conditions can be in principle modified to mimic other specific environmental relevant conditions. For comparing the dissolution rate of different nanomaterials, the chosen test conditions for those nanomaterials should be the same.

30.   With current scientific knowledge and the co-dependence of the dissolution rate on solubility, thickness of the nanomaterial's boundary layer and specific surface area/particle size, exact cut-off values for applying a dynamic dissolution test cannot be given. From current experiences the dynamic test would be suggested if the solubility of the nanomaterial is between 0.1 and 10 mg/L. For most of these nanomaterials constant values for dissolution rate were achieved within less than 12 hours. Values for dissolution rate should be determined with sufficient narrow measurement intervals until constant values are achieved. The investigation of nanomaterials with lower solubility is currently limited by the ability to detect the low concentration of released ions. The applicability range of the method may be broadened when test conditions such as flow rate and amount of material in the test are adapted to the solubility of the material.

31.   In addition, the test procedure can be adapted in such a way that the application of the investigated nanomaterial can be injected directly as a dispersion into the system at a location between the pump and the filter membrane (MWCO between 3-10 kDa). A schematic overview of the dynamic test system is presented in Figure 2. Filter membranes with low MWCO can help to avoid the passage of small particles. However, care should be taken as the use of low MWCO filter membranes can lead to a built up of ions in front of the filter. This results into a measurement of a lower a dissolution rate (see also paragraph 20 and 24).

32.   Similar to the batch test, in principle the flow-through test procedure may also be applicable for testing of non-metal nanomaterials, but the analytical possibilities still limit these options. In consequence, the selection of an analytic method also needs to include considerations of e.g. the detection limit of the method in relation to the investigated nanomaterial or the necessary effort to distinguish between the investigated nanomaterial and the media compound (e.g. NOM and carbon-based nanomaterials). Thus, as for the batch test, the decision on an appropriate analytic method currently can only be done case-by-case.
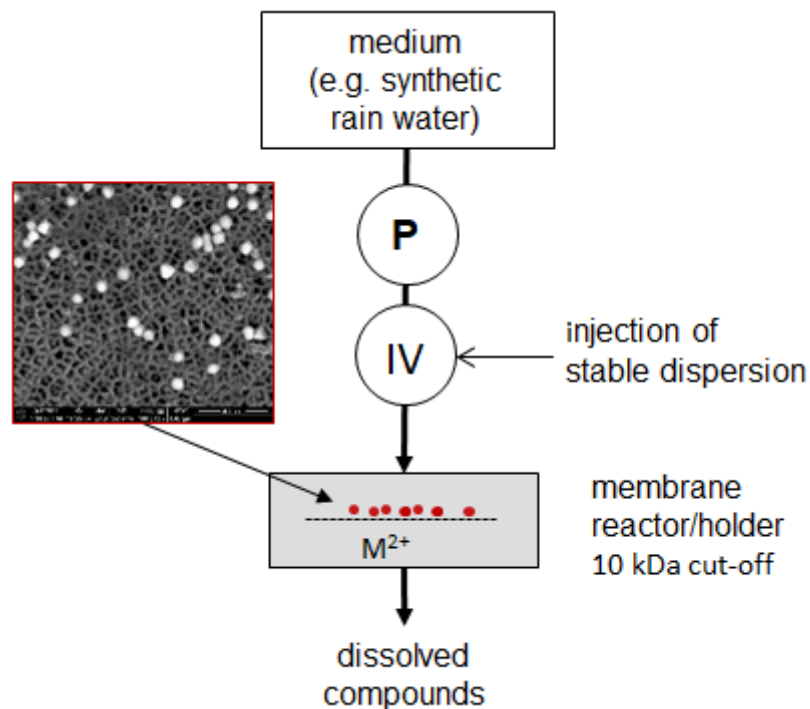
Figure 2. A draft scheme for dynamic testing of dissolution rate (von der Kammer 2018, personal communication, Stetten et al. in preparation). (P = pump, IV = Injection Valve).

2.3 Data evaluation and reporting from dissolution testing

33. The mass concentration of the dissolved fraction should be measured during the test and plotted versus time. The result of the solubility test has to be expressed as mg/L of the ions formed as a consequence of dissolution. Expression of solubility in % alone (without also providing results in mg/L) is discouraged as this is difficult to interpret and to compare with other data.

34. For most existing nanomaterials, dissolution follows (pseudo-)first order kinetics. In a given medium, the determined dissolution rate, expressed initially as increase of dissolved ions concentration per time (mg $L^{-1}s^{-1}$), will depend on the initial mass of nanomaterial in the test, the specific surface area of the nanomaterial, the solubility of the nanomaterial, and the test conditions (shaken, stirred or not agitated at all). This can be calculated to the loss of solid nanomaterial over time and expressed as:

Equation 1:

$$\text{Dissolution rate } = \frac{d\text{m}}{d\text{t}} = -k_{\text{diss}} \cdot \text{m}$$

where m is mass of the nanomaterial, t is time, and k_{diss} is dissolution rate constant. The dissolution rate is of interest in most cases. However, a value given as mg $L^{-1}s^{-1}$ is of little use, because this value will depend largely on the initial mass of the nanomaterial in the test system. Hence, the dissolution rate should be normalised to either the mass or the surface area of investigated nanomaterial. Both have different characteristics. Results normalised to mass would only be valid for the investigated nanomaterial. Dissolution rates normalised to mass cannot be transferred to other forms of the same substance (e.g. of different specific surface area or different particle size). For this purpose, dissolution rate normalised to surface area will provide a remedy. The dissolution rate should be expressed as mg $kg^{-1}s^{-1}$ or, preferably, when specific surface area of the material is available, as mg $m^{-2}s^{-1}$. Since the dissolution rate is depending

on the solubility of the nanomaterial, the available surface area of the nanomaterial in the test system, and the countering concentration of surrounding dissolved ions of the nanomaterial the rate can be derived by using the Noyes-Whitney equation:

Equation 2:

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

where D is the diffusion coefficient of the dissolved species in the medium, A is the surface area of the nanomaterial, h is the thickness of the diffusion layer, c_S is the saturation concentration (solubility limit), and c is the concentration of dissolved ions in the test medium. The thickness of the diffusion layer will be dependent on the test condition e.g. the composition of test media or potential agitation of the nanomaterial during the test performance and can only be estimated, what limits the applicability of the approach.

35.   It should be noted that there are nanomaterials that might follow zero or second order dissolution kinetics and thus, it has to be reviewed how models relate to nanomaterials of different shape and surface area. Care should be also taken for nanomaterials with broad size distribution as the smaller particles tend to dissolve faster than the larger ones. This could lead to an incorrect choice of fitting models or impede the application of a correct reaction order at all.

36.   Based on the dissolution rate constant halftime (when half of the nanomaterial is left and half is dissolved) can be estimated (for information on calculation model see e.g. chapter 11.5 of ISO 2017b).

37.   Results on solubility and dissolution rate have to be reported together with test conditions like media composition, temperature, test duration, and characteristics of the investigated nanomaterial (e.g. size distribution, shape, and composition including information on surface coating/modification). Regarding environmental exposure modelling, one of the important nanomaterial characteristics required is the dissolution rate constant (k_{diss}) and not the dissolution rate itself. The use of k_{diss} for exposure modelling is discussed in Chapter 5.

3 Testing of dispersion stability with TG 318

38.   OECD TG 318 (OECD, 2017) describes a method for determining the dispersibility and dispersion stability of nanomaterials in aqueous media of different, yet environmentally relevant hydrochemistry. A prerequisite for the experimental approach was to enable the investigation of the dispersion behaviour in a small number of relatively simple tests within a time frame suitable for standard laboratory routine. The hydrochemical conditions in the tests cover those parameters and parameter ranges which are (a) representative for natural waters and (b) are recognized drivers for agglomeration of nanomaterials within the concentration range of the TG (Monikh et al., 2018). Hence the composition of the test media reflects only those compounds in surface water that are relevant for the agglomeration process and in a concentration range where they can become relevant.

39.   Dispersion stability as measured using TG 318 actually determines homoagglomeration (attachment of nanomaterial to each other) under consideration of environmental parameters which have a major influence on the dispersion stability of nanomaterials over a fixed time-span of 6 hours. This enables a direct comparison of nanomaterials with each other and how they will behave in test systems. For comparison with media which differ in composition from the test media in TG 318, the agglomeration-relevant compounds in the media should be compared. These are the concentrations of divalent cations and anions, the pH, and the concentration of natural organic matter.

40.   The kinetics of the homoagglomeration processes are depending on the number concentration of the nanomaterials and the progression of agglomeration. To be able to directly compare results among different nanomaterials and also to finish the test over a period of 6 hours, the starting concentration must be set to a fixed particle number concentration. In this way the agglomeration process is almost independent of particle size and density. Comparisons have shown that the starting concentration in particle number of 10^{12} particles/L should not vary more than one order of magnitude between different nanomaterials (i.e. roughly plus or minus half an order of magnitude). To obtain the required mass concentration of the nanomaterial, the mass concentration of the nanomaterial in the stock dispersion has to be converted into particle number concentration by using the average particle diameter and material density as described in TG 318. It is acknowledged that, especially for nanomaterials with a broad size distribution, the average particle size will not convert correctly into the particle number concentration. However, the influence on the test outcome appears to be small (order of magnitude accuracy required) so that the additional effort for precisely determining the particle size distribution and considering it in the number calculation appears not necessary. However, if precise data on the particle size distribution are available, it is advised to use this information. For an example see Figure 3.
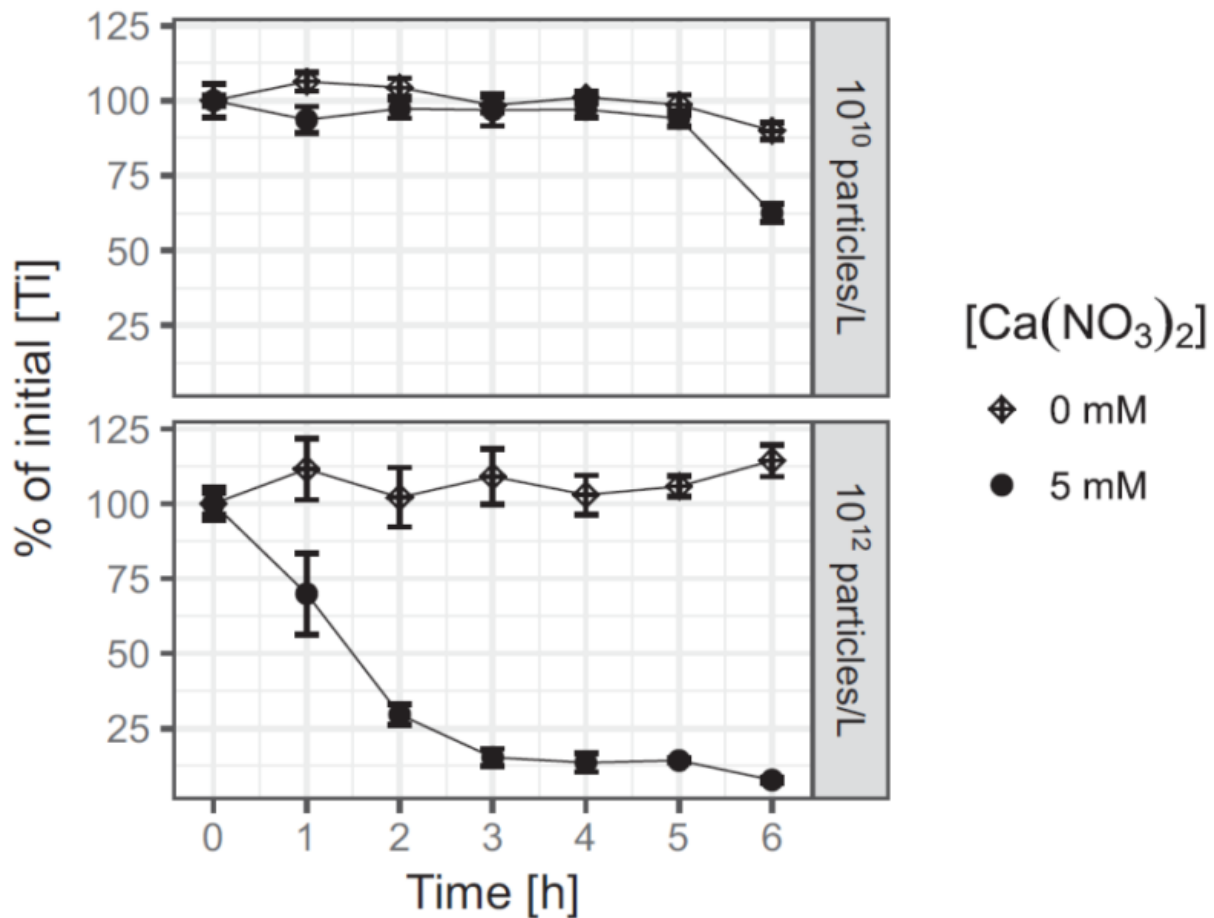
Figure 3. Example of experimental data over the test period of 6 h for TiO₂ NM-105 nanomaterials under stable (0 mM Ca(NO₃)₂; open squares) and destabilizing (5 mM Ca(NO₃)₂; filled circles) conditions, and two different starting particle number concentrations (10^{10} and 10^{12} particles/L) affecting agglomeration kinetics (Monikh et al. 2018).

41.   With progressing agglomeration and sedimentation of the agglomerates, the concentration in the supernatant will decrease over time. The sedimentation velocity, and thus also the removal of nanomaterial from the supernatant by sedimentation is depending on many factors, e.g. the density of the primary particles, the apparent density of the formed agglomerate, the structure of the agglomerate, the surface chemistry, and how the water flows around or through the agglomerate. To eliminate at least the effect of density, the last step after 6 hours is a centrifugation step where the run conditions of the centrifugation are set to achieve a size cut-off at > 1 µm. TG 318 describes how to calculate the centrifugation conditions and an Excel spreadsheet-tool is accompanying TG 318 for those calculations. After the centrifugation step, the remaining concentration of the nanomaterials in the supernatant of the dispersion is analysed. The centrifugation step after 6 hours is best suited to compare different materials with each other, while the hourly measurements between 1 to 5 h show the behaviour of the material in a water column.

42.   Apart from the intrinsic properties of the nanomaterial, the composition of the medium is the driver for the stabilisation or destabilisation of the dispersions. Therefore, the test considers the concentration ranges of the composition of natural waters that are dominating this process and are sufficiently abundant in natural waters to become relevant in the process, i.e. divalent ions, natural dissolved organic matter and pH (Ottofuelling et al. 2011). The concentration ranges of these compounds were set to represent about 95% of the conditions found in natural waters. The various effects of different media components on dispersion stability are listed in Table 1.

Table 1. Role and effect of the selected components in the synthetic water. The Table shows representative compounds in natural water that mainly influence nanomaterial's stability in aqueous media. Furthermore, it highlights the effects of these compounds on the stability of nanomaterials and the strength of these effects as well as the abundance of the compounds in natural water.

compound	represents	effect	effect strength	abundance
Na^+	monovalent cations not included	destabilisation	low	medium
Ca^{2+}	divalent cations	destabilisation stabilisation when adsorbing to surfaces of positively charged materials	medium	high
Mg^{2+}	divalent cations less complexed by NOM	destabilisation relevant when NOM is high and complexes Ca^{2+}	low	low
NO_3^-	monovalent anions	destabilisation non-adsorbing non-complexing	low	low
SO_4^{2-}	divalent anions	destabilisation, especially positively charged particles	medium	medium
Al^{3+}	trivalent cations not included	destabilisation	high	very low
SR-NOM	dissolved organic matter	stabilisation through surface adsorption with electrostatic (negative) and steric effect charge reversal (positive to negative) through complexation of destabilising cations destabilising when adsorption to positive surfaces reduces net charge of surfaces	high	medium

43. A decision tree is presented in the TG 318 to determine whether the nanomaterial of interest requires only a screening procedure or if an in-depth testing has to be performed. The decision tree allows nanomaterials to be categorised as generally stable in dispersion under all test conditions, non-dispersible or dispersible depending on the hydrochemical conditions. The screening test is performed in the presence of natural organic matter, which will generally stabilise most nanomaterials against agglomeration. This will put many nanomaterials into the category "dispersible, no detailed testing in TG 318 necessary".

44. The NOM added to the test vial has three roles in the test: (a) it acts as a pH buffer stabilizing the adjusted pH, especially at pH 9, (b) it complexes Ca^{2+} and reduces thereby the activity of the destabilising Ca^{2+} ion, (c) it adsorbs to the surface of the nanomaterial and adds to the negative charge density, thereby reducing the net positive charge that can lead to destabilisation. If present in sufficient amounts, it eventually may reverse the charge to negative and can increase the magnitude of the negative charge density. The amount of NOM to be used is standardised to 10 mg/L DOC in 40 ml (400 µg DOC) and a calculation tool for the required minimum DOC is provided in the TG 318. It should be considered that under certain conditions (e.g. NOM composition) NOM can lead to a destabilisation. In addition, some nanomaterials may not adsorb to NOM under surface-water like conditions (Hedberg et al. 2017a, Hedberg et al. 2017b, Pradhan et al. 2017)

45. Applicability of TG 318 for different nanomaterial types should be considered based on the available data on similar nanomaterials, e.g. based on their size and shape (spherical, rod, platelets, fibre-like). Three considerations are important to judge a priori if the test can be applied to a nanomaterial: (a)

the nanomaterials density must be > 1 g/cm³, (b) the mass concentration calculated for the required particle number concentration must be at least 10 times above the quantification limit of the analytical method, (c) an analytical method with sufficient sensitivity for the nanomaterial or an equally distributed component is required. The requirement (b) stems from the consideration that even with 90% removal from the water column the nanomaterial should still be quantifiable in the supernatant. Especially for small, low density particles, the mass concentration originating from the recommended number concentration might challenge the ICP-OES based methods and at some point, also routinely operated ICP-MS methods. The required quantification limit (10% of initial concentration) for a SiO_2-NP of 15 nm would be ~0.2 µg/L (Si).

46.   Regarding the analytical approach to quantify the nanomaterials in the supernatant in principle any method/instrument (e.g. ICP-MS, GFAAS, polarography) can be applied which offers the required detection limits for the nanomaterial or a relevant component of it. Besides a dispersion stability measurement, zeta potential as calculated from electrophoretic mobility can provide an indicator for nanomaterial stability. Measurement of zeta potential over time could provide indications for heterogenous nanomaterials (like Figure 7c). However, this method will not provide information on the amount and behaviour of the (un-) stable fraction.

47.   As an alternative method to determine dispersion stability, TG 318 provides information on the measurement using UV/VIS photometry. The continuous monitoring of the agglomeration/settling process in a UV/VIS photometer will yield to quantitative different results compared to the standard test procedure of TG 318. This is due to a different detection principle, a different location of analysis (close to the bottom of the vial and not at the top of the supernatant) and different test vessel size and geometry. Also, the centrifugation step is missing and therefore low-density nanomaterials could agglomerate without being settled out. A direct comparison of quantitative results obtained from both methods is therefore impeded.

48.   Changing cuvettes could multiplex the experiment, giving the opportunity of several parallel tests in cuvettes and placing them in the spectrometer one after the other for absorbance determination. However, moving the cuvettes can disturb the settling process and introduce errors.

49.   The wavelength of the UV/VIS signal needs to be in correlation with the properties of the investigated nanomaterial. It should be noted that the change of absorbance can be caused by processes other than settling-out of agglomerates. When the nanomaterial agglomerates the change in size distribution also changes the optical density and hence the absorbance reading even before settling starts. Dissolution would reduce the absorbance without agglomeration taking place. This would underestimate the stability, contrary to the standard test with determination of the remaining fraction in the supernatant, where dissolved ions would lead to an overestimation of stability in case the dissolved fraction is not quantified. Transformation of the nanomaterial might reduce e.g. the plasmon resonance of metallic nanomaterials and incorrectly decrease the absorbance reading. Therefore, it should be evaluated with care, if measuring dispersion stability using UV/VIS spectroscopy is an appropriate approach for the investigated nanomaterial.

3.1 Data presentation and evaluation for TG 318

50.   When the dispersion stability of a nanomaterial is tested according to TG 318 either the screening test is already sufficient, or a full testing is required.

51.   There are various ways to present the outcomes of the screening test or to visualize the influence of electrolyte concentration, pH, and presence of NOM. Examples of data presentation from dispersion

stability studies using TG 318 are shown in Figure 4, 5, and 6. A spreadsheet tool[1] is accompanying this GD to facilitate such a harmonized presentation of the data.

52.   Figure 4 shows a schematic example on dispersion stability for results of the screening test with the three possible outcomes according to the TG 318 decision tree.

53.   In Figure 5 the output of the tool for illustrating the full tests' results (following Monikh et al. 2018) is presented. Here the results are presented in a three-dimensional matrix considering various hydrochemical conditions and the resulting dispersion stability. Higher colloidal stability (less agglomeration/sedimentation) is reflected by a higher remaining concentration (brighter shade in the plots) in the dispersion relative to the starting concentration (0-100%).

54.   The full test provides information on dispersion stability and the underlying processes leading to the removal from the water column can be elucidated. Figure 6 presents an example of a dispersion stability plot of the full test for one test condition where the dispersion stability measured at each hour is plotted over the time. Possible interpretations of removal processes based on the removal function are shown in Figure 7. The interpretations shown here may also help designing testing strategies (Section 4 of this GD).

55.   Besides usual information on test material and test media data, reporting from the UV/VIS method should include details of the UV/VIS method (instrument type, detection wavelength in nm, type of cuvette used), the recorded absorbance over time profile, and the remaining fraction after 24h given as absorbance reading after 24h divided by the absorbance reading at the start of the test.

Figure 4. Example outputs from the spreadsheet tool for the screening test at different Ca^{2+} concentrations (y-axis) and pH-values (x-axis) in the test medium. (The numbers in the boxes indicate the % of initial concentration of the test material left in the supernatant at the end of the test). Left panel: all tests are completed with ≥90 % of the nominal (initial) concentration left in the supernatant after 6 h (indicating high stability). Middle panel: some conditions lead to stable (≥90 %), some to intermediate stability (≥10 % and ≤ 90%), further testing is required in a full test. Right panel: all conditions lead to ≥ 90 % sedimentation from the water column (≤10 % stability), indicating an unstable nanomaterial under tested conditions. A green tick indicates those cases where a nanomaterial is highly stable and a red cross where a nanomaterial is unstable under the respective conditions. A yellow exclamation mark indicates cases were a nanomaterial shows intermediate stability.
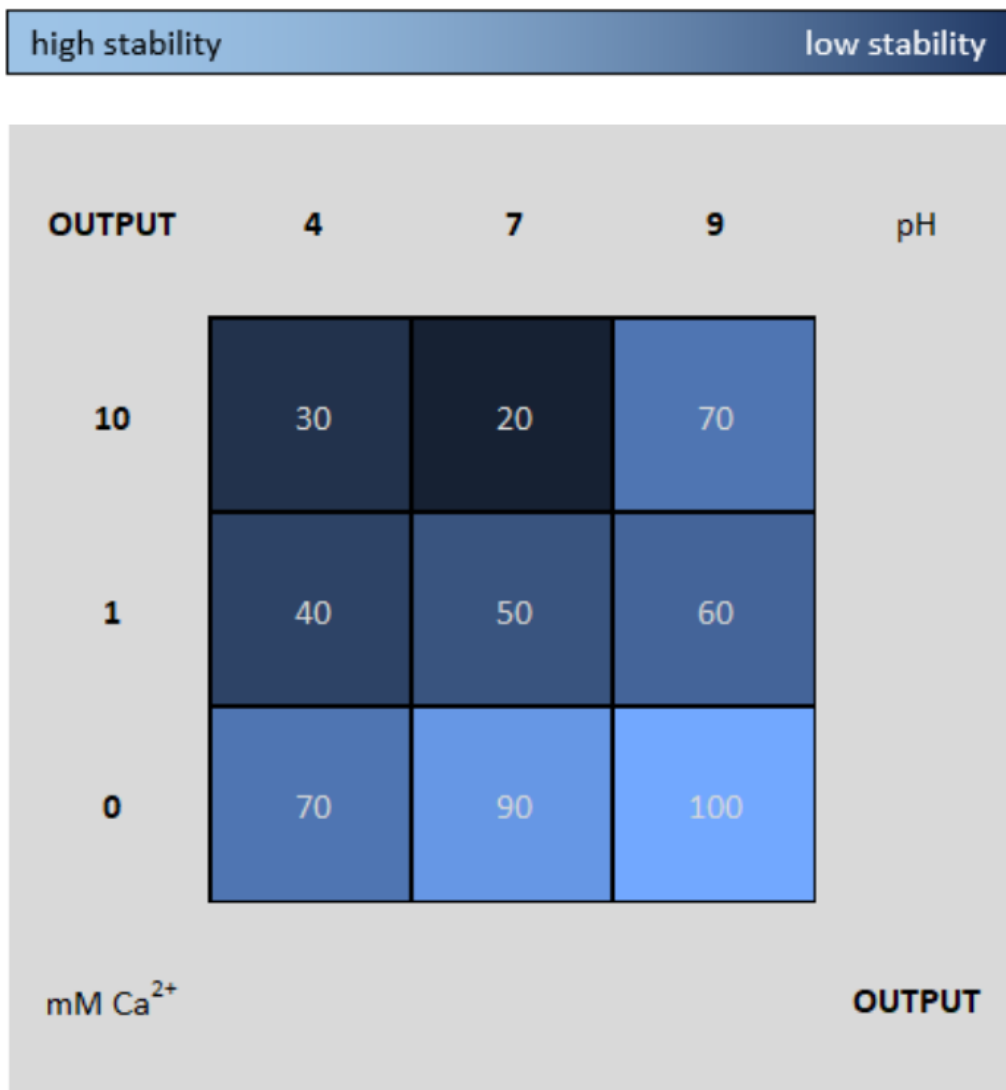
Figure 5. Dispersion stability regarding different environmental conditions (Ca^{2+} concentration: y-axis, pH-value: x-axis). Higher stability (less agglomeration/sedimentation) is reflected by a higher remaining concentration of test material (brighter shade in the plots) in the dispersion relative to the starting concentration (0-100%). The numbers in the boxes represent the percentage of the nanomaterial remaining in the dispersion, thus the dispersion stability in %. (from Monikh et al., 2018).
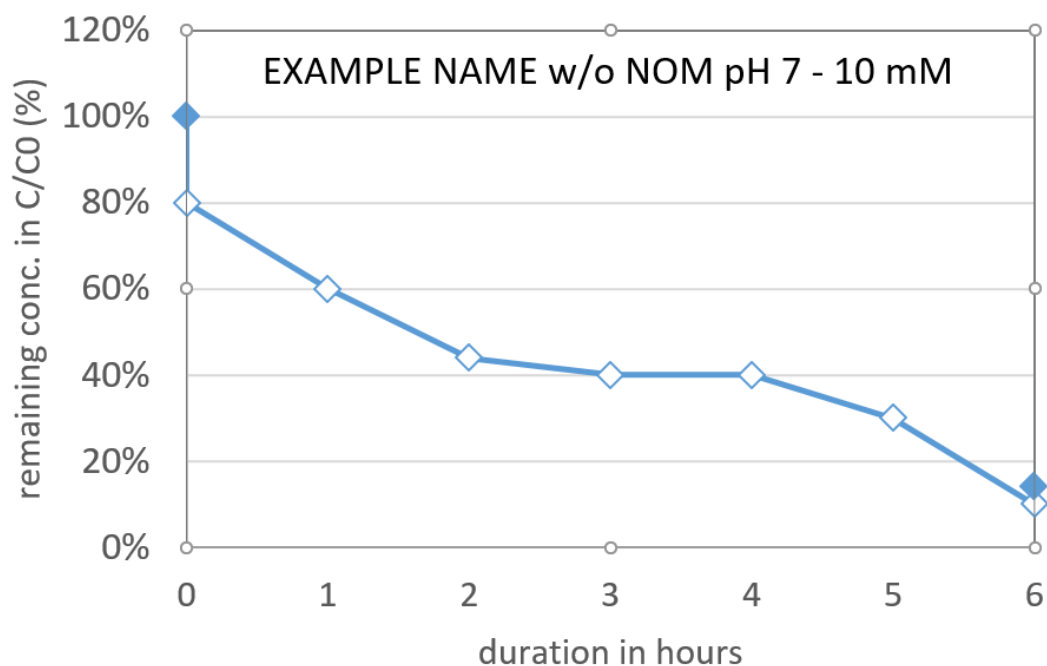
Figure 6. An example of dispersion stability plot as percentage of nanomaterials remaining in water phase compared to initial concentration (y-axis) against time in hours (x-axis) of the full test for one (here not further specified) test condition. The closed data point at 0 hours represents the nominal concentration, and the open data point at 0 hours is the measured concentration at the start (evoked by e.g. incomplete dispersion, fast sedimentation or loss to vessel; see paragraph 25 of OECD TG 318). The closed data point at 6 hours represents the measured concentration before the centrifugation step while the open point represents the measured concentration after the centrifugation step. Differences between the measured concentrations at point 6 hours are caused by density effects (see also Figure 7B). The values are normalised to the nominal concentration at 0 hours.

A	fast agglomeration and removal agglomerates are much denser than water
B	agglomeration of particles with low density difference to water centrifugation step considers low density and removes agglomerates
C	heterogeneous nanomaterial. A fraction agglomerates quickly another fraction is stable under the applied conditions
D	no agglomeration, stable material could also point to total dissolution of the material, check with DLS or consult dissolution test data
E	removal at the beginning points toward non-ideal dispersion slow, continuous agglomeration complete removal of material in centrifugation step
F	losses from nominal concentration to initial time point at 0h indicate incomplete dispersion, rapid agglomeration in stock or attachment to vessel walls

Figure 7. Possible shapes of the removal function over time and suggested interpretations.

3.2 Alternative test conditions

56.   TG 318 covers only synthetic waters that resemble the bandwidth of agglomeration-relevant components found in surface waters. In principle TG 318 can also be performed with other media than those used in TG 318, e.g. ecotoxicological test media. When using any other test media, it should be assured that the addition of the nanomaterial to the medium does not significantly change media conditions, e.g. the pH. A good estimate can be drawn from results of TG 318 with synthetic waters if the agglomeration-relevant components of the test media (sum of divalent cations, sulphate) are comparable with TG 318 conditions (the full test without NOM). When using alternative test conditions compared to TG 318 it is of utmost importance to characterise and report the test media compositions and conditions and also to compare those with test media compositions and conditions of TG 318 for the data evaluation and interpretation. This will also enable retrospective analysis of studies compared with new data produced in the future.

57.   TG 318 can be used with natural waters to investigate the dispersion stability and agglomeration behaviour in these waters. To prevent a situation where homo- and heteroagglomeration takes place in an uncontrolled way, the water sample should be filtered over a filter membrane with pore size equal to or smaller than 0.1 µm or subjected to ultracentrifugation to remove all sorts of natural particles, microorganisms, µm-sized debris, colloids and nanomaterials from the sample. It should also be taken into account that the obtained result is a (very precise) descriptive value for this one sample only, representing

a unique and constantly changing situation regarding hydrochemical composition and type and concentration of NOM. Whether it is possible to transfer this one result to the sampled surface waterbody in general depends on the spatial and temporal variability of the waterbody. If the unfiltered sample shall be tested with TG 318 the presence of natural suspended particulate matter will make it necessary to apply the variant of TG 318 that deals with heteroagglomeration (see section 3.3).

58.   The transferability of the data from TG 318 studies to higher tier testing (e.g. ecotoxicological studies) should be carefully evaluated as the used simulated environmental media differs from ecotoxicological test media which aims to promote animal vitality rather than to mimic natural habitats. When possible, it would be beneficial to test the dispersion stability with TG 318 using the test media used in the ecotoxicological test as recommended in the OECD draft GD for Aquatic (and Sediment) Toxicological Testing of Nanomaterials (WNT project 2.51). However, in many ecotoxicological tests, the test organism will change the test media composition, e.g. by algal exudates or photosynthesis in a 72 hours algae toxicity test according to TG 201. This will result in pH shift, various ionic compositions and different NOM characteristics and thereby lead to changes in dispersion stability as well as dissolution rates.

59.   NOM has an enormous variability in structure, molecular weight distribution, conformation, composition and purity. The type and quality of the used NOM or even NOM in natural waters or test media with intrinsic NOM will have effects on dispersion stability. This should be taken into account for data evaluation and when choosing test conditions that represent environmental relevant conditions. One has to distinguish between processed commercial products resembling NOM or unprocessed natural NOM as part of a natural water sample. The commercial products are more or less close to reality regarding their properties and are more likely to enable repeatability of the results. In contrast, natural NOM might trigger a behaviour of the nanomaterial (e.g. formation of "ecological corona") that is linked to the composition of the NOM, which might be very unique in time and space and not fully resemble the surface water the NOM was sampled from. In TG 318 2R101N Suwannee River NOM (SRNOM) is recommended as standardised and purified material. Due to differences in the composition of NOM from different sources, it is difficult to compare results obtained by the use of different types of NOM. Therefore, the alternatively used NOM should be characterised as much as possible, at least the minimum DOC content after properly cleaned from ions and ash should be determined. DOC content and treatment should be reported together with the test results. It is advisable to always test the nanomaterial according to the conditions presented in TG 318 in order to obtain comparative "benchmark" data.

60.   There are nanomaterials that are extremely hydrophobic and thus not miscible with aqueous media. For those nanomaterials the use of a dispersion agent (e.g. surfactants) could help to enhance the dispersibility in the stock dispersion. However, such an approach won`t deliver data on the dispersion stability of the pristine material. Depending on the scope of testing, the use of a dispersion agent for the dispersion stability testing could help to understand the fate of extremely hydrophobic nanomaterial when entering into the aquatic environment together with a dispersion agent (e.g. from a product).

3.3 Testing of heteroagglomeration

61.   The heteroagglomeration of nanomaterials with suspended particulate matter (SPM), which is ubiquitous in natural surface waters, is a crucial process affecting the environmental transport and fate of nanomaterials (Praetorius et al., 2014a; Quik et al., 2014; Therezien et al. 2014; Gao and Lowry, 2018).

62.   Many studies report measurements of different endpoints reflecting heteroagglomeration behaviour (e.g. agglomeration rate, sedimentation, attachment efficiency) with diverse nanomaterials under various conditions (e.g. Praetorius et al., 2014a; Labille et al., 2015; Velzeboer et al., 2014; Huynh et al., 2012; Quik et al., 2014; Geitner, 2017; Barton et al., 2014). However, the heteroagglomeration

attachment efficiency (α_{hetero}, for definition see section 3.4) has been found to be the most suitable parameter to inform fate modelling and risk assessment (Praetorius et al., 2014b).

63.   The need for consideration of heteroagglomeration as an aspect of nanomaterial fate has been discussed already during the development of TG 318 (Baun et al., 2017). TG 318 is in principle fit for purpose to also investigate heteroagglomeration and roughly estimate α_{hetero}. However, methods are not yet progressed enough to develop a fully validated TG for heteroagglomeration testing. Thus, based on the available scientific knowledge and methods, guidance for heteroagglomeration testing and presentation of data is provided in this GD. Necessary considerations and modifications of the test setup are explained in the following paragraphs.

64.   A major necessary adaptation of TG 318 to test for heteroagglomeration is the addition of SPM as agglomeration counterpart. Due to the complexity and diversity of natural SPM and the number of possible interaction mechanisms of nanomaterials with it, it seems on one hand impossible to decide on a representative set of SPMs for standardisation. On the other hand, restricting the types of SPM (e.g. to a simple mineral analogue) could lead to disproportional uncertainty in the estimation of α_{hetero} values (Walch et al., in preparation).

65.   Nanomaterial attachment to SPM results in a change of their transport regime (Hofmann and von der Kammer 2009) and possibly their bioavailability. Thus, the determined α_{hetero} has to be precise enough to be able to estimate the necessary timeframe (seconds-hours, days-weeks, months or more) until the majority of free nanomaterials has become SPM-attached nanomaterials. In most cases an orders-of-magnitude based category of α_{hetero} (e.g. "low", "medium" and "high") will be sufficient to address this point. Such α_{hetero} categories can serve as indicators for the expected half-life ($t_{1/2}$) of free nanomaterials under certain conditions (e.g. SPM concentrations). An example for CeO_2 is shown in Table 2 (Walch et al., 2019). In multimedia fate modelling a similar regime of sensitivity to α_{hetero} for predicted environmental concentrations is observed (Meesters et al., 2019). For further explanation see paragraph 71.

Table 2: Relationship of attachment efficiency (α_{hetero}) and expected half-life ($t_{1/2}$) of free nanomaterials (5ppb CeO_2, d = 25nm) in presence of 1-150ppm SPM (Walch et al. 2019).

heteroagglomeration attachment efficiency	expected free nanomaterial (5 ppb CeO_2, d =25 nm) half-life range for 150 ppm – 1 ppm SPM (d_n =1.5 µm, ρ =1.5 g/cm³)
$\alpha_{hetero} \approx 0.1 - 1$	$t_{1/2} \approx$ seconds – 1 day
$\alpha_{hetero} \approx 0.01$	$t_{1/2} \approx$ hours – days or (few) week(s)
$\alpha_{hetero} \approx 0.001$	$t_{1/2} \approx$ day(s) – month(s)
$\alpha_{hetero} \approx 0.0001$	$t_{1/2} \approx$ week(s) – (few) year(s)

66.   The hydrochemical background conditions suggested in TG 318 with regards to electrolyte compositions and concentrations can be equally applied to heteroagglomeration, as the mechanistic principles are the same. It is however suggested to use the "alternative medium" as indicated in TG 318 including SO_4^{2-} to cover the effects of divalent anions on agglomeration, especially if one of the components (SPM or nanomaterials) is expected to display a positive surface charge.

67.   Obviously, the introduction of an agglomeration counterpart into the test system is required. Hence, selecting suitable SPM analogues is the first crucial step. Such analogues need to be stable (for the test duration), reproducible (among test runs) monodisperse and well characterised in terms of composition, size (size distribution), shape and density (to allow a good approximation of the number-based SPM concentration needed for estimation of α_{hetero}, see section 3.4. The options may range from very simple mineral analogues (e.g. quartz particles) up to the use of well-characterised natural samples

(e.g. river waters or sewage sludge) and the choice of a relevant SPM type depends on the aim of the study.

68.   For a general comparative assessment of nanomaterials with regards to their heteroagglomeration behaviour in freshwaters, a simple mineral analogue would be too simplistic, whereas natural water samples do not allow for generalisations. Therefore, in an EU project "Nanomaterial Fate and Speciation in the Environment" (NanoFASE) researchers aimed to create a "model SPM" (complex floc-like SPM analogues that represent process-relevant characteristics of natural SPM) and a standard procedure for its production. These SPM flocs are composed of naturally occurring minerals, selected based on a trade-off between representing the dominant mineral mass fractions and covering a broad range of physicochemical surface properties (e.g. surface charge) in realistic mixing ratios, which are typically encountered in natural freshwater SPM. The mix includes quartz, illite, hematite and organic macromolecules associated with microbial activity (Walch et al. in preparation). This could be one "standard SPM" to be used in the test, with regards to the requirements for suitable SPM analogues mentioned above.

69.   In order to assess the fraction of "free", not heteroagglomerated nanomaterials after a certain or several (necessary to estimate α values) specified interaction time(s), a separation step needs to be introduced. Separation should be fast and non-intrusive, to allow removal of the SPM-attached nanomaterials from suspension while minimising artefacts on the "free" nanomaterial fraction. That can be achieved by centrifugation at each time interval. If the analytical detection limits allow, dilution before centrifugation helps to avoid non-attached nanomaterials being removed via "screening" by the SPM during centrifugation. Gravitational separability of nanomaterials and SPM hence becomes a prerequisite, meaning that the size and/or density of the SPM needs to exceed that of the tested nanomaterials, to an extent at which (at a selected centrifugation speed and time) the SPM will be removed from suspension, while free nanomaterials will not. This can be ensured by employing centrifugation cut-off calculations and verified by preliminary testing. If significant sedimentation of the SPM over the test duration is likely, agitation by shaking or stirring during the reaction time might be necessary. However, it cannot be ruled out that shaking and stirring affects the apparent rates of heteroagglomeration. Stirring should be effected in a controlled way to allow at least an approximate calculation of shear forces in the system (shear rate G), which is needed for collision frequency calculations (see equation 4 in section 3.4 below). Principles of stirred batch reactor design (Zlokarnik 2001) can be applied. Changes in shear force from ~40-180 s^{-1} only had minor impact on the size of mentioned model SPM analogue flocs (results from laboratory pre-tests). The shear force necessary to avoid sedimentation, being the dominant collision mode, depends on the diameters of the nanomaterials and SPM employed and can be calculated using equations 3 (section 3.4 below).

70.   The selection of the nanomaterial mass concentration needs to be based on the analytical limits in the matrix (as a rule of thumb, the quantification of remaining "free" nanomaterials should be possible down to ~10 % of the initially added nanomaterials). One can quantify either the elemental mass concentration or the particle number concentration in the supernatant by ICP-MS (after digestion) or single particle ICP-MS, respectively.

71.   Next to analytical limitations, there are process-determined limitations in selecting nanomaterial and SPM concentrations to optimise the system for selective heteroagglomeration testing. Both, homo- and heteroagglomeration kinetics are driven by the particle number concentration in the system (for heteroagglomeration more precisely the number ratio of nanomaterials and SPM (Labille et al. 2015)), as well as the collision rate constant, which depends on the nanomaterial and SPM size and density and the G in the system (see equations 3 in section 3.4). Shear forces are not part of the original TG 318, as particles in the nm-size range are not affected by shear forces and diffusion is the dominant transport mechanism (Elimelech 1995), meaning that nanomaterial homoagglomeration is independent of G. In the μm-size range typical for SPM flocs, however, shear forces start playing a role. Since heteroagglomeration is the process of interest, the selection of the nanomaterial/SPM number concentrations and shear forces

needs to ensure that potentially simultaneously occurring homoagglomeration[2] remains negligible (in case of unfavourable hydrochemical conditions for homoagglomeration or very low expected α_{homo}) or is at least significantly dominated by heteroagglomeration (in case of homoagglomeration not being negligible). Such an optimisation of a stirred test system for heteroagglomeration can be based on model calculations (see equations 3 in chapter 3.4) depicting the interplay between SPM and nanomaterial sizes and densities, their number concentrations and applied shear forces (Figure 8). Knowing (or approximating) the size[3] and density of both nanomaterial and SPM, the mass-corresponding number concentrations can be calculated assuming (if not known) spherical shape for both. To allow defining suitable conditions, it is suggested to take the necessary minimum nanomaterial number concentration determined by analytical limitations as a starting point, and modify the SPM number concentration and the G-value to "worst case" conditions, i.e. assigning the highest attachment efficiency value of unity for both, α_{homo} and α_{hetero} (where each collision results in attachment). An example is shown in Figure 8, where homoagglomeration of 5 ppb 30 nm CeO_2 is certainly dominated by heteroagglomeration when 45 ppm SPM (d = 6 µm, ρ = 1.5g/cm³) is used at stirring which effected a G = 100s^{-1}. Additionally, the model gives a first indication of the necessary temporal resolution.

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[2] Even if homoagglomeration takes place, homoagglomerates may be too small to be removed from suspension by centrifugation.

[3] Note that e.g. light-scattering-based size measurements are biased towards larger sizes and applying such particle diameters may lead to a significant underestimation of the particle number.
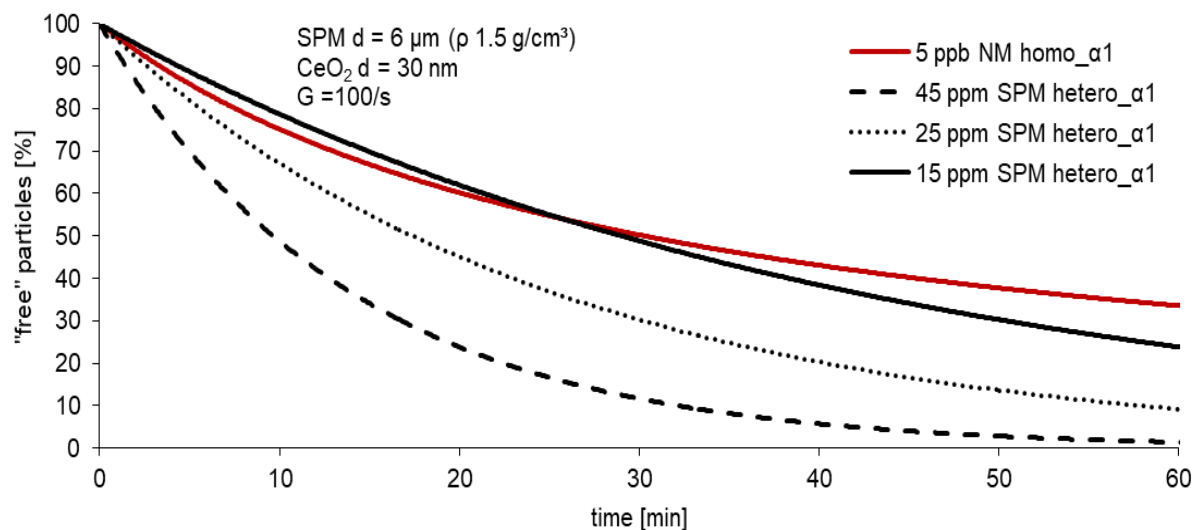
Figure 8. Model calculations employing equation 3 (see section 3.4) to find suitable SPM concentrations at which heteroagglomeration is dominating over homoagglomeration in case of both α_{homo} and α_{hetero} being set to one. CeO_2 has been chosen as example for low analytical limits (ICP-MS), allowing experiments at 5 ppb (10% remaining would still be > 5 × limit of quantification). Number concentrations were converted from mass concentrations assuming spherical shape for both SPM and nanomaterial and the given diameters and densities (CeO_2: 7.22 g/cm³). In both the homo- (red line), and heteroagglomeration case (black lines) the initial nanomaterial concentrations were 5 ppb. With increasing SPM concentrations in the heteroagglomeration case, the nanomaterial-SPM number ratio changes and at 45 ppm SPM the decrease of "free" nanomaterials due to heteroagglomeration will clearly dominate over homoagglomeration, whereas at 15 ppm SPM homoagglomeration might have a similar share (unless $\alpha_{homo} \ll \alpha_{hetero}$) in total removal of free nanomaterials.

72.   In order to support the model calculation and the assumption of negligible loss of nanomaterials other than by heteroagglomeration, an additional control test should be conducted. This is done using the same concentration of nanomaterials in the same background hydrochemistry (pH, electrolytes, NOM) but without any SPM. Sampling and sample treatment should be performed in the same way as for the heteroagglomeration test to measure free nanomaterial. Digesting the remaining samples (after centrifugation and sampling supernatant aliquots for free nanomaterial quantification) can serve to close the mass balance and account for losses to the vessels or tubes.

73.   The test duration window needs to capture the nanomaterial removal over time, which depends on the expected α_{hetero} value, the nanomaterial and SPM number concentrations and collision frequency (equations 3 in chapter 3.4). Hence, the selected particle number concentrations, test-duration and the decision if agitation is necessary or not, can be optimised. Model calculations may also serve to get an idea about the relevant reaction-time window to be investigated. The more complex and heterogeneous the chosen SPM, the more likely it seems that heteroagglomeration is a very fast process. Thus, it is recommended to aim for a high time-resolution of the initial agglomeration phase (e.g. every few minutes during the first 30 minutes of the test). Intervals can be steadily increased up to e.g. 6 hours and a final "stable" nanomaterial fraction can be determined after e.g. 24 hours.

3.4 Data evaluation and derivation of attachment efficiency

74.   Attachment efficiencies, beside the dissolution rate constant, are considered an important parameter for exposure modelling of nanomaterials. Recently, scientists developed fate models for nanomaterials that use attachment efficiencies as input parameters to predict nanomaterial concentrations in environmental compartments (e.g. SimpleBox4Nano (Meesters et al., 2014), models provided by EU Horizon Project NanoFASE available at: http://nanofase.eu/show/fate-and-exposure-modelling-of-nanomaterials-the-nanofase-model-catalogue_1963/#]). These nanospecific models will provide more reliable and relevant predicted environmental concentrations (PECs) for nanomaterials than the conventional fate models that rely on equilibrium partitioning and will thus contribute to an improved nanomaterials risk assessment.

75.   Based on Smoluchowski's agglomeration theory (Elimelech,1995), the heteroagglomeration attachment efficiency (depicted as α_{hetero}) can be defined as the fraction of nanomaterial-SPM collisions resulting in nanomaterial attachment to SPM. α_{hetero} can take values from 0 to 1 to modulate the collision rate constant between nanomaterials and SPM reflecting their physicochemical surface affinity for each other (under the given hydrochemical conditions). Assuming irreversible attachment, the rate of free nanomaterial removal by heteroagglomeration can be calculated according to equation 1. With a constant number of SPM particles, heteroagglomeration turns out to be a pseudo first-order reaction (n_{SPM} can be included into k_{het}, further on denounced as k_{het}^*) (Praetorius et al., 2012). The collision rate constant (k_{coll}) combines the three modes of transport of nanomaterials and SPM towards each other (diffusion, differential sedimentation and advection), assuming spherical nanomaterial and SPM shapes and rectilinear collisions (Praetorius et al., 2012). Transport by advection is incorporated employing the shear rate or the velocity gradient (G) introduced by Camp and Stein (1943) for stirred vessels. It is defined as the square root of the energy (e.g. added by stirring) dissipated by turbulence per unit time, accounting for the volume and viscosity of the considered fluid. The sensitivity of the system to changes in shear is relatively low compared to other parameters. To avoid sedimentation, we suggest a value of G around 40-100 s^{-1} for a stirred batch system. Shear rates in rivers are typically > 10 s^{-1} (Arvidsson et al., 2011).

Equations 3: Rate of "free" nanomaterials removal from suspension due to heteroagglomeration over time (based on Smoluchowski; Praetorius et al. 2012)

$$\frac{dn_{\text{NM}}}{dt} = - k_{\text{het}}\, n_{\text{NM}}\, n_{\text{SPM}}$$

k_{het}: heteroagglomeration rate constant [m³/s]
n_{NM}: number concentration of nanomaterials [1/m³]
n_{SPM}: number concentration of SPM [1/m³]

$$k_{\text{het}} = \alpha_{\text{het}}\, k_{\text{coll}}$$

α_{het}: attachment efficiency between nanomaterials & SPM
k_{coll}: collision rate constant between nanomaterials & SPM [m³/s]

Equations 4: Calculation of collision frequencies due to diffusion (perikinetic), advection (orthokinetic) and differential sedimentation (DS) assuming spherical particles, laminar flow and straight streamlines (rectilinear collisions)

perikinetic collisions

$$k_{\text{coll}_{\text{perikin}}} = \frac{2kT}{3\mu}\, \frac{(r_{\text{SPM}} + r_{\text{NM}})^2}{r_{\text{SPM}}\, r_{\text{NM}}}$$

orthokinetic collisions

$$k_{\text{coll}_{\text{orthokin}}} = \frac{4}{3} G (r_{\text{SPM}} + r_{\text{NM}})^3$$

$$\text{with} \quad \overline{G} = \sqrt{\frac{\epsilon}{\nu}} = \sqrt{\frac{P}{\mu V}}$$

collisions due to differential sedimentation

$$k_{\text{coll}_{\text{DS}}} = \pi (r_{\text{SPM}} + r_{\text{NM}})^2 |v_{\text{SPM}} - v_{\text{NM}}|$$

$$\text{with} \quad v_{\text{SPM; NM}} = \frac{2}{9} \frac{\rho_{\text{SPM; NM}} - \rho_{\text{L}}}{\mu} g\, r^2_{\text{SPM; NM}}$$

summed-up to total collision frequency:

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

k: Boltzmann's constant [m²kg/s²K]
T: absolute temperature [K]
μ: dynamic viscosity of dispersion medium [Ns/m²]
r: particle/aggregate radius [m]
G: shear rate (velocity gradient) [s⁻¹]
g: gravity acceleration [m/s²]
ρ_S: density of particles [kg/m³]

ρ_L: density of liquid [kg/m³]
$u_{\text{SPM; NM}}$: settling velocity of SPM or nanomaterials [m/s]
ϵ: power input per unit mass [Nm/skg]
v: kinematic viscosity (μ/ρ_L) [m²/s]
P: power dissipated by liquid turbulence [W]
V: volume of liquid [m³]

76.   These equations may on the one hand be used to design the tests (SPM and nanomaterial number concentrations, test duration and time resolution, see above paragraph 73) and on the other hand to approximate α_{hetero}. With heteroagglomeration being a pseudo first-order reaction (in the early agglomeration phase), the natural logarithm of the particle concentration plotted over time will give a linear relation (see example in Figure 9). The slope equals the pseudo first-order reaction rate ($k_{het}^{*} = \alpha_{het}\, n_{SPM}\, k_{coll}$) and α_{hetero} can be calculated.

77.   As there will be many collisions between nanomaterials and SPM, only nanomaterials with very low attachment efficiencies are likely to remain "free". Many results (e.g. Praetorius et al., 2012) have shown α values (attachment efficiencies) that result in almost immediate nanomaterial-SPM associations.

The exception is, if the SPM number concentration is very low. In the latter case homoagglomeration will be dominant (see Figure 8). Assuming pseudo first-order kinetics, the half-life of free nanomaterials can be calculated $(t_{1/2} = \ln(2)/k_{het}{}^*)$. Plotting the expected half-lives at given SPM/nanomaterial concentrations and shear rate, over a range of α_{hetero}-values employing three different SPM concentrations (Figure 10) shows that only starting from small α_{hetero} <0.01-0.001 significant numbers of free nanomaterials will remain after just one day, except for very low SPM concentrations (5 ppm and lower).

slope (k_{het}) = - 2.44*10^{-4} s^{-1}

k_{coll} = 7.31*10^{-16} s^{-1}
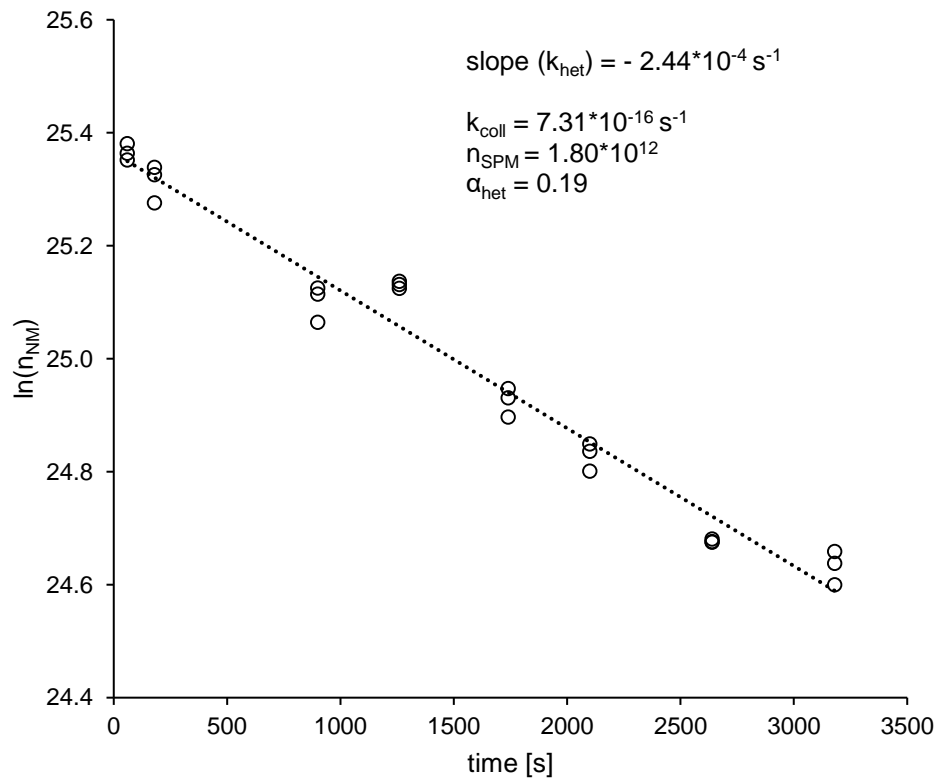n_{SPM} = 1.80*10^{12}
α_{het} = 0.19

Figure 9. An example of pseudo first-order kinetics of heteroagglomeration.
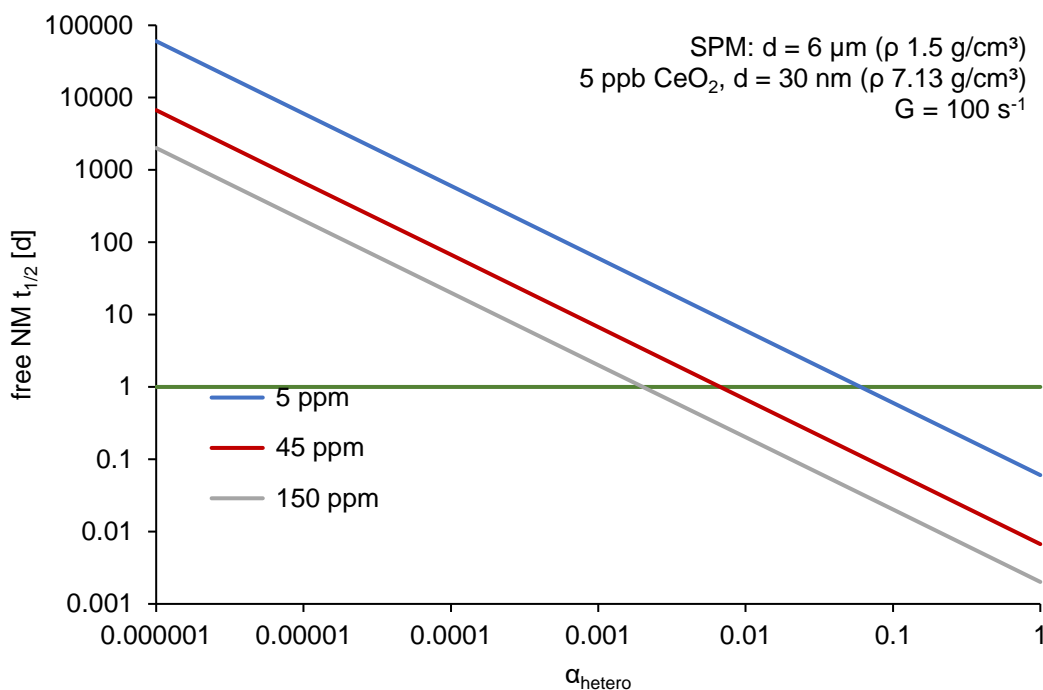
Figure 10. Half-life time of free nanomaterials based on heteroagglomeration model calculations with the example of 5 ppb CeO_2 nanomaterial (30 nm) and three different concentrations of SPM (5 ppm blue line, 45 ppm red line, 150 ppm grey line) are shown against α_{hetero}. The number of SPM (n_{SPM}) and k_{coll} were calculated with the given parameters assuming spherical shape. For half-lives longer than one day (i.e. those above the horizontal green line), α_{hetero} needs to be smaller than 0.1-0.001.

78.   Based on the considerations above, the test duration of 6 hours defined in TG 318 will likely also work for heteroagglomeration. For conditions where heteroagglomeration is the dominant process, transport-relevant αhetero values of 0.01 and lower are only to be expected if there is a significant fraction e.g. 10-20 % of nanomaterials remaining after 3 (conservative) to 6 hours. With further experimental data providing proof of principle, this relationship between the order of magnitude of αhetero and the test duration can be further refined for given nanomaterial/SPM concentration ratios.

79.   If it is aimed to compare heteroagglomeration behaviour of various nanomaterials under same conditions, a tiered approach is suggested that could look as following: In a first tier, the nanomaterials which are prone to quickly heteroagglomerate should be identified. Those can be assumed to immediately attach to SPM, making kinetic considerations obsolete. Other nanomaterials should be subjected to a second-tier kinetic study to determine αhetero. The first tier could consist of two-point measurements, where the free nanomaterial fraction left in the supernatant after centrifugation and removal of the SPM-attached fraction at t_1 is compared to the initial free nanomaterial concentration at t_0[4]. If the remaining

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[4] A comparison to t_0 might be substituted by a comparison to a "homoagglomeration" control (containing the same amount of nanomaterials with the same background hydrochemistry and DOM concentration, but without SPM). However, using the t_0-heteroagglomeration concentration as reference for comparison ensures that, at both time points, the same fraction of free nanomaterials was removed by "screening-effects" (SPM will physically catch and remove some nanomaterials from dispersion during centrifugation). This helps to avoid a misinterpretation of a screening-induced removal of nanomaterials from suspension as a heteroagglomeration-induced removal. However,

nanomaterial fraction at t_1 exceeds a selected threshold value, indicating a low α_{hetero} and potential stability of free nanomaterials, a kinetic study should be conducted. As shown by calculations above, correlations between α_{hetero}, $t_{1/2}$ and the fraction of free nanomaterials left after a certain time may be used to establish general threshold values to categorise αhetero values into "high", "medium" and "low". The following example shall illustrate the principle: If less than 10% of the nanomaterials is left after 2 h (meaning fast heteroagglomeration), an αhetero larger than 0.1 and $t_{1/2}$ less than 1 d is expected; if more than 10% are left after 2 h (meaning slower heteroagglomeration), a kinetic study to determine α_{hetero} should be conducted. These relations are, however, strongly dependent on the chosen nanomaterial/SPM number concentrations and sizes. Thus, establishment of threshold values needs to be tied to robust experimental procedures, which still have to be developed and systematically validated. A crucial point here is the selection of t_1 (2 h in the example) since the influence of respective nanomaterial/SPM number-ratio needs to be taken into account (see Figure 10). Furthermore, setting a threshold for the requirement of a kinetic study based on the remaining fraction of free nanomaterials (10% in the example) should be done under careful consideration: Studies found that the removal of nanomaterials can reach a plateau, leaving a stable fraction of the initially added nanomaterials (Quik et al. 2012 and 2014). This can either be due to a very stable sub-fraction present in the nanomaterial sample (i.e. "impurities" exhibiting different intrinsic surface properties from the bulk), or due to an in-situ stabilisation of a sub-fraction of nanomaterials by compounds of the test media (e.g. NOM). Thus, a certain remaining fraction after a defined reaction time does not necessarily indicate slow or fast heteroagglomeration. Hence, another useful step is the determination of the "final" stable fraction (e.g. after 24 h) to avoid misinterpretation. Since this fraction is unlikely to attach to SPM in the long run, it should be considered "free" nanomaterials when it comes to transport regimes or bioavailability.

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if heteroagglomeration is very fast, a homoagglomeration control might provide a more reliable initial concentration (but only if the screening effect can be considered negligible, e.g. when very low SPM concentrations are used).

4 Use of data on dissolution and dispersion stability for further testing and assessment strategies

80.   Both dispersion stability and dissolution (rate) are considered to be endpoints of major importance for describing environmental behaviour and fate of nanomaterials. Thus, for fate assessment as well as for exposure assessment both endpoints need to be taken into account, and they will also be key information for ecotoxicity testing.

81.   While TG 318 for dispersion stability has been published (OECD, 2017), currently no OECD TG on solubility and dissolution rate of nanomaterials is available. However, existing guidance documents such as the GD 29 (OECD 2001) may provide a basic orientation (see Chapter 2). Other TG and GDs as well as ongoing WNT projects are also dealing with areas of dissolution (see Chapter 2 and Chapter 6).

82.   There is not just one single optimal strategy on how to determine solubility, dissolution, and dispersion stability. The initial nanomaterial concentrations, the media composition, and timeframe that is relevant to use will always depend on the purpose and objective of testing (see Section 4.1). Likewise, the testing strategy employed depends on whether the data is needed to inform consideration of what further ecotoxicity testing is needed, as input data for modelling of environmental fate, or as basic data on environmental behaviour (see Section 4.2).

83.   Validity and correct interpretation of dispersion stability, and dissolution rate or solubility measurements are interdependent. For instance, the measurement of dissolution in a suspension where agglomeration and sedimentation has occurred will underestimate dissolution (as agglomerated/precipitated material will have less surface available for dissolution). When dissolution has occurred, determination of dispersion stability based on measurement of the remaining particulate fraction only will underestimate dispersion stability as any nanomaterial missing through dissolution will be interpreted as precipitated. Determination of dispersion stability of dissolving nanomaterial based on remaining total concentration in the supernatant (as described in TG 318) leads to an overestimation of dispersion stability in case the dissolved fraction is not quantified. Therefore, the quantification of the dissolved fraction of the stable part is required in TG 318 when dissolving nanomaterials are investigated. Further situations where such caution must be shown when interpreting the data and where both types of data must be viewed in concert are highlighted with some outline application examples in Section 4.3.

4.1 Purpose

84.   Dissolution (rate) and dispersion stability are relevant triggers needed for the safety assessment of nanomaterials. Regulatory information requirements for solubility, dissolution rate and dispersion stability of nanomaterials exist in some chemical legislation such as REACH (EU 2018). These parameters are also relevant as triggers for further assessment strategies and decisions. Given the current worldwide development in chemicals regulations to manage nanomaterials, it is anticipated that other chemicals legislations (e.g. US EPA, 2016) meet the same challenges.

85.   The understanding of environmental fate and behaviour of nanomaterials is one of the key aspects for environmental exposure assessment. Furthermore, it supports the knowledge about uptake by, interaction with, and effects on environmental organisms. Therefore, dissolution, (hetero-) agglomeration and sedimentation are important drivers for environmental fate, behaviour and effects of nanomaterials.

86.   Furthermore, given the numerous manufactured nanomaterials already on the market and many more expected in future, the effort for the individual investigation of hazard and risk would be enormous and virtually impossible. To overcome this challenge, grouping and read-across approaches of nanomaterials has been identified as one critical issue by OECD (OECD, 2016). Grouping and read-across approaches are already established for chemical substances to meet regulatory data requirements by deviation from the requirements of performing standard tests for years (OECD, 2007; 2014b). The aim of these approaches is to predict the physico-chemical, toxicological, and fate-related properties of chemical substances based on structural similarities. If sufficient evidence for similarity is available between two chemical forms, then it should be possible to transfer available data on the fate or hazard of one chemical substance to another one. These approaches are intended to reduce the amount of testing necessary to determine the behaviour and effects of all individual members of a group.

87.   Grouping and read-across based on molecular structural similarity alone is not sufficient for any chemical. This is of special importance for nanomaterials, as nanoforms of the same chemical composition but deviating in physico-chemical parameters can show differences in effects and behaviour. To allow an adequate assessment of nanomaterials using grouping and read-across further parameters are necessary to justify such an approach. In that context, beside chemical composition, parameters like morphology, surface properties, and shape as well as reactivity, and fate descriptors like dissolution rate or dispersion stability in relevant media are discussed for consideration (e.g. ECHA Guidance R6.1 (ECHA 2019)).

88.   TG 318 for dispersion stability and the available methods for solubility and dissolution rate can address the respective endpoints and might be used to compare similarities or differences of nanomaterials regarding these endpoints at given conditions and thus support the development of grouping and analogue approaches for nanomaterials as part of integrated testing and assessment approaches (IATAs).

89.   For further (hazard) testing knowledge on dispersion stability and dissolution behaviour can inform on the test set-up and necessary measurements for exposure characterisation during the testing. Furthermore, knowledge on dispersion stability and dissolution behaviour in a test system can facilitate better interpretation of test results (e.g. whether effects seen can be attributed to nanomaterials or free ions only).

4.2 Testing strategy

90.   Based on the purpose of testing, information from solubility, dissolution rate and dispersion stability testing can inform a further testing strategy. The suggested approach to do so is outlined in Figure 11. The approach starts with a screening batch test on dissolution followed by a dynamic dissolution test if needed. The approach furthermore includes the testing of dispersion stability for nanomaterials that do not fully dissolve within a relevant time scale. This approach also provides further information to guide on the effort needed for appropriate analytics in further testing. Before entering into the tiered approach, sufficient characterisation of the investigated nanomaterial (e.g. chemical composition, size distribution, morphology, and surface modifications) provides information to prepare the testing, and interpret test results.
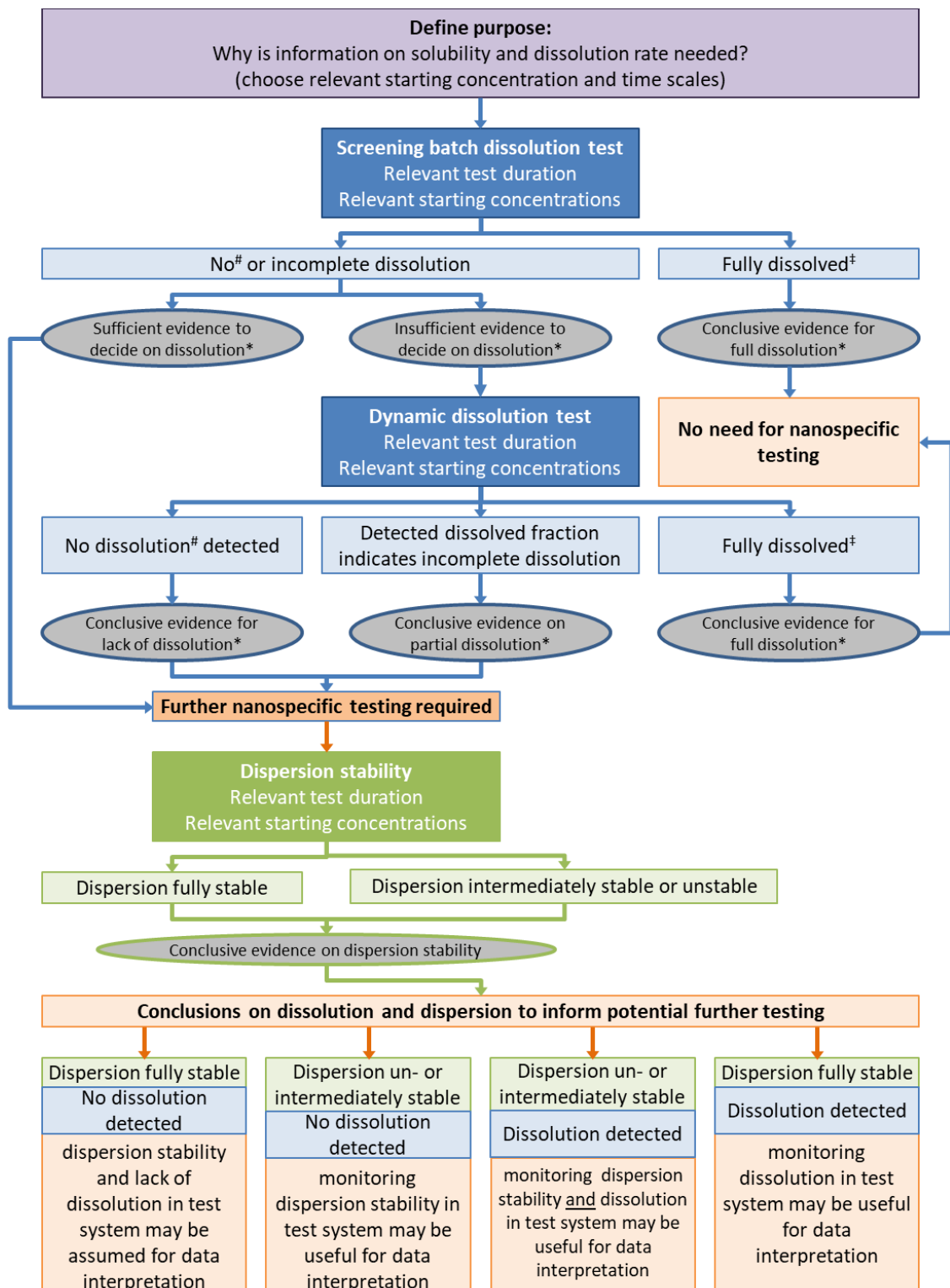
Define purpose:
Why is information on solubility and dissolution rate needed?
(choose relevant starting concentration and time scales)

Screening batch dissolution test
Relevant test duration
Relevant starting concentrations

No# or incomplete dissolution

Fully dissolved‡

Sufficient evidence to decide on dissolution*

Insufficient evidence to decide on dissolution*

Conclusive evidence for full dissolution*

Dynamic dissolution test
Relevant test duration
Relevant starting concentrations

No need for nanospecific testing

No dissolution# detected

Detected dissolved fraction indicates incomplete dissolution

Fully dissolved‡

Conclusive evidence for lack of dissolution*

Conclusive evidence on partial dissolution*

Conclusive evidence for full dissolution*

Further nanospecific testing required

Dispersion stability
Relevant test duration
Relevant starting concentrations

Dispersion fully stable

Dispersion intermediately stable or unstable

Conclusive evidence on dispersion stability

Conclusions on dissolution and dispersion to inform potential further testing

Dispersion fully stable	Dispersion un- or intermediately stable	Dispersion un- or intermediately stable	Dispersion fully stable
No dissolution detected	No dissolution detected	Dissolution detected	Dissolution detected
dispersion stability and lack of dissolution in test system may be assumed for data interpretation	monitoring dispersion stability in test system may be useful for data interpretation	monitoring dispersion stability and dissolution in test system may be useful for data interpretation	monitoring dissolution in test system may be useful for data interpretation

Figure 11. Overview of a testing strategy building on dissolution and dispersion stability data. # Potential dissolved fraction is below detection limit; ‡ All relevant mass of the investigated nanomaterial is in the dissolved fraction; and * The evidence takes relevant time scales into account. The testing strategy and elements that need to be considered for the development of such a strategy is further explained in section 4.3 and section 4.4.

91.   Depending on the nanomaterial under investigation and the objective of the testing, appropriate starting concentrations and test durations need to be chosen for the initial screening dissolution batch test.

When addressing triggers for safety assessment and decision making for further testing, there might be the need to test concentrations which are above environmentally relevant concentrations. Therefore, it is advisable to test the nanomaterials with test conditions of TG 318 and the upcoming TG for dissolution, respectively, to gain comparable and reliable data. Furthermore, it might be challenging to analytically verify test concentrations under (mimicked) realistic environmental conditions.

92.   In case the batch test gives conclusive evidence for full dissolution (all relevant mass in dissolved fraction) of the investigated nanomaterial in a relevant time scale (relevant refers to the actual question for which the test strategy is set up), further dissolution testing by dynamic methods might not be needed. In this case it can also be considered to refrain from nanospecific testing. For nanomaterials that show incomplete dissolution or dissolution below detection limit, it should be decided if the information from the batch test provides sufficient evidence to enable a decision on the dissolution for the purpose defined. For example, information on dissolution based on the batch test could provide sufficient supplementary information for a subsequent ecotoxicity test. In contrast, information from the batch test might be insufficient to e.g. inform exposure relevant testing regimes in which dissolution against an infinitive sink is of interest. Based on the question also the appropriateness of the analytic equipment (e.g. concerning sensitivity) should be considered.

93.   In case information from the batch test is sufficient to conclude on the dissolution of the investigated nanomaterial one could directly proceed with further nanospecific testing. If the information from the batch test is insufficient, testing of dissolution with a dynamic method should be considered. For nanomaterials that do not fully dissolve in a dynamic dissolution test within a relevant time scale, again further nanospecific testing is needed. For further nanospecific testing a first suggested step is testing of dispersion stability.

94.   For testing dispersion stability, it is recommended to use OECD TG 318 as it is described. Deviation from test conditions might be possible in order to inform higher tier testing. In the latter case it is suggested to choose appropriate test duration and concentration based on the objective. Information from dispersion stability testing can provide an indication about the analytical needs (monitoring of dissolved fraction, monitoring of dispersion stability) to be performed for or during potential further nanospecific testing.

95.   In addition, results from solubility, dissolution rate and dispersion stability testing can provide information to facilitate the choice of appropriate test performance of potential further testing (e.g. frequency of water renewal rate, (semi)static vs. flow-through test conditions) to maintain stable conditions.

4.3 Points to consider when applying information on dispersion stability and dissolution for potential further testing

96.   The methods presented in this GD will provide relevant data on solubility, dissolution rate and dispersion stability. Moreover, in principle these methods can also be performed with test media (e.g. natural water, ecotoxicological test media) that differ from the ones presented in the respective test guideline or recommended here. However, as these endpoints are influenced by properties of the nanomaterial in question as well as interactions between the nanomaterial and its surroundings, results between tests may differ depending on nanomaterial modification(s) and the test media used.

97.   Consequently, the repeatability, reliability, and robustness should be taken into account as much as possible, when using alternative test conditions. In these cases, it is prerequisite to characterise and report the test media conditions as detailed as possible to enable data evaluation and interpretation of data (and consider simultaneous testing). This will also enable future analysis of existing studies, e.g. to compare existing results with new data from new studies.

98.   While information from these methods might be directly applicable for exposure estimation, conclusions for a further testing strategy on hazard should be considered carefully. The relevance of the information as well as the decision how to consider data generated on these endpoints for further testing will always depend on the individual question and purpose of testing.

99.   Solubility and dissolution rate inform about in which state (particulate, completely or partly dissolved) a nanomaterial is present under respective conditions. Regarding solubility and dissolution rate the main question is when it is possible to refrain from nanospecific considerations in hazard assessment (i.e. when dissolution is considered complete and fast enough compared to the duration of the corresponding (eco)toxicity test). For an appropriate decision on the relevant hazard assessment, various aspects need to be considered like the regarded nanomaterial (e.g. chemical composition), endpoint under investigation (e.g. long term vs. acute testing), and test performance (e.g. media type, water renewal frequency). In the evaluation of an aquatic hazard endpoint supporting information from discrete dissolution testing (solubility, dissolution rate) in the respective aquatic test media and with respective concentrations could help to decide if it might be possible to refrain from nanospecific testing. That could be the case if the nanomaterial under investigation fully dissolves in dissolution testing within a timeframe and for concentrations relevant for the respective aquatic endpoint. The chosen test parameter should allow to conclude if the contribution of the particulate form to the eco-toxicological impact is insignificant and do not very likely change the results of the ecotoxicity testing. Furthermore, hazard test data for the solute needs to be available.

100.   Regarding dissolution it should be considered that the dissolution kinetic of some nanomaterials (e.g. certain metals or metal oxides) is sensitive to light/darkness, with generally a greater dissolution at visible light or UV-irradiated conditions as compared to darkness. Therefore, the light/dark-conditions should be reported where relevant (Hedberg et al., 2012; Li et al. 2014).

101.   OECD TG 318 provides information on how stable a nanomaterial dispersion is in aqueous media at given conditions. There is sufficient evidence that nanomaterials tend to agglomerate and settle out of the water phase during sufficient long periods and thus sediment, sludge and soil are expected to be the main environmental components in which the nanomaterials end up (Hartmann et al.; 2014). Thus, if nanomaterials show low stability in the water phase, inclusion of data relating to the sediment or soil compartment into environmental risk assessment of nanomaterials may become relevant.

102.   However, agglomeration and sedimentation should not be equated per se with lack of exposure in water column. For hazard testing, existing data show that agglomeration and sedimentation do not necessarily prevent availability to the organisms in aquatic test systems. Due to the permanently agitated test system used for testing algae toxicity, test organisms also can be affected by nanomaterials unstable in the test dispersion. In addition, unstable dispersed nanomaterials can be taken up by pelagic organisms like daphnia (Botha et al., 2016; Tan et al., 2016; Hund-Rinke et al., 2017). Unstable nanomaterials furthermore can be available to benthic deposit-feeders and filtering organisms (Tella et al., 2014; Kuehr et al., 2020). In case of ion releasing nanomaterials, released ions might get back into the water phase, affecting test organisms while the particulate fraction remains deposited at the bottom. Strictly speaking this may not be considered nanomaterial toxicity, but by constantly replenishing the ion concentration in the water phase, the particulate fraction strongly influences the outcomes of the test. Also, under environmental conditions it is conceivable that deposited or heteroagglomerated nanomaterials are consumed from biofilms, ground or plant surfaces or taken up via sediment or natural suspended matter, respectively (Geitner et al., 2018; Perrier et al., 2018). In addition to dissolution and agglomeration, bioavailability and uptake of the various nanomaterials by aquatic organisms depends on other aspects of the nanomaterials like size, chemical composition, synthesis methods, or nature of coating of the investigated nanomaterial (Lead et al., 2018). However, the understanding how these aspects influence bioavailability and uptake is still far from complete.

103.   When using data from dispersion stability and dissolution testing for exposure assessment it has to be taken into account that not only a single event but also a continuous entry into the environment is of relevance. This can lead to a situation that even if a nanomaterial dissolves relatively fast a mixed exposure of particulate and dissolved forms can take place.

104.   For nanomaterials that release ions or dissolve over a sufficient long period of time, it is important to monitor in-depth the ion concentration in the test system to inform on the amounts of particulate and dissolved forms being present. Depending on the purpose of testing and investigated nanomaterial, it can be also of importance to know how the particulate form changes in surface or core composition or size distribution. Changes in composition might be relevant for e.g. nanomaterials that consist of various substances (e.g. core-shell nanomaterials) with different dissolution behaviour and should be determined where relevant. Knowledge on changes in size distribution (e.g. loss of smallest particle, decrease of particle size across the distribution, formation of new particles) can become relevant to decide on further hazard and fate testing strategies.

105.   The joint consideration of the endpoints determined by the TG 318 and available methods on solubility and dissolution rate is of expedient importance as these endpoints strongly influence each other and interpreting one in absence of the other is difficult. The dispersion state of a nanomaterial will affect its dissolution kinetic, while ion releasing of the nanomaterial will alter the outcome of dispersion stability testing for dissolving nanomaterials.

106.   Dissolution leads to an increase of ion concentration that subsequently can lead to either an increased or a decreased dispersion stability. Furthermore, readily soluble nanomaterials can show a loss of particles during TG 318 performance. Therefore, care should be taken when analysing and interpreting results from TG 318 to not mistake dissolved nanomaterials with stable fraction. In TG 318 it is described how dissolution needs to be evaluated during testing for ion releasing nanomaterials. On the other hand, agglomeration/sedimentation will lead to a decrease in available surface, which in consequence will result in a decrease in dissolution.

107.   Next to (hetero-) agglomeration, sedimentation and dissolution, potential nanomaterial transformations (e.g. sulphidation, oxidation/reduction reactions and coating transformations) influence the fate of nanomaterials in the environment and thus should be considered when evaluating the data. An OECD project is under development on transformation of nanomaterials in aquatic environmental media to provide advice on ways to determine abiotic transformations of nanomaterials in the environment.

5 Use of data on dispersion stability, solubility and dissolution rate in exposure modelling

108.   Both data from TG 318 and the currently available methods for determining solubility and dissolution rate may support the derivation of appropriate input parameters for exposure modelling of nanomaterials. As a starting point for environmental fate modelling, basic data as collected using TG 318 and adapted test performance of GD 29 might be used as input for deriving screening level estimations of predicted environmental concentrations (PECs), e.g. to compare potential exposure of various nanomaterials. This basic data can be a ranking in stable or instable nanomaterial dispersion (Figure 4) according to TG 318, which can be linked to the degree of agglomeration expected. Choosing a low or high attachment efficiency that coincides with stable or instable conditions can be based on the PEC sensitivity reported by Meesters et al. (2019). Similarly, such basic data on the dissolution or transformation of nanomaterials (e.g. GD 29) can be used to choose a relevant order of magnitude of this rate constant based on the PEC sensitivity reported by Meesters et al (2019). For refinement of such data, further testing like using dynamic test designs to determine dissolution (rate) and/or alternative media (e.g. in order to determine heteroagglomeration with TG 318) will help to improve the PEC estimations in various environmental compartments.

5.1 Use of data on dissolution rate in exposure modelling

109.   Data on dissolution of nanomaterials is essential in environmental exposure modelling to enable the distinction between the dissolved and the particulate fraction of a nanomaterial. This distinction is included in current environmental fate models for nanomaterials, where dissolution is a process by which nanomaterials are transformed from the particulate form to the dissolved form (Meesters et al. 2019). As noted in Chapter 2 dissolution data can be used to estimate the dissolution rate constant. This is an input parameter in environmental fate models to calculate the predicted exposure concentration. Assuming first order kinetics, the dissolution rate constant can be calculated from experimental data as:

Equation 5:

$$k_{\text{diss}} = \frac{ln\left(\frac{[\text{NM}_0] - \left([\text{M}^{n+}] - [\text{M}_0{}^{n+}]\right)}{[\text{NM}_0]}\right)}{t}$$

where [NM$_0$] is the nanomaterial concentration in mg/L at the start of the experiment ($t = 0$), [M^{n+}] is the dissolved ionic metal concentration in mg/L at time t, [M$_0{}^{n+}$] is the dissolved ionic metal concentration in mg/L at the start of the experiment ($t = 0$), (k_{diss}) is the dissolution rate constant, and t is time of measurement. The dissolved ion concentration is operationally defined as the portion passing a filter of 3,

5 or 10 kDa MWCO at a certain media composition. If the medium contains complexing agents such as NOM and the NOM-metal ion complex can penetrate the membrane, the measured dissolved concentration is composed of the free metal ions and the complexed metal ions. In case multiple data points (i.e. concentration measurements) in time are available for $[M^{n+}]$, k_{diss} can be derived by fitting the relationship between $[M^{n+}]_t$ and time by using the following equation:

Equation 6:

$$[M^{n+}]_t = [M_0^{n+}] - [NM_0] + ([NM_0] \cdot e^{k_{diss} \cdot t})$$

110. As k_{diss} is only derived from ions appearing in solution over time, it is essential that the measurement technique used enables a distinction of the ionic fraction from the other metal fractions. It should be considered to use the ion concentration in the untreated medium as $[M_0^{n+}]$ to ensure that this value is not influenced by NM_0. Some caution is needed where rapid dissolution occurs at the beginning of the experiment, as this may result in artificially low k_{diss} when measurements do not capture this (e.g. when measurements are only available for 0, 24, and 48 hours).

6 Links to other relevant TGs and GDs

111.   Currently there are various OECD projects underway to develop nanospecific TGs and GDs. Solubility, dissolution (rate) and dispersion stability are important parameters that need to be considered in these activities as they influence the outcome of the respective methods (ProSafe, 2017). This chapter will give a short overview on how these parameters are interlinked with the ongoing environmental relevant OECD projects for nanomaterials as well as existing methods for solubility and dissolution.

6.1 OECD TG 105 - Dissolution in water

112.   TG 105 (OECD, 1995) describes a column elution method and a flask method to investigate solubility of compounds.

113.   During the column elution method of TG 105, the solubility of a solid chemical is determined while attached to an inert substrate in a column. The solubility equals the plateau concentration of a chemical dissolved from the test material reached in the eluate of the column.

114.   The flow-through testing for dissolution rate described in this GD is similar to the column elution method of TG 105 in that sense that the simulated media in contact with the nanomaterials is continuously refreshed and the concentration of dissolved substances is measured continuously. However, it is not clear how nanomaterials could be attached to such a substrate. In this GD, the nanomaterials are therefore attached to an ultrafiltration membrane or contained in a compartment formed by ultrafiltration membranes that are continuously in contact with the medium.

115.   The data interpretation in this GD is fundamentally different from that in TG 105. TG 105 describes how the solubility of compounds can be obtained, whereas this GD describes how a dissolution rate can be obtained.

116.   The flask method in TG 105 resembles the static batch test method described in this GD. However, TG 105 prescribes that solution and solid material must be separated using centrifugation after 24 hours of equilibration after which the solubility is measured. This GD prescribes using ultrafiltration as a separation technique, because centrifugation does not guarantee separation of nanomaterials and dissolved substances. Moreover, this GD encourages dissolved concentrations to be measured at several time points allowing to fit a kinetic model and calculating the dissolution rate.

6.2 OECD TG 106 - Adsorption - Desorption Using a Batch Equilibrium Method

117.   TG 106 (OECD, 2000) describes a batch method where a substrate, usually a dried and homogenised soil, is equilibrated with a chemical dissolved in water for a determined time. The chemical then adsorbs to the substrate and this process is quantified in the form of a partitioning coefficient (K_d). In general, TG 106 will not provide reliable results for nanomaterials as they do not establish a thermodynamic equilibrium with any substrate (Praetorius et al., 2014b). This GD does not apply to dissolved chemicals but to nanomaterials. An attachment efficiency is deducted in this GD using a kinetic approach instead of a partitioning coefficient (see chapter 3).

118.   Finally, SPM is recommended as a substrate in this GD, whereas a soil is usually used for TG 106.

6.3 OECD GD 23 - Guidance Document on Aquatic Toxicity Testing of Difficult Substances and Mixtures

119.   OECD GD 23 provides guidance for the testing of chemicals that are difficult to test for the purposes of determining their aquatic toxicity. This includes guidance for the testing for instance of poorly or sparingly water-soluble substances, substances degrading in test system, or hydrophobic substances.

120.   In paragraph 5 of OECD GD 23 (OECD, 2019) it is stated: "Consistent with the guidance presented herein, this document is generally not applicable to aquatic tests which include the undissolved phase of a test chemical, except where noted in this guidance for test chemicals which form stable dispersions. It should be noted that specific OECD guidance documents are under development for aquatic toxicity tests of test chemicals considered as nanomaterials, which may include both dissolved and undissolved fractions in the exposure."

121.   Guidance for testing of nanomaterials in aquatic ecotoxicity can be found in the GD on Aquatic and Sediment Toxicological Testing of Nanomaterials (see also section 6.5) while this GD provides guidance for the testing of the fate endpoints solubility, dissolution rate, and dispersion stability on basis of homo-, and heteroagglomeration.

6.4 OECD GD 29 - Guidance Document on Transformation/Dissolution of Metals and Metal Compounds in Aqueous Media

122.   As in this GD, GD 29 (OECD, 2001) describes a screening level and one full dissolution/transformation test. A high loading of a solid compound is equilibrated for 24 hours in a test medium during the screening test followed by a single measurement.

123.   The dissolved fraction is determined in a 0.20 μm filtrate using GD 29, differing from this GD where ultrafiltration is used for separation, because nanomaterials may pass a 0.20 μm filter. Further, indications on adapted needs when considering nanomaterial testing can be found in chapter 2.

124.   In the full test described by GD 29, three different concentrations of a solid compound are equilibrated at times up to 28 days, where the test medium is refreshed every 24 hours. Refreshing the medium would not be possible for nanomaterials in this way, because they are suspended in the medium and would be removed during refreshing.

125.   This GD recommends continuous refreshing, more in line with TG 105 and ISO TR 19057 (ISO 2017b). As in the screening test, ultrafiltration is recommended in this GD.

6.5 OECD GD 317 - Aquatic and Sediment Toxicological Testing of Nanomaterials

126.   Information on nanomaterial dissolution and dispersion stability should be used in addition to the guidance given in the GD on aquatic (and sediment) toxicity testing of nanomaterials in order to interpret the determined aquatic and sediment toxicity data. In the present GD (Chapter 4) a testing strategy is suggested for solubility, dissolution rate, and dispersion stability to inform about in which state (particulate, completely or partly dissolved) a nanomaterial is present under conditions of an ecotoxicity study and help to interpret test results regarding the contribution of dissolved or undissolved fraction to the toxicity.

127.   Information on dispersion stability helps to identify the main routes of exposure within the test system (e.g. via water phase in case of stable dispersion, via possible dietary exposure of unstable dispersion, via sediment).

128.   In addition, results from solubility, dissolution rate and dispersion stability testing can provide information to facilitate to decide on the analytic needs and the choice of appropriate test performance of potential further testing (e.g. frequency of water renewal rate, (semi)static vs. flow through test conditions).

129.   Data on heteroagglomeration obtained using this GD may also support interpretation of ecotoxicological data of standard test results. SPM of different forms are present in high enough concentrations in some standard tests and can affect the nanomaterial that organisms are exposed to. Attachment to fish feed, if it occurs, determines how fish are exposed to nanomaterials, via gills or via the gut (Handy et al., 2018). Extracellular polymers e.g. algae exudates may occur in standard aquatic tests and heteroagglomeration of nanomaterials and such polymers affects the exposure and consequent toxicity of nanomaterials to the organisms (Yang et al., 2018).

130.   Even though tests in soil systems are not included in the GD on aquatic and sediment tests, the recommendations mentioned above also apply to soil organisms. Soil pore water is expected to be an important exposure pathway of nanomaterials to soil organisms. Heteroagglomeration and dissolution in soil pore water is therefore also determining the toxicity to soil organisms. Attachment to inorganic particles such as clays or iron oxides is likely in standard soils (Miglietta et al., 2015) and determines whether organisms are exposed to individual nanomaterials and/or to heteroagglomerated nanomaterials, which may affect toxicity (Gupta et al., 2017).

131.   To broaden the significance of data on dispersion stability or dissolution for interpretation of nanomaterial behaviour in ecotoxicity test media, the simulated environmental media used in TG 318 and for dissolution testing may be amended to determine the dispersion and dissolution behaviour directly in the relevant test media for ecotoxicity testing.

132.   Comparing information on dissolution (rate) and dispersion (stability) of different nanoforms of a substance prior to ecotoxicity testing of those nanoforms, can support the development of hypotheses on expected similar behaviour and thus may guide grouping and read-across.

6.6 OECD TG for Nanomaterial Removal in Wastewater[5]

133.   This TG aims to provide a standard method for characterising the interaction between nanomaterials and wastewater treatment sludge. In this case the heteroagglomeration is specific biomass sampled from a wastewater treatment plant.

134.   The approach prescribes mixing biomass with nanomaterials for one hour followed by gravitational settling. The test is done at different total nanomaterial concentrations and sorption isotherms are calculated based on the nanomaterial concentration measured in the supernatant after settling. In the case of this GD, we recommend separating free from heteroagglomerated nanomaterials by means of accelerated sedimentation using centrifugation.

135.   The parameter reported from this GD in terms of heteroagglomeration is attachment efficiency which is a dimensionless parameter that is used in kinetic models, thus models that assume equilibrium do not apply. In contrast sorption isotherms reports isothermal trends of partitioning coefficients and the underlying model of these parameters assume a continuously equilibrium or steady-state situation. Different parameters are thus determined.

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[5] Under development.

6.7 OECD GD to Assess the Apparent Accumulation Potential of Nanomaterials during Fish Bioaccumulation Studies[6]

136.   As described earlier, dispersion stability and ion dissolution will affect what the organisms are exposed to, both in standard tests as well as in realistic environments. Interpretation of bioaccumulation testing therefore likewise needs information on dispersion stability, heteroagglomeration and dissolution of the nanomaterial in question as, for instance, proposed for fish bioaccumulation testing (Handy et al. 2018).

137.   A dissolution rate rather than solubility is needed for bioaccumulation testing given the kinetic nature of bioaccumulation.

138.   The information from dissolution, dispersion stability, and heteroagglomeration could also be used for decision making prior to testing, or bioaccumulation test media could be used in an adapted protocol of both endpoints in order to investigate dispersion and/or dissolution under experimental settings relevant for bioaccumulation tests.

6.8 OECD GD to support the Use of TG 312 (Leaching in Soil Columns) for Nanomaterial Safety Testing[7]

139.   A GD on how to conduct column tests to determine transport of nanomaterials in porous media based on TG 312 (OECD, 2004) includes recommendations on how to calculate a global attachment efficiency (α_{global}) for a specific nanomaterial in a specific soil. This GD provides guidance on calculating the attachment efficiency for interaction between a specific nanomaterial and specific SPM (α_{SPM}).

140.   It has to be noted that these attachment efficiencies are not the same, even if SPM material is used that is relevant for the specific soil studied. (α_{global} is a global parameter grouping many different processes that reduce transport (and thus increase (α_{global}) in soils (Baalousha et al., 2016). One of these processes may be heteroagglomeration with natural SPM in the pore water, the rate quantification of which may occur as described in this GD.

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[6] Under development.

[7] Under development.

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8 Terminology, definitions and abbreviations

141.   One important issue is the **harmonization of terminology and definitions** in various TGs and GDs for environmental fate testing of nanomaterials. Currently the published OECD TG 318 (OECD 2017) for dispersion stability already contains agreed and adopted terminology and this should be taken into account when future TGs and GDs are developed, in case applicable.

Agglomeration – Process of contact and adhesion whereby dispersed particles are held together by weak physical interactions ultimately leading to enhanced sedimentation by the formation of particles (agglomerates) of larger than colloidal size. In contrast to aggregation where particles held by strong bonds like sinter bridges, agglomeration is a reversible process.

Attachment Efficiency – Fraction of nanomaterial-SPM collisions resulting in nanomaterial attachment to SPM.

BET – Gas-adsorption method commonly using nitrogen (N_2) gas to determine the specific surface area of powders according to the Brunauer–Emmett–Teller theory (ISO 2010).

Concentrations – Concentrations of the particles and NOM in the samples are given in grams per litre (g/L), milligrams per litre (mg/L) and micrograms per litre (µg/L). In case of NOM it is given in mg/L of DOC. Concentrations of electrolytes ($Ca(NO_3)_2$ and $NaHCO_3$) present in the samples are given in millimole per litre (mM/L).

DOC – Dissolved Organic Carbon: the organic bound carbon in a water sample which passes a 0.45 µm filter, a part of dissolved organic matter (DOM). DOM in the form of humic substances have ~50 % DOC.

Dispersibility – is the condition of particular material of being dispersible or a measure to which extent it is dispersible in a dispersing medium or continuous phase. Dispersion stability refers to the ability of a dispersion to resist change in its properties over time.

Dissolution – Process under which a substance dissolves.

Dissolution rate – The amount of substance dissolved (solute) into a solvent over time.

Heteroagglomeration – Agglomeration of particles (here nanomaterials) with other particles (synthetic or natural).

Homoagglomeration – Agglomeration of particles (here nanomaterials) with each other

MWCO - pore diameter of ultrafiltration is expressed in terms of molecular weight cut-off (MWCO), i.e. the molecular weight of different molecules in the filtration process that are retained for 90 % by the membrane.

NOM – Natural organic matter: the organic substances present in surface or ground water. NOM covers humic and non-humic fractions as e.g. polysaccharides. NOM is operationally divided into Dissolved Organic Matter (DOM) and Particulate Organic Matter (POM). DOM passes a 0.45 µm filter, POM is retained by the same filter. POM as defined herein should not be confused with purgeable organic carbon.

Shear rate (G) – The shear rate or the velocity gradient (G) was introduced by Camp and Stein (1943) for stirred vessels. It is defined as the square root of the energy (e.g. added by stirring) dissipated by turbulence per unit time, accounting for the volume and viscosity of the considered fluid.

Size – Size of the particles, aggregates or agglomerates is given in micrometres (μm) or nanometres (nm). Particle size usually refers to the particle size distribution. The method for particle size determination and relevant parameters of the particle size average should be reported.

Solubility - The quantity of solute that dissolves in a given quantity of solvent to form a saturated solution.

SPM – Suspended particulate matter: finely divided solids dispersed in an aqueous environment

SRNOM – Suwannee River NOM: a standard surface water NOM material of the International Humic Substance Society (IHSS), isolated from the Suwannee River (US) by reverse osmosis and purified according to the procedures of the IHSS. The material can be purchased from the IHSS.