A common European approach to the regulatory testing of nanomaterials

Technical Guidance

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Physicochemical characterisation of manufactured nanomaterials (MNMs) in aquatic environmental fate and ecotoxicity exposure media

Version 2

Date: 19 October, 2015

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1. Background

1.1. Goal

To prepare input for a document on 'procedures for quantification of MNM exposure and fate in dispersions for aquatic ecotoxicological studies'.

1.2. Prerequisite data and information

The use of the following Technical Guidance Document (TGD) is recommended in conjunction with the following documents prepared and validated for aquatic ecotoxicology studies as part of the EU FP7 Project NANoREG:

- Probe Sonication Calibration SOP
- NANoREG ECOTOX Dispersion SOP

Guidance for the preparation of MNM dispersion for aquatic ecotoxicity testing is given in Figure 1. For aquatic ecotoxicity, stock dispersions of any test MNM should be prepared according to the NANoREG ECOTOX Dispersion SOP document. Prior to preparation of MNM stock dispersions using this SOP, the probe sonicator should be calibrated using the Probe Sonication Calibration SOP. MNM stock solutions for aquatic ecotoxicity testing are prepared in MilliQ water (or suitable equivalent) prior to further dispersion at relevant concentrations in the appropriate aquatic media for the species being tested.

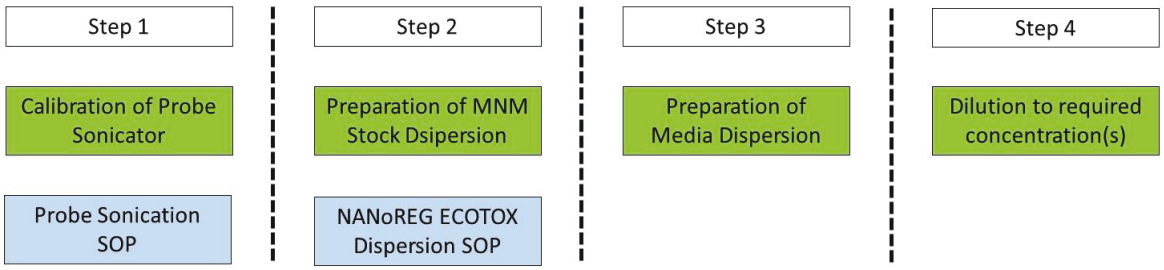

Figure 1. Schematic showing the preparation steps required for producing reproducible MNM dispersion for aquatic ecotoxicity testing. Recommended SOP are given in the blue boxes.

The use of the following TGD is recommended in conjunction with the prior characterisation of the pristine form of the test MNM to determine the following parameters:

- Particle size and morphology (e.g. using transmission electron microscopy)
- Particle specific surface area (SSA) (e.g. using BET methodology)
- Any other relevant physicochemical properties such as chemical composition and impurities should also be considered.

1.3. Information/data provided by the TGD

This TGD will provide a recommendation for conducting aquatic ecotoxicology studies with MNMs whereby the following information and data will be generated:

- Characterisation of the test MNMs at the start and end of the exposure study in order to determine changes in particle size and morphology over time
- The nominal concentration of MNM ($C_{nominal}$) in both mass on MNM (e.g. mg/L) and SSA (cm^3/L)
- The total concentration of MNM (C_{total}) present in the water phase of the exposure at both the start and end of the experiment (in SSA and mass)
- The concentration of both dissolved MNM ($C_{dissolved}$) and particulate MNM ($C_{particulate}$) in the water phase (actual) present in the water phase of the exposure at both the start and end of the experiment (in SSA and mass)
- Estimation of the amount of test MNM which has either sedimented out of the water phase or adsorbed to the surfaces of the exposure system (in SSA and mass)

An overview of the relationship between the terms outlined above is given in Figure 2.

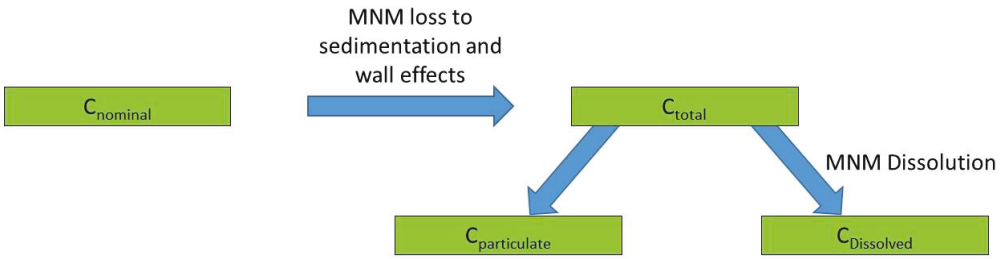

Figure 2. Schematic showing the relationship between the terms $C_{nominal}$, C_{total}, $C_{dissolved}$ and $C_{particulate}$.

1.4. General points for consideration

The following are important considerations when using or consulting this Technical Guidance Document.

- The TGD contains a detailed overview of both the **'Minimum'** and **'Additional/Desirable'** requirements for exposure and fate in ecotoxicity studies
- Media characterisation should be conducted as a part of ecotoxicity tests, as the exposure of organisms (and the resultant toxicity) can vary depending on the media and species being tested.
- No time points for any study are proposed in this Technical Guidance document as this will be test specific depending on the organisms being used.

2. Experimental approach and sampling regime

2.1. Size and morphology characterisation

MNM size and morphology should be determined in order to identify possible changes in these parameters in the exposure media during the exposure period. Size and morphology should be determined in the following sample types and at the following times (Figure 3):

- Stock MNM dispersion
- Pristine media dispersion (Time = 0)
- Pristine media dispersion (Time = end)
- Media dispersion with organisms (Time = end)

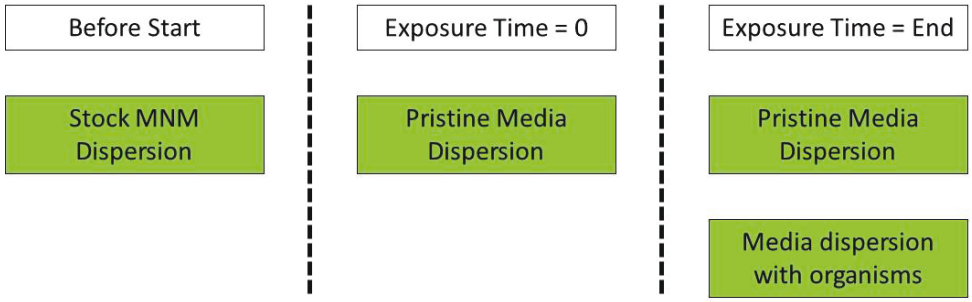

Figure 3. Schematic showing the sampling time and sample types necessary for assessing changes in MNM size and morphology.

MNM size and morphology analysis does not need to be conducted on every sample in triplicate. The aim is to determine what changes to the particles (size, morphology etc.) have occurred during the exposure period. However, as this may be a concentration dependent process, measurements should be conducted at every concentration used in the study.

2.2. Nominal concentration of MNM ($C_{nominal}$) in the water phase

An initial MNM stock dispersion is prepared in MilliQ according to the NANoREG ECOTOX Dispersion SOP. From this stock dispersion, a secondary dispersion is then made by dilution of the stock dispersion in the appropriate media required for the selected ecotoxicity test. The total concentration of MNM ($C_{nominal}$) is defined as the theoretical concentration of MNM present in the secondary dispersion and is based upon the assumption that 100% of the MNM used in preparation of the stock dispersion remains in suspended in water phase. It is recommended that $C_{nominal}$ is defined at all times based on both mass and SSA:

- mass of MNM (e.g. mg/L)
- SSA of MNM (cm^3/L)

2.3. Total MNM concentration (C_{total}) remaining dispersed in water phase

The total MNM concentration (sum of $C_{dissolved}$ and $C_{particulate}$ fractions) present in the water phase of should be determined. This will provide more accurate information regarding the chemical form of the MNMs which are present in the test system, and that will determine the exposure to the selected test species. Recommended methods for determining C_{total} are provided in Section 3.3. C_{total} should be determined in the following sample types when conducting aquatic ecotoxicity studies:

- In all samples and controls
- At all time points
- In all sample replicates

The method for determination of C_{total} will be dependent upon the type of MNMs being studied. Methods for the determination/quantification of MNMs are presented in detail in Section 3.3.

2.4. MNM pre-tests for dissolution and aggregation

If possible, it is recommended to combine the proposed MNM aggregation, sedimentation and dissolution pre-tests described below into a single study.

2.4.1. MNM pre-test for aggregation and sedimentation

An understanding of the aggregation and settling behaviour of test MNMs in the water phase of aqueous exposure solutions is necessary for all aquatic ecotoxicity studies. This TGD document recommends the implementation of simplified 'pre-study' to determine how relevant the MNM aggregation process is for a particular test MNM in a particular ecotoxicity media. By determination of C_{total} at each sampling point, an indication of the loss of MNMs to settling (sedimentation from the aqueous phase) and adsorption to equipment (e.g. walls) is gained. This permits documentation of the MNM concentration and approximate size that the test organisms have actually been exposed. The following MNM aggregation and sedimentation pre-test should be conducted prior to any planned aquatic ecotoxicity study involving MNMs. The MNM aggregation pre-test provides an initial look at aggregation behaviour in the appropriate exposure media without the presence of any organisms. The pre-test should be conducted using the following approach:

- Conducted in the pure ecotoxicity media for a minimum of the planned aquatic ecotoxicity test duration
- Conducted at each concentration planned for exposure study (not necessary to do this for triplicates)
- Should determine MNM aggregation and sedimentation behaviour using dynamic light scattering (DLS), and if possible, C_{total} MNM concentration, in the water phase at the start and end of the planned aquatic ecotoxicity test duration for each concentration

Methodology for the determination of MNM aggregation is described in detail in Section 3.1. If MNM aggregation in the pre-test is found to remain within 20% of the average particle size (z-ave) in the initial dispersion (Time = 0) the dispersion is considered stable and further monitoring of MNM

aggregation during the full aquatic ecotoxicity test is unnecessary (Figure 4). If MNM aggregation at the end of the pre-test (Time = end) is found to be more than 20% of the z-ave value measured in the initial dispersion (Time = 0) the dispersion is considered unstable and further determination of MNM aggregation during the full aquatic ecotoxicity test is necessary (Section 2.5.1).

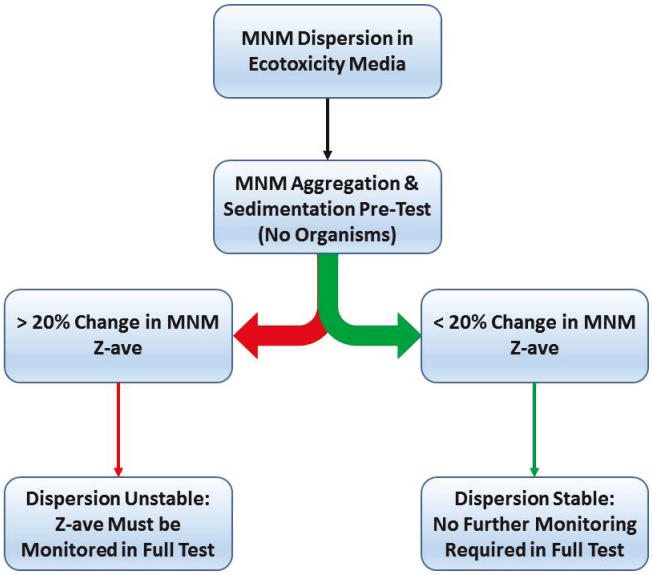

Figure 4. Decision tree for assessing the degree of monitoring of MNM z-ave required in ecotoxicity studies.

Methodology for the determination of MNM C_{total} is described in detail in Section 3.3. If MNM C_{total} in the pre-test is found to remain within 20% of the value determined in the initial dispersion (Time = 0) the dispersion is considered stable. If MNM C_{total} at the end of the pre-test (Time = end) is found to be more than 20% of the value measured in the initial dispersion (Time = 0) the dispersion is considered unstable.

2.4.2. MNM pre-test for dissolution

An understanding of the distribution of test MNMs between the dissolved and particulate phase of aqueous exposure solutions is necessary for all aquatic ecotoxicity studies. This TGD document recommends the implementation of simplified 'pre-study' to determine how relevant the MNM dissolution process is for a particular test MNM in a particular ecotoxicity media. The following MNM dissolution pre-test should be conducted prior to any planned aquatic ecotoxicity study investigating MNMs. The MNM dissolution pre-test provides an initial look at MNM dissolution in the appropriate exposure media without the presence of any organisms. The pre-test should be conducted using the following approach:

- Conducted in the pure ecotoxicity media for a minimum of the planned aquatic ecotoxicity test duration

- Conducted at each concentration planned for exposure study (not necessary to do this for triplicates)
- Should determine the dissolved MNM concentration ($C_{dissolved}$) in the water phase at the start and end of the planned aquatic ecotoxicity test duration for each concentration

Determination of C_{total} allows an estimation of the loss of MNM through settling and adsorption. To determine in which chemical form (dissolved or particulate) MNMs are present at a certain C_{total} within the water phase, measurement of $C_{dissolved}$ and $C_{particulate}$ in the water phase should be conducted. Methodology for the determination of $C_{dissolved}$ and the calculation of $C_{particulate}$ is described in detail in Section 3.2 and Section 3.3.

If dissolved species are known to not exert toxicity and MNM dissolution in the pre-test is found to be less than 10% of C_{total} in all concentrations of the exposure media tested, then only a limited monitoring of MNM dissolution during the full aquatic ecotoxicity test is considered necessary (Figure 5). If dissolved form is known to exert a toxic effect, and dissolution in the pre-test is found to be more than 20% of C_{total} in any of the concentrations, then dissolution of the MNM in the full aquatic ecotoxicity test must be determined in detail (Section 2.5.2).

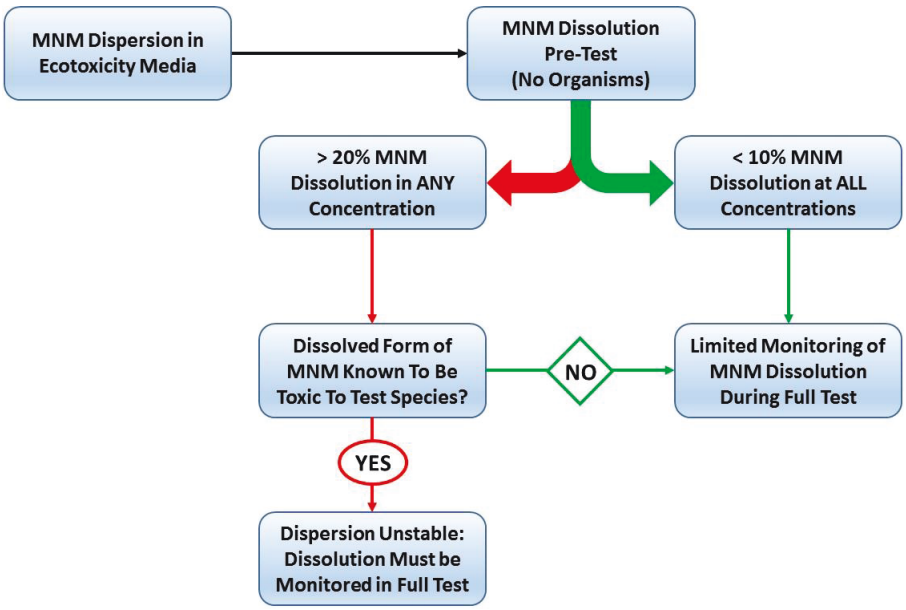

Figure 5. Decision tree for assessing the degree of monitoring of MNM dissolution required in ecotoxicity studies.

2.5. MNM sampling for dissolution and aggregation in ecotoxicity tests

2.5.1. MNM sampling for aggregation determination

Where a pre-test has indicated that a selected MNM undergoes significant aggregation in the relevant ecotoxicity media, determination of this parameter must be conducted as part the full aquatic ecotoxicity study. The following MNM aggregation determination strategy should be conducted as part of any aquatic ecotoxicity study investigating MNMs:

- Sampling conducted at the start (Time = 0), end (Time = end) and at any other sampling points during the ecotoxicity test
- Should be conducted for one sample at each concentration planned for exposure study (but not necessary to do this for all replicates)

Methodology for the determination of MNM aggregation is described in detail in Section 3.1. Methodology for the determination of MNM C_{total} is described in detail in Section 3.3.

2.5.2. Determination of dissolved ($C_{dissolved}$) and particulate ($C_{particulate}$) MNM fractions in the water phase

Where a pre-test has indicated that a selected MNM undergoes significant dissolution in the relevant ecotoxicity media, determination of this parameter must be conducted as part the full aquatic ecotoxicity study. The following MNM dissolution determination strategy should be conducted as part of any aquatic ecotoxicity study investigating MNMs:

- Total MNM concentration (C_{total}) in the water phase should be determined in all samples and replicates (no filtration required for this approach)
- If MNMs are considered 'non-dissolvable' in the pre-test (less than 10% dissolution), a limited number of sampling points for determination of $C_{dissolved}$ and $C_{particulate}$ should be included within the final exposure study (i.e. conduct at highest and lowest concentrations used).
- If MNMs are considered toxic or 'dissolvable' in the pre-test (greater than 10% dissolution), then determination of $C_{dissolved}$ and $C_{particulate}$ needs to be conducted at each sampling point (start, end and others) and for each exposure concentration.

The above sampling strategy will provide more detailed information on MNM chemical form and concentration along the experiment. This will allow for a better understanding of sample concentration effects and any dissolution process which occurs, offering the possibility of correlating this information with measured effects on test organisms.

3. Quantification methodologies

3.1. Methods for determining MNM aggregation and sedimentation

DLS is considered the most appropriate approach for the determination of MNM aggregation and settling behaviour in aquatic ecotoxicity tests. Water samples should be collected from either pre-

test or full ecotoxicity test exposures and transferred directly to relevant sample vessels (e.g. cuvettes). DLS analysis and data interpretation should be conducted according to standard approaches. Where available, zeta potential (ZP) determination is also recommended in order to give a direct measurement of MNM dispersion stability in aqueous ecotoxicity. Samples should be collected as for DLS measurements and transferred to the appropriate measurement vessels. Measurements should be conducted as soon as possible after sampling, preferably within a few hours maximum.

It should be noted that DLS can be limited at lower MNM exposure concentrations due to sensitivity issues. Furthermore, DLS accuracy can also be reduced due to interference from organisms (e.g. unicellular species such as algae and bacteria) and the presence of organic matter and proteins in the exposure media. In such cases where DLS does not prove to be a viable measurement technique for MNM aggregation, a simple filtration step using a 0.22 µm syringe filter followed by MNM concentration determination (See Section 3.3) can be used to give a basic indication of aggregates present above this size.

A possible alternative approach is to collect a drop of the MNM dispersion, place it on a SEM stub or a TEM grid. After a proper drying process, SEM or TEM can be used to obtain an indication of MNM aggregate size and morphology. However, when employing these approaches, it is important to be aware of the possible influence of the drying process on sample aggregation. Such sample preparation does not typically have an impact on particle morphology.

3.2. Methods for determining MNM dissolution

3.2.1. Dissolution - Minimum requirement

Ultrafiltration (3-10 kDa) is the most straightforward approach to monitor dissolution, and can operate over a wide range of concentrations. Detection limits are determined by the methods available for analysis of the metal or carbon concentrations in the filtrate. A variety of different techniques are available depending on the volume of the sample available from the test, and all have their advantages and disadvantages. Crossflow ultrafiltration can handle volumes of over 1 litre rapidly and with less clogging than standard methods; small centrifugation/ultrafiltration tubes can handle samples from 1-20 ml, are relatively cheap and easy to apply, but are more susceptible to clogging and sorption, especially if particle load is high. In all cases it is important to condition the equipment before taking samples (i.e., first filter and discard a sub-sample). The equipment and method should have been calibrated for filtration recovery of ionic solutions, as well as cut-off performance with standards. Lower mass cut-offs are more susceptible to clogging, so the filter size should be chosen according to the size of the MNM. Since media exposure solutions can be unstable, ultrafiltration should be carried out as soon as possible after sample collection, usually no longer than a few hours.

3.2.2. Dissolution - Additional/Ideal approaches

A number of alternative approaches with different associated costs and processing times are also available and might offer a more accurate determination of MNM dissolution. These approaches are considered as useful alternatives to the minimum requirement outlined above.

- High speed ultracentrifugation can be applied to separate the dissolved fraction, but needs prior calibration to ensure the parameters for particles removal, and careful control for resuspension of fine particles.
- Voltammetry can be applied as a validation or backup of the filtration efficiency, but usually only applicable on higher concentration samples.
- Potentiometry can be applied as a validation/backup of the filtration efficiency.
- Field Flow Fractionation (FFF) can be applied as a supporting method to follow both aggregation and dissolution.

3.3. Quantification of NM in exposure media

3.3.1. Metal and metal oxide MNMs

- ICP-MS/ICP-OES/ICP-AES can be used for elemental analysis of MNM concentration (total, dissolved, particulate) is dispersion samples.
    - Digestion of the sample in an appropriate acid should be employed when necessary (e.g. samples containing particulates).

Additional approaches which can be considered

- UV-vis spectroscopy is also a relevant technique for MNMs comprised of noble metals which show a strong absorption peak (due to the surface plasmon resonance).
- X-ray fluorescence spectrometry (XRF) is also a relevant technique for metal and metal oxide MNMs.

In all cases, quantification should be based upon a generated calibration curve. The calibration curve can also be used to determine the individual instrumental detection limits for each MNM type.

3.3.2. CNTs and other carbon-based MNMs (CNMs)

- UV-vis spectroscopy can be used for determination of CNM concentration (total, dissolved, particulate) is dispersion samples.
    - This approach may be limited when attempting to quantify CNMs in media which contains other carbons sources (e.g. natural organic matter). In this case, unique absorbance wavelengths for the tests CNMs should be identified.

Additional approaches which can be considered

- Thermogravimetric Analysis (TGA) is also a relevant technique for CNMs, but again can be limited if other carbon sources are present in the sample.
- Raman spectroscopy is also a relevant technique for CNMs
- X-ray fluorescence spectrometry (XRF) is also a relevant technique for CNMs.

In all cases, quantification should be based upon a generated calibration curve. The calibration curve can also be used to determine the individual instrumental detection limits for each MNM type.