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OECD GUIDELINE FOR THE TESTING OF CHEMICALS

DISPERSION STABILITY OF NANOMATERIALS IN SIMULATED ENVIRONMENTAL MEDIA

INTRODUCTION

1. The increased production and wide usage of manufactured nanomaterials suggest a higher probability of finding them in natural systems, where transport and distribution in the environment will take place especially via aqueous media. This has raised the question and lead to discussion as to whether the existing regulatory testing protocols are sufficient and adequate to assess the fate of manufactured nanomaterials and their impact on the natural system(s) of interest. Dispersion stability of the nanomaterials was identified as important parameter affecting the environmental behaviour of nanomaterials. This parameter depends on the physicochemical characteristics of the nanomaterial itself, the physicochemical characteristics of the suspension medium, suspension preparation, concentration of the nanomaterial and concentration of other substances and particles in the suspension. The dispersion stability is also highly dynamic in many cases, because it is controlled by kinetics (energy barriers) rather than thermodynamic equilibrium. Therefore information on nanomaterials dispersion stability and agglomeration behaviour is, beside e.g. dissolution rate, one prerequisite for a robust and reliable further testing of nanomaterials.

2. This test guideline describes a test procedure to gain information on dispersion stability of manufactured nanomaterials in simulated environmental media. Based on the results it is possible to categorise tested nanomaterials into three different classes according to their dispersion stability in tested aqueous media. These classifies nanomaterials of low dispersion stability or high dispersion stability under given conditions, and nanomaterials with condition-depending dispersion stabilities. To represent natural environmental conditions the test guideline prescribes testing under different hydro-chemical conditions representing the stability relevant composition of many fresh waters with varying parameters such as pH, ionic strength, and presence of dissolved natural organic matter (NOM/DOM). The data on dispersion

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stability is important for the assessment of the fate of nanomaterials in the aquatic environment and can help to predict the behaviour of nanomaterials in eco-toxicological tests.

3. This test guideline provides a simple and effective tool that can analyse the dispersion stability in aqueous media under conditions representative for natural surface water chemistry with regard to their stability controlling components. The experiments shall be performed with the purpose of investigating the fate of nanomaterials in natural waters, potential behaviour in test media and their impact on the environment based on the criteria listed above.

4. Definitions and units can be found in Annex 1.

INITIAL CONSIDERATIONS AND LIMITATIONS

5. *Background and purpose of the test guideline* - The main purpose of this guideline is to assess the ability of a nanomaterial to attain a colloidal dispersion and to conserve this dispersion under environmentally relevant conditions. The test procedure involves a dispersion of the nanomaterial with the aid of a calibrated sonication procedure and the determination of the mass concentration of the nanomaterial in a set of test vials while the particles undergo homoagglomeration and settling in environments of different hydrochemistry. The test procedure addresses the question if a nanomaterial can be dispersed under the chosen hydrochemical conditions and to which extent it maintains dispersed over time. Dispersion stability will be determined in percentage after 6 h and as function of time, respectively. The test is operationally defined and allows materials to be compared with each other. This means it does not produce quantitative parameters as agglomeration rates, particle size as a function of time or attachment efficiencies. Furthermore, the test procedure is intended to be simple and to be conducted in standard laboratory conditions. This approach was developed based on the proposal of von der Kammer (1) and further elaborated and checked for environmental relevance by Ottofuelling et al. (2). Accordingly, the procedure is based on the construction of a multi-dimensional test matrix including environmentally relevant test parameters predominantly influencing nanomaterial dispersion stability. These parameters include presence of various cations and anions of natural waters, natural organic matter (NOM) and pH, and represent compositions of parameters found in 90-95% of natural fresh water bodies. Such an approach is utilized in the present guideline and represents a more universal and simpler procedure than testing all (relevant) fresh water types and considering all contingencies (e.g. seasonal changes). Theoretical background, selection of media and parameters which need to be considered to be important to influence the dispersibility and dispersion stability are presented in paragraphs 6-16.

6. *Stability of an aqueous dispersion* - In an aqueous dispersion particles undergo Brownian motion resulting in particle-particle collisions. If the particles adhere to each other as a result of such a collision they form agglomerates. While for small particles the sedimentation in the aqueous medium is countered by the Brownian displacement and net sedimentation rate is negligible, the formed agglomerates eventually reach particle sizes at which the sedimentation rate is greater than the Brownian displacement causing the particles to sediment: the dispersion is not stable over time. The particle size at which the sedimentation becomes the dominating process is depending on the density and shape of the primary particles, the size, structure and effective density of the agglomerates and temperature. It should be noted that some of these parameters will not have a single fixed value (i.e. size), but a distribution of values (i.e. size distribution). Due to the collisions of particles, dispersions of solid particles are inherently unstable and the particles eventually agglomerate. However, an energy barrier, resulting from the interplay of various particle-particle interaction forces can prevent the attachment of particles to each other and therefore prevent or slow down the process of agglomeration. Among the various forces existing between particles, there are three major forces of different nature controlling the particle interactions. These are the forces of Van der Waals interaction (in most cases attractive, short ranged), electrostatic interactions (attractive or repulsive) and steric forces emerging from surface bound macromolecules (repulsive or attractive). In aqueous dispersions, the resulting interaction force between particles is predominantly controlled by the particle

composition, the surface chemistry and the hydrochemistry. A stable dispersion is created if the resulting interaction forces are repulsive and cannot be overcome by the momentum of the approaching particles and therefore represents a non-equilibrium situation which is kinetically hindered from reaching equilibrium in the agglomerated state.

7. *Selection of media parameters* - Since the aim of the test is to assess nanomaterials behavior in relevant natural conditions, the choice of the composition of the simulated environmental media for the test is crucial for the relevance of the test. The media shall represent natural surface waters and the choice of individual parameters and parameter ranges must be based on (i) the effectiveness of the parameter to influence colloidal stability and (ii) the likelihood that the parameter has a magnitude in natural waters which results in an important impact on colloidal stability. For example, sodium as a monovalent cation typically influences the colloidal stability of dispersions only at a concentration not often encountered in natural freshwaters. At the same time it is likely that calcium, which has more than an order of magnitude higher effect on colloidal stability because of its divalent character, is found often at concentrations where it influences colloidal stability. The pH influences the surface charge (and hence the zeta potential) of many nanomaterials. NOM has a relative universal effect in stabilizing dispersions against agglomeration, although this depends on the type of nanomaterial and its coating, the concentration and type of NOM, and the hydrochemistry. Thus the key parameters for the test media are pH, concentration of divalent ions and of NOM. These are applied at concentrations which represent 90-95% of natural surface waters (according to FOREGS Database (3)). $Ca(NO_3)_2$ has been chosen as major electrolyte because of the dominant effect of multivalent cations (Ca^{2+}) on particle aggregation as compared with the less effective monovalent ions and less abundant trivalent ions and because it is present in relatively high concentrations.

8. *Alternative medium* – The media parameters described above are the stability- influencing components of the media. This is adequate and simulates natural conditions for particles which are negatively charged under the test conditions and interact with NOM. In this case the calcium ions represent the near total sum of divalent cations in natural waters and therefore small differences due to the presence of magnesium can be neglected. When particle surfaces are positively charged, the nitrate in the primary test medium is, as a monovalent anion, less effective in de-stabilizing the dispersion than sulphate would be. Hence, a more realistic, but also more complex, medium would also consider magnesium and sulphate, which both appear in natural waters in concentration ranges which are able to control stability. Magnesium is less efficiently complexed by NOM, in a situation where NOM is not adsorbing to the surface of the tested nanomaterial, the complexation of the divalent cation by NOM (thereby reducing its ion activity) is an important stabilization mechanism. Since Mg^{2+} is less complexed than Ca^{2+}, a test medium containing calcium and magnesium would represent the natural situation better. It has been observed that adsorption of sulphate to TiO_2 nanoparticles increases the negative zeta potential, but at the same time de-stabilizes the dispersion considerably (4). Therefore, for an additional alternative testing with a more realistic medium the following salt ratio can be used. The ratios of molar concentrations in the alternative medium should approximately follow the ratios on average found in nature: Ca^{2+}/Mg^{2+} 4:1, nitrate/sulphate 10:1. This is matched most easily (although not exactly) by using $Ca(NO_3)_2$ and $MgSO_4$ salts to prepare the medium with a molar ratio of Ca^{2+}/Mg^{2+} 4:1. The testing of the dispersion stability in the alternative medium was not part of the validation study underlying this guideline.

9. Ionic strength (IS) –a measure of the concentration of all ions in a solution. The ionic strength is calculated as:

$$IS = 0.5 \sum_{i=1}^{n} c_i z_i{}^2$$

with *c* the molar concentration of the *i*-th ion and *z* the valence of this ion, both anions and cations are accounted for. The ionic strength relates directly to the thickness of the Electric Double Layer. In the presence of an electrolyte in an aqueous medium an Electric Double Layer (EDL) around the particles is formed. The EDL is the region near the particle surface where the electric potential of the surface is balanced by counter-ions in the solution. The higher the electrolyte concentration and ion valence, the smaller is the distance from the particle surface at which the potential vanishes. Apart from many other forces the electrostatic interaction is controlling the stability of the dispersion. For particles with the same polarity of their surface charge, low electrolyte concentration promotes stability, while high electrolyte concentration promotes their agglomeration and sedimentation. A compact, thin EDL (at high electrolyte concentration) allows particles to approach closer to each other and interact more effectively based on short-range Van der Waals attractive forces. When particles with the opposite surface charge are considered, low electrolyte concentration leads to faster particles agglomeration and sedimentation. In this case increased thickness of EDL enables particles to approach each other and interact faster through forces of electrostatic attraction.

10. *pH* – pH has an effect on the surface charge of particles carrying variable charges which can be protonated or de-protonated at the surface. These variable charges can have their origin i.e. from the mineral surface or from ionisable organic surface coatings. Hence a change in pH can change the magnitude and polarity of the surface charge. Dispersions of particles which are predominantly electrostatically stabilized are most unstable at the pH of Point of Zero Charge (PZC). At this pH the net charge at the particle surface that forms the EDL is zero, what diminishes the electrostatic repulsion. Due to specific adsorption of potential determining ions (i.e. calcium, carbonate) or NOM molecules from the test solution to the particle surfaces the PZC must not equal the IEP of the particles in the test medium.

11. *Concentration of Dissolved Organic Matter (DOM)* – DOM, the < 0.45 µm fraction of the NOM, influences the stability of particle dispersions through adsorption on the particle surfaces. A positive surface charge can be reversed to negative charge due to the adsorption of DOM which has in general an anionic character, uncharged or negatively charged surfaces can experience an (increasing) negative potential, thereby providing enhanced stability. Particle dispersions generally become more stable due to such interactions. However, when the adsorbed DOM reduces or only balances a positive charge on the particles surfaces, agglomeration could be observed. DOM molecules in the liquid phase also act as a buffering agent, keeping the pH of particle dispersion at a constant level and DOM complexes cations which e.g. reduces the activity of calcium in the medium, thereby reducing the de-stabilizing effect of calcium.

12. *Particle concentration* – For dispersions of particles, the particle concentration is often given in mass concentration of particles per volume of dispersion liquid. However, not the mass but the number concentration of particles is key for the number of particle-particle collisions in a dispersion per unit time and therefore determines the rate of the agglomeration process and hence the rate at which the agglomeration and phase transfer proceeds. To achieve comparable results from nanomaterials of different particle size in the given time frame of the experiment, a range of particle number concentrations is defined (0.5×10^{12} to 5×10^{12} particles/L) which should be met when preparing the test dispersions. Particle

number concentrations are related to mass concentrations via particle density and size (and geometry of the particles).

13. *Particle nature and surface chemistry* – The surfaces of various nanomaterials are terminated by surface functional groups which determine the surface chemistry of the nanomaterials in the dispersion. The surface density and type of surface groups determine the extent in which particles are able to develop surface charge and build an EDL in an aqueous medium. Permanent charge acquired by isomorphic replacement and pH-dependent, variable charge are two major factors to consider. Additionally surface-bound molecules (coatings) may introduce a non-electrostatic stability factor, the steric stabilization, where the molecules prevent attachment of the particles by creating a steric barrier. When the particles interact with components of the aqueous phase (i.e. ions and NOM) the surface chemistry will be modified compared to the pristine state. Surface charge will change and the IEP will no longer be identical to the PZC. Adsorbing molecules of the NOM might replace or overcoat an existing technical coating of the particles, which also changes its surface chemistry and can add a component of steric stabilization.

14. *Particle size and particle density* – Particles < 100 nm in an aqueous medium undergo rapid Brownian motion and have small settling velocities. When Brownian motion compensates for settling, particles stay in dispersion as long as they do not agglomerate. Density difference to that of water of course plays a role (see next paragraph). Large particles of the same density sediment faster than smaller one. Since particle size has a larger effect on particle settling than density, agglomeration leads to faster particle settling even when the apparent density of the agglomerate becomes smaller than the density of the particles it is formed of. With increasing particle or agglomerate size the settling becomes faster and the effect of Brownian motion vanishes, and phase separation occurs, resulting in the dispersion that is not stable. As a rule of thumb settling overwhelms Brownian motion between 100 nm and 1 µm, depending on density of the particles or effective density of the agglomerates. Also, particles with a density close to that of water might agglomerate, but not settle during the experimental time of 6 h. This is accounted for in the final centrifugation step in which the material density (not the effective density of the agglomerates because this is difficult to determine) and a particle size cut-off of 1 µm is considered. Particles with high density (e.g. Gold-Nanoparticles) show considerable sedimentation rates even in the non-aggregated form (e.g. 100 nm Gold-NPs settle by 4.3 mm/day at 20 °C)

15. *Limitation of the Test Guideline* – This guideline is applicable for nanomaterials having a density > 1 kg/L. The density difference of particles to the dispersing medium and their size (buoyant mass) are the significant factors influencing their sedimentation rate, especially at the stage of sample's centrifugation. Nanomaterials with the density smaller than 1 kg/L (dendrimers, polymers, etc.) will not settle regardless of their size or even can accumulate at the air/water interface in the vessels. For these nanomaterials alternative methods from those based on sedimentation have to applied, e.g. the time-dependent increase of particle size measured in a DLS device. Such methods are beyond the scope of this TG.

16. It is recommended to consult the OECD document *ENV/JM/MONO(2012)40 Guidance on Sample Preparation and Dosimetry for the Safety Testing of Manufactured Nanomaterials* where useful additional information on physico-chemical characterisation can be found.

PRINCIPLE OF THE TEST

17. The tests are designed in the form of a dispersion – agglomeration – settling procedure in 50 mL centrifuge vials, where the phase separation, resulting from particle agglomeration and settling, is assessed via particle concentration measurement(s) in the upper part of the supernatant (Figure 1).

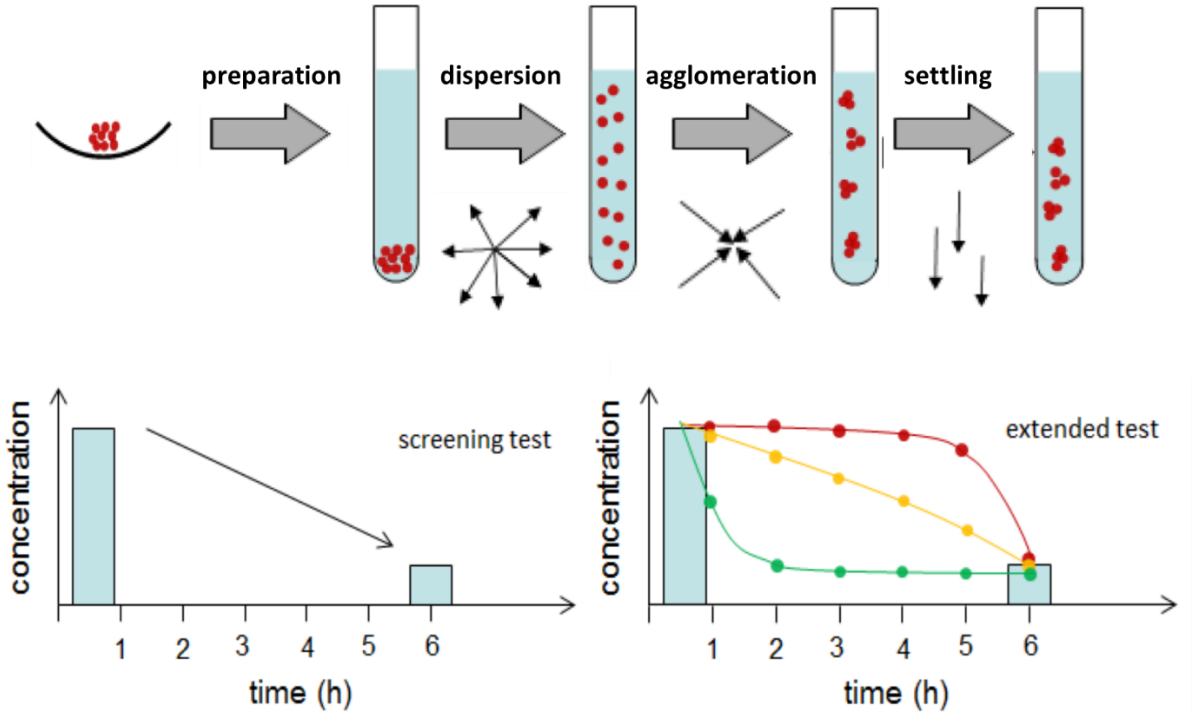

Figure 1: Principle of the testing scheme (upper part), and possible outcome of the screening and the extended testing (lower part; see text and figure 2). The red line represents a nanomaterial that has a small density difference to water, it agglomerates, but does almost not settle. In step 6, a centrifugation is performed which takes the density of the nanomaterial into account and performs a particle size cut-off. Here, the lightweight agglomerates are removed into the deposited fraction because they are larger than the cut-off value. The yellow line represents a nanomaterial that continuously agglomerates and settles out. The green line represents either a nanomaterial that quickly agglomerates and settles (high density) or a heterogeneous nanomaterial that contains two different fractions. For the first, the number concentration in the top of the vial is reduced so that the further agglomeration is slowed down to a point where it becomes virtually stable (not enough collisions in the timeframe). For the latter, one fraction is unstable and settled out within 2 h, another fraction is highly stable and does not agglomerate.

18. The experimental matrix of hydro-chemical conditions covers environmentally relevant conditions which are influencing nanomaterial agglomeration in natural waters, such as pH, concentration of electrolyte (as $Ca(NO_3)_2$) and dissolved natural organic matter. To check the dispersion stability in the

prepared test samples, the agglomeration and resulting sedimentation of nanomaterials is probed by determining the remaining particle concentration in the top 0.5-1 cm layer of dispersion. Before the determination at hour 6, samples are furthermore subjected to a centrifugation. For a fully stable dispersion, the concentration of particles in the top 0.5 -1 cm volume of the test vial is not supposed to change over time, whereas agglomeration and (as a consequence) settling shall gradually reduce the concentration of particles in the top volume of the sample. The possible losses of material during dispersion and test vial preparation procedures are assessed through the calculation of the ratio between the concentration of material detected during 0-hour measurement and the expected concentration value based on the mass of the dispersed material and should be recorded (expressed in %). The 0h value is then set to 100 % for further calculations and plotting, thereby losses during the preparation of the dispersion and setting the hydro-chemical conditions in the test vial are neglected.

19. The test procedure is split into two parts (see figure 2). A comparably quick screening test (first part) which covers a range of electrolyte concentrations, pH and NOM and where the concentration in the supernatant is only measured at two time points: at the beginning (0 h) and after 6 h plus a centrifugation step with a particle size cut-off of 1 µm (calculation tool provided for download). The NOM supports pH stability through its inherent buffering capacity and promotes dispersion stability. This test may therefore be seen as a scenario using optimal conditions for nanomaterials in aqueous media with regard to stable dispersions (although not all particles might experience stabilization by NOM against agglomeration). If the nanomaterial does not show low dispersion stability (≤10 %) or high dispersion stability (≥90 %), respectively, under all test conditions an extended test has to be conducted where a sub-sample for analysis is taken every hour during the time period of 6 hours (0-6 hours). The extended test (second part) is performed in the prescribed condition range and in presence and in the absence of NOM. To counter pH changes in the virtually un-buffered dispersions without NOM, sodium bicarbonate is added at 5 mM to supply buffering while still being a compound present in all natural waters at the tested pH conditions.

20. Addition of NOM solution to the samples serves two purposes. Primary purpose is the mimicking of greater dispersion stability achieved through the adsorption of NOM molecules at the particle surfaces, which simulates the interaction of the nanomaterial with NOM present in natural waters. This stabilization originates predominantly from electrostatic and steric effects of the deprotonated, negatively charged, carboxylic groups in the NOM through the increase of the negative charge on the particles surfaces (stability from -/- repulsion). In the case of positively charged particle surfaces (stability from +/+ repulsion) the addition of NOM first reduces the positive charge on the particles, thereby reducing particle stability. If a too small amount of NOM is added the charge reversal (+/+ → -/-) is not reached or not sufficient to re-establish stability and particles will show dispersion instability from the NOM addition. This can be avoided using a minimum NOM concentration, which is (simplified) depending on the specific surface area of the particles. Secondary purpose of NOM addition is the pH buffering of the dispersion at elevated pH against the effect of absorption of CO_2 from air without the need for any further buffering additives which might influence stability of the dispersion. The added NOM also interacts with the calcium ions through complexation; the stability of the particles is hence also increased by a reduction of the calcium ion activity.

21. One important factor for the comparability of results among different nanomaterials is a standardized particle number concentration at the beginning of the test. The particle number concentration directly determines the speed at which the agglomeration and settling process proceeds. The test looks at this process in fixed time intervals. If identical mass concentrations are used for particles of different size and density, results at fixed time points will vary just because of different number concentrations at the beginning of the test. The effect of particle number concentrations on the outcome of the test has been

investigated on the example of TiO_2 (NM-105 (4)). It was apparent that the test is insensitive to the particle number concentration in a range of about one order of magnitude. Average particle number concentration should be calculated from the particle density and a suitable particle size measurement, ideally provided from physico-chemical testing of the respective nanomaterial. The choice of suitable particle size measurement method should be made based on knowledge about the characteristic of the particles under investigation to facilitate the needed accuracy of the number concentration calculation. If those data are not available a DLS analysis according to ISO 22412:2008 of the prepared stock dispersion is recommended. Present guideline is applicable to dispersions with the approximate particle concentration of 10^{12} particles/L (±1/2 order of magnitude, what defines the required accuracy of the particle size analysis). The number of particles in the dispersion can be calculated according to the formula:

$$N = \frac{M}{\rho \times V}$$

where N is the number of particles in the dispersion, M is the mass of the powder to prepare the dispersion, ρ (kg/m3) is the density of the particle material (known value), V (m3) is the volume of particles taken from existing material information or calculated from DLS size-measurements. For the latter, use the formula for the volume of a sphere and the first mode (most frequent diameter) of the intensity weighted distribution of the autocorrelation function), not the Z-average[1]. A Microsoft® Excel test preparation tool is available on OECD website for calculation of particle number concentration from mass concentrations based on the model of a homogeneous solid sphere with material density and average particle diameter.

22. The experimental design as well as typical experimental results for TiO_2 (NM-105) and Ag (NM-300K(5)) nanomaterials, presented for comparison, can be found in annex 2B.

[1] The **Z average** is the intensity weighted mean hydrodynamic size of the ensemble collection of particles measured by dynamic light scattering (DLS).

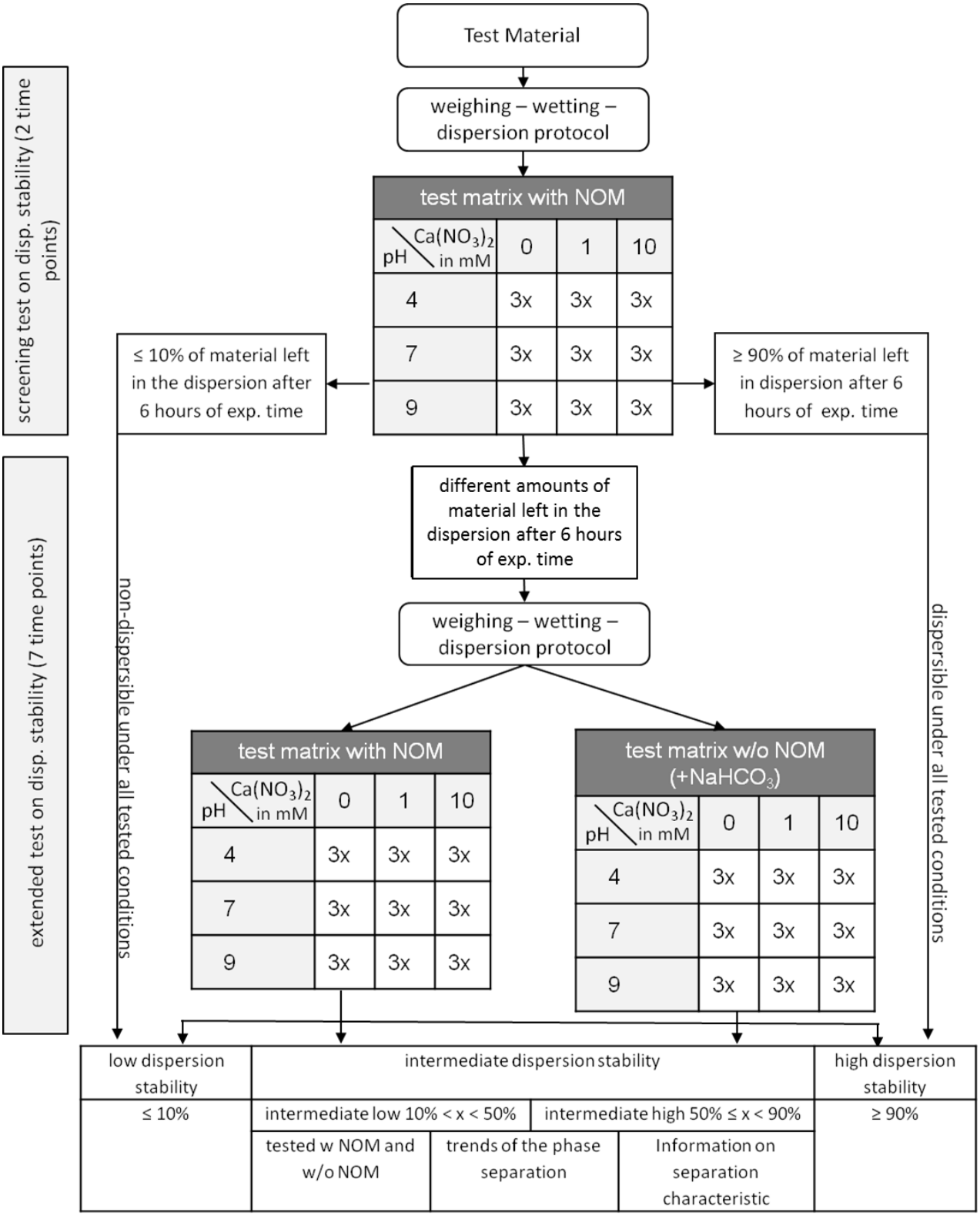

Figure 2: Decision-tree layout of the tiered testing for dispersion stability. For nanomaterials which are not high dispersible or low dispersible, respectively, under all test conditions of the screening test the extended test is required. The extended test will give also

timely resolved information on the trend of settling and by this information about the underlying sedimentation process. $Ca(NO_3)_2$ concentrations in the figure are given in mmol/L.

INFORMATION ON THE TEST MATERIAL

23. For a sound test performance and interpretation of test results information on nanomaterials composition, density, particle size, specific surface area, IEP, and dissolution (rate) is needed before conducting the test and should be reported together with the results. Density and particle size is needed to calculate the required sample mass in the test to obtain the desired particle number concentration. Information on these physical chemical parameters is also needed to identify if the nanomaterial under investigation fulfils the validity requirements of the test (paragraph 25-27).

RECOMMENDED NANOMATERIALS FOR VALIDATION ON THE TEST

24. Three nanomaterials from the JRC nanomaterial repository were used to underpin the applicability of the proposed experimental routines. Following three nanomaterials can be used as controls for routine validation of this test guideline as representatives of specific dispersion stabilities under the given conditions: Ag (NM-300K) (5) as completely stable over time and all conditions, carbon nanotubes CNTs (NM-400) (6) as not stable in all test conditions, and TiO_2 (NM-105) (4) as intermediate stability, strongly dependent on test conditions (see also Annex 2B). It is recommended to perform the test with the stated nanomaterials at least once in case there are no experiences with the test system or the particles under investigation, respectively.

CONSIDERATIONS FOR APPLICABILITY OF THE TEST

25. Apart from reduction of particle concentration in the supernatant by the agglomeration -and subsequent settling of dispersed nanomaterials, deviation from expected starting concentration can occur also due to:

(a) Incomplete dispersion of the analysed nanomaterial
(b) Fast sedimentation of large particles present in the analysed nanomaterial
(c) Attachment/adhesion of the nanomaterials on the walls of experimental tubes

26. While (a) and (b) are not considered a failure of the test, because the test would correctly report the dispersion stability of the whole tested nanomaterial, c) has no link to dispersion stability. Therefore, losses to vessel wall needs to be determined and reported. Particle losses to vessel walls can be identified by visual inspection and by analysing the total dispersion volume including any precipitates after the test for the nanomaterial amount in the liquid part. A mass balance will then reveal losses due to wall adsorption. Loss to vessel wall should be avoided as far as possible. The use of a different vial material (glass, polycarbonate, Teflon) instead of the polypropylene centrifugation vials specified for this test guideline can help to reduce these losses. Deviation of vessels should be reported.

27. If the tested nanomaterial dissolves in a considerable amount (this information might be available from the results of the dissolution test guideline) a false positive result will be received from this test guideline since the released ions stay in the supernatant. In such a case the sample taken from the supernatant must be analysed in two different ways: 1) for the total nanomaterial content; and 2) for the dissolved content after filtration trough a filter with small pore size. Filtration through 10.000 g/mol (Dalton) molecular weight cut-off membranes in 15 mL centrifugal filtration units may be used. The membrane cut-off equals approximately 2 – 3 nm particle size. The filtrate chamber of the device should be pre-loaded with 50 µL of 6 M nitric acid to prevent losses of the ions to the vessel walls. This volume needs to be considered when calculating the concentration of ions. Amount of dissolved ions should be reported.

DESCRIPTION OF THE METHOD

Apparatus and chemical reagents

28. Standard laboratory equipment, including but not limited to:

(a) Calibrated pipets for sample preparation (5 mL, 2 mL, 0.1 mL volume)
(b) Ultrasonic probe ½" or 13 mm diameter (with regulated power output) for producing the particle dispersion
(c) pH-meter with 3 mm ∅ micro-electrode to measure the pH of the dispersion
(d) DLS device for measuring the particle size if information from other physico-chemical characterization is not available.
(e) 250 ml glass beaker to prepare stock dispersion
(f) Amber glass container to store stock dispersion or aluminium-foil wrapped polypropylene container to shield from sunlight
(g) 50 ml conical bottom polypropylene tubes (50 mL conical bottom centrifuge tubes, 3 cm in diameter, 11.5 cm tall) to perform the agglomeration experiments

(h) Ca. 10 ml polypropylene tubes to prepare the samples for Inductively Coupled Plasma Mass Spectrometry Device or Inductively Coupled Plasma Optical Emission Spectrometry analysis (as required by the autosampler of the instrument)
(i) Standard lab centrifuge, capable of >3000 relative centrifugal force (rcf)
(j) Inductively Coupled Plasma Mass Spectrometry Device or Inductively Coupled Plasma Optical Emission Spectrometry device for analysis of nanoparticle dispersions

29. Materials:

(a) Water (H_2O) – ultrapure de-ionized water (18 MΩ resistivity) is used for all preparations and dilutions
(b) Sodium Hydroxide solution (NaOH) – 0.1 M solution, for establishing pH
(c) Nitric acid (HNO_3) – 0.1 M solution, for establishing pH
(d) NOM solution of Natural Organic Matter (1 g/L of DOC as a target value, precise value to be determined with a DOC measurement)
(e) Calcium Nitrate ($Ca(NO_3)_2$) – 0.1 M solution to establish the different electrolyte concentrations
(f) $NaHCO_3$ solution with a concentration of 0.1 M
(g) Analysed nanomaterial in the form of dry powder or in a form of aqueous dispersion

General conditions

30. All experiments should be performed in triplicates at conditions about standard (298.15 K (25 °C, 77 °F), 101.3 kPa (14.7 psi, 1.00 atm, and 1.013 bar).

Preparation of nanoparticle stock dispersion

31. In case the tested nanomaterial is provided in the form of dry powder, it should be pre-wetted in ultrapure water and left in the form of wet-paste for 24 h to insure the proper interaction of nanomaterial surface with ultrapure water. After 24 h of pre-wetting the resulted wet paste is dispersed into known volume of ultrapure water, thus providing a stock dispersion with known nanomaterial concentration. The concentration of the stock dispersion shall be sufficient to allow the further dilution to the required particle number concentration (0.5×10^{12} to 5×10^{12} particles/L) within the analysed samples. The mass concentration of the nanomaterial in the stock dispersion should not be too high to avoid facilitation of nanomaterial agglomeration in the stock before dilution into the test vessels. Thus the recommended concentration of nanomaterial within stock dispersion shall not exceed the concentration of nanomaterial within analysed samples more than 20 times. Usage of a calibrated sonication probe[2] is mandatory. Sonication probes are widely available and allow comparably easy calibration of the energy input to the dispersion. A Microsoft® Excel test preparation tool supporting the calculation of delivered sonication energy via sonication probe is available on the OECD website. Sonication of the dispersion should be performed as follows:

(a) Place a 250 mL glass beaker in an >1 L ice bath and secure against moving.

[2] Description of calibration process of sonication probe can be found in annex 2A

(b) Add 125 mL of ultrapure water and the required amount of nanomaterial.
(c) Place the tip of the ultrasonic probe (½" or 13 mm diameter), in the centre of the beaker, 2.5 cm below the surface.
(d) Add 40 W output power for 10 min.
(e) Store the prepared dispersion in an amber glass container at 4 °C (do not freeze!).

32. Sonication of high aspect ratio nanomaterials should be performed with care since they tend to break during high energy input. If applicable, high aspect ratio nanomaterials should be sonicated with a method that allows dispersion as far as possible while avoiding destruction of the nanomaterial under investigation. Appropriate dispersion routines, e.g. like for bath sonication, can be found in literature. However, it should be noted that bath sonicators are difficult to calibrate and suffer from a non-uniform distribution of the ultrasonic energy in the bath. As other techniques baths sonicate through the walls of the test vessels which absorb an unknown amount of the sonication energy through damping effects.

33. In case the investigated nanomaterials are delivered in a form of stable stock dispersion, the stock dispersion is directly diluted to the concentration, convenient for further sample preparation. No additional sonication is required in this case.

34. The undiluted stock dispersion should not be stored for longer than 14 days and after more than 3 h a re-dispersion by probe sonication according to (§28 (c) and (d) (but place the sonication probe in the bottle of the stock suspension) has to be performed.

Preparation of NOM stock solution

35. Prior to the observation of dispersion stability in presence of NOM, a stock solution of corresponding well characterised and purified NOM (properly cleaned from ions and ash) shall be prepared and the DOC content determined. Chemical composition needs to be reported. 2R101N Suwannee River NOM (SRNOM) (8) is recommended as an example for these purposes as standardized and purified material (Tables 5 and 6, annex 2B). The stock solution of NOM is prepared by addition of required amount of NOM powder to the ultrapure water. After adjustment of pH to 8 to accelerate dissolution, NOM solution is left under vigorous stirring or on a shaker for 24 hours. After that the pH is measured and adjusted to pH=8 if needed using either 1 M HNO3 or NaOH solutions. The solution is filtrated using a vacuum filtration set-up, consisting of vacuum pump and sterile bottle-top 0.2 µm polyether-sulfone (PES) filter. Thereafter the solution of NOM is analysed for the DOC content (e.g. ISO CEN EN 1484). The NOM solution is maintained at 4 °C in amber glass bottles avoiding exposure to the light. Due to the aging of NOM followed by precipitation, it is recommended to store the NOM stock solution no longer than 4 weeks.

Dispersion stability in presence of electrolyte at different pH in the dispersions with and without NOM

36. Following the tiered approach shown in figure 2 a screening test scheme can be first performed. This covers nine different situations with a variation in pH and ionic strength in the presence of NOM (see also figure 3 for the testing scheme). The analysis program is reduced to two samplings at 0 h and after 6 h plus following centrifugation to a size cut-off of 1µm taking into account the nanomaterial density. The extended test scheme is performed in the absence and presence of NOM at again nine different conditions of pH and $Ca(NO_3)_2$ concentrations and requires sampling at every hour up to 6 h followed by the centrifugation step described above. The extended test does not need to be performed if the nanomaterials

show low dispersion stability or high dispersion stability, respectively, under all conditions of the screening test (see figure 2).

37. The aliquots of sonicated stock dispersion (paragraphs 31-34) are transferred into 50 ml polypropylene tubes and diluted with ultrapure water to the volume of 20 ml. Dilution of dispersion of tested material is performed to minimize the contact of particles in dispersion with concentrated solution of NOM or buffer and electrolyte, added in the next steps (see figure 3 for the testing scheme).

38. If the test shall be performed in the presence of NOM, the necessary amount of NOM stock solution (paragraph 35) is added to the samples to achieve the desired concentration of DOC. In general, the concentration of DOC in the final test volume of 40 mL shall be 10 mg/L. In principle a mass concentration value for the NOM is not straight forward, as for providing a uniform stabilization effect for nanomaterials of different particle size and therefore different specific surface area, the added NOM should scale with the surface area of the nanomaterials in the test vial. Therefore, a minimal concentration of needed DOC shall be determined by the surface area of nanomaterial in the sample (SA) that is calculated as follows:

$$SA=C_s \times V_s \times SSA$$

where C_S is the concentration of the sample (in g/L), V_S is the volume of the sample (in L) and SSA is the specific surface area of the nanomaterial (m^2/g) by e.g. the BET method. While a concentration of 10 mg/L DOC was found sufficient for the TiO_2 (NM-105) material, this might not be the case for nanomaterials with larger specific surface area. Here it is assumed that a minimum of 1 DOM molecule per nm^2 on the entire surface area of analysed nanomaterial would be a minimum requirement. The recommended minimal concentration of DOC is 0.004 g per SA=1 m^2 of the nanomaterial in the sample.[3] A Microsoft® Excel test preparation tool is available on the OECD website to aid in calculation of the required amount of DOC in samples.

39. If the test is performed without the addition of NOM, a buffer is required to stabilize the pH in the different pH settings. Therefore, instead of the NOM, a 5 mM sodium bicarbonate buffer is provided by adding 2 mL of the 0.1 M stock solution.

40. Next, the adjustment of ionic strength is performed by adding 0, 400 µL and 4 mL of the 0.1M $Ca(NO_3)_2$ stock solution added to achieve the final concentrations of 0, 1, and 10 mM respectively in the final volume of the sample. Afterwards the volume is adjusted to 35 mL by addition of water.

41. For setting the pH, values of 4, 7 and 9 +/- 0.2 pH are recommended (9). The pH of the samples is established using HNO_3 and NaOH in relatively high concentration (0.01 or 0.1 M, whatever is more convenient regarding dosage), so that the total volume of the samples could remain fairly constant. For equilibration purpose especially with slow buffering NOM the test dispersions are equilibrated within a time period of 1 hour. The tubes should be kept closed at all times (unless sampling takes place) to minimise the influence of atmospheric CO_2 on the pH of the samples. The use of a micro-electrode with 3 mm electrode diameter is recommended.

[3] For the derivation of the value please see annex 1B

42. After addition of DOM or buffer, the electrolyte and the pH adjustment, the volume of the samples is adjusted to 40 ml. Afterwards each sample undergoes a 30 second sonication in an ultrasonic bath to ensure the particles in the dispersion are dispersed and well mixed before the start of the test.

43. Each test condition should be tested at least in triplicates.

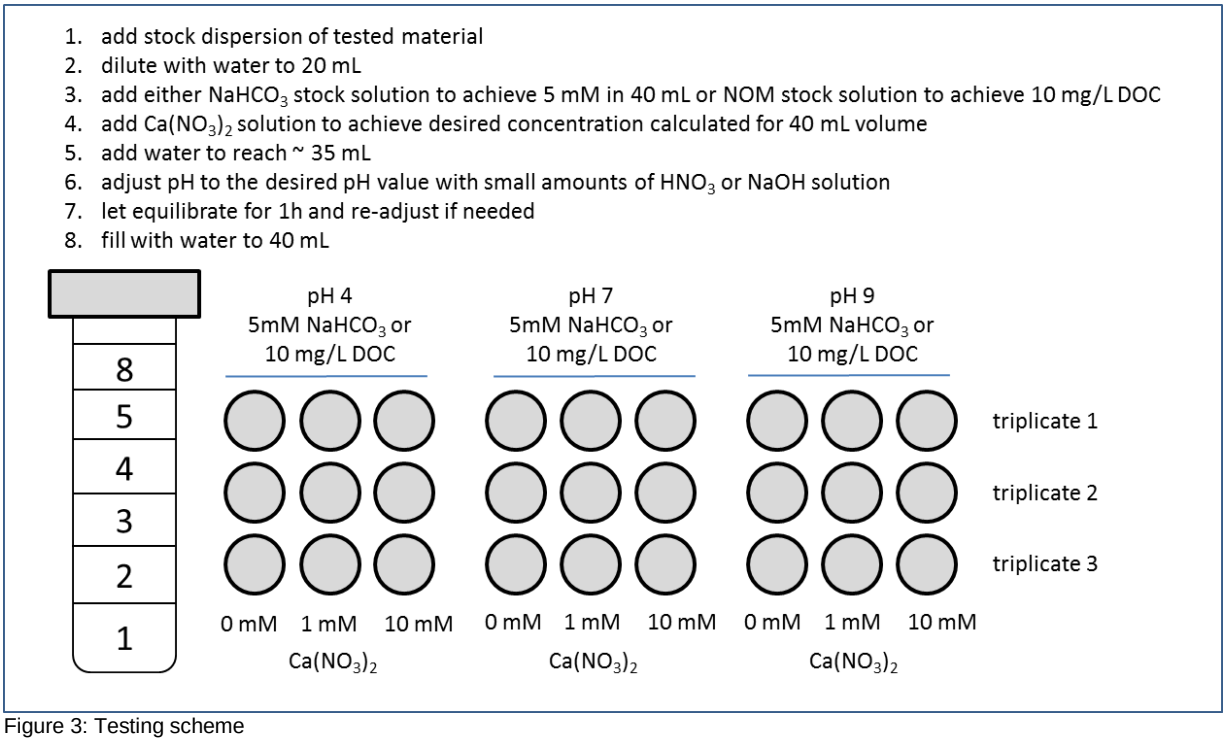

Figure 3: Testing scheme

Sampling and analysis of the supernatant from the test vials

44. The aliquots of the supernatant of the test vials prepared as described in paragraphs 36-43 are taken from the top 0.5-1 cm volume of dispersion. In some cases particles will stick to the air/water interface and caution has to be taken not to get those into the sample aliquot. The recommended volume of taken aliquots is 0.5 mL. The aliquots are taken from the same test vial every hour for the extended testing, starting hour 0 and finishing hour 6, and at hour 0 and 6 for the screening test. Before the sub-sampling at hour 6, the samples undergo centrifugation to enhance the particle sedimentation independent of their different densities. A Microsoft® Excel test preparation tool is available on the OECD website to aid calculation of required centrifugation time based on the particle size cut-off (recommended value 1 µm) and their density as well as parameters of the centrifuge, such as speed of rotation and the radius of the sample liquid surface in the vial and the sampling point. Final sample should be taken 2 cm below the surface of the dispersion and the centrifugation parameters should be calculated accordingly.

45. The sub-samples taken from the supernatant are diluted for further analysis by addition of 9.5 mL of ultrapure water. Resulting samples are vigorously shaken by hand or on a vortex shaker after preparation and right before the analysis, to ensure their proper homogenization. Samples are analysed by Inductively Coupled Plasma Mass Spectrometry (ICP-MS) or Inductively Coupled Plasma Optical Emission Spectrometry (ICP-OES) methods, depending on the expected concentrations. For digests of the particles containing samples (see paragraph 46) or if it can be expected that the particles will be fully atomized, also

graphite-furnace atomic absorption spectrometry might be used. For nanomaterials constituted by elements not detectable by those instruments (e.g. CNTs or fullerenes) other analytical methods have to be used. For sampling and analysis of dissolving particles paragraph 27 should be taken into account.

46. To ensure reliable results, samples prepared as described in paragraphs 44 and 45 should be stored at in the dark at 4°C and analysed within 24 hours after preparation. Samples of acid-soluble nanomaterials might be stabilized through dissolution by acid addition such as concentrated HNO_3 and later analysed in the form of the dissolved ions. If a direct measurement of the nanomaterials s shall be performed (either out of practical reasons or to avoid a time consuming sample digestion) common practices of slurry analysis shall be applied (10). Nanomaterials then should be stable as dispersion or measures to provide stability should be applied. Samples then should be diluted with ultrapure water sufficiently to ensure additional dispersion stability or suitable stabilizing agents need to be added (e.g. surfactants, tetra sodium diphosphate or other, as appropriate). If nanomaterials cannot be stabilized against losses due to sedimentation or attachment to vessel walls, a complete solubilisation of the sample is required, typically in the form of a microwave assisted acid digestion. When varying pH, IEP shall not be crossed, as this will cause nanomaterials agglomeration and sedimentation during storage and analysis.

Possible complications during sample preparation and analysis

47. Test dispersions as prepared in paragraphs 36-43 have to be handled with care to avoid re-mixing them before sampling the supernatant. Even very little shaking can result in undesired sample mixing especially in the top 0.5-1 cm layer of the sample.

INVESTIGATION OF DISPERSION STABILITY BY MEANS OF ULTRAVIOLET-571 VISIBLE (UV-VIS) SPECTROPHOTOMETRY

48. Continuous monitoring of the settling process can be achieved by using UV-VIS spectrophotometry. Samples for analysis of dispersion stability by UV-VIS are prepared in the way described in paragraphs 36-43 in regards to sample composition. Standard 1 cm cuvettes are used and filled with 3.5 mL of sample. For precious samples low-volume cuvettes may be used but need to be filled to the same height compared to the 1 cm cuvettes at 3.5 mL filling volume. The Samples of interest shall be analysed against blank samples in a double-beam photometer. Blank samples are prepared as the samples of interest, containing all solutes, but in absence of the investigated nanomaterials. As UV-VIS spectrophotometers allow the analysis of only one sample at a time, the analysis of only one experimental condition at a time can be performed. Thereby, the preparation of only one sample and one corresponding blank sample at a time is required. All samples have to be prepared directly before UV-VIS measurements to avoid particle agglomeration followed by enhanced sedimentation.

49. Prior to the measurements the baseline of UV-VIS spectrometer is set to zero by measuring two blank samples against each other. After this, the sample of interest is inserted to the device and analysed against the blank sample for recommended time period of 24h, the spectrometer software is configured so

that a single measurement is being performed each 15 or 30 min. Depending on the manufacturer's specific layout of the used spectrometer the analysis is performed at the bottom of the UV-VIS cuvette (typically in the lowest 1 cm), thereby lengthening the analysis time from 6h (supernatant analysis) to 24h in the UV-VIS spectrometer. The analysis of the samples is recommended at the wavelength of maximum light absorption or scattering taking into account background signal from the blank. Too high background signal (> 0.1 AU) should be avoided. Spectral properties of the nanomaterial and the test solutions, especially in the presence of NOM, need to be considered in the selection of the measurement wavelength.

Complications during UV-VIS measurements

50. Typical UV-VIS spectrometer allows analysis of only one sample in 24 hours and therefore the measurements by this technique might be time consuming. Thus to study the nanomaterial dispersion stability in all hydrochemical conditions, without performing any duplicate measurements, 30 full measurement days are required for the extended test.

51. Particle concentration can be monitored by a UV-VIS spectrometer based on an on-axis turbidity measurement where the attenuation of the incident beam by light scattering of the particles is used as a concentration signal or light absorption of the particles in case the particles specifically absorb light on a certain wavelength. The on-axis measurement of turbidity (light source, sample and detector are situated on one optical axis) comes with some drawbacks, what is the reason why turbidity measurements are typically conducted in turbidity meters off-axis where the light scattered by the particles is recorded in an angle, typically 90° to the incident beam. Care should be taken to avoid influence of background absorption originating from the NO_3^- ion or the added NOM. The final results are recommended for presentation in the form of relative to the first measurement of absorption, (A/A_0). The used wavelength must be reported.

52. One shall take into account the possible photocatalytic properties of the investigated nanomaterials. If the particles under investigation reveal photocatalytic activity, the analysis of NOM containing samples shall be performed at the wavelength ≥ 380 nm or at least outside of the absorption band of the photocatalyst. Otherwise, the interactions of analysed particles with UV irradiation may cause the (partial) destruction of NOM molecules via radical formation. For the analysis of dissolving particles paragraph 27 should be taken into account.

53. Special attention should be paid to the choice of cuvettes for UV-VIS measurements. Plastic cuvettes are recommended only when UV irradiation is undesirable.

54. Finally, the cleanness of UV-VIS cuvettes should be considered. Since particles can likely attach to the cuvette walls, cuvettes have to be prewashed with 5 N HNO_3 prior to each measurement and rinsed several times with ultrapure water afterwards. A mechanical cleaning of surfaces might be necessary to avoid carry-over if non-disposable cuvettes are used. Deposition of particles at the surfaces of the cuvettes can interfere with the stability measurement by falsely giving a higher concentration of the nanomaterial in the dispersion than is present.

DATA AND REPORTING

Data Treatment

55. Results on dispersion stability of the samples prepared as described in paragraphs 36-43 and analysed as described in 50-52 are presented in the form of plots, where the percentage of nanomaterial left in the supernatant after 6 h plus centrifugation is given (2-point screening test) or in plots where the X-axis represents the time of sampling (0 to 6 hours in the extended test) and Y-axis represents the percentage (%) of the concentration of analysed nanomaterial relative to the initial concentration (0 h measurement) of the material in the samples of interest ($C_{measured}$/ $C_{initial}$) ×100 (%). The data of the pH and ionic strength dependency (each 3 experiments) are plotted together in one graph to visualize the effect of both parameters. Standard deviations should be calculated based on the performed triplicate measurements of each time point and could be displayed in the graphs. The measurement at 0h may differ from the calculated expected concentration after the dilution of the stock due to losses in the dispersion routine and during sample preparation (setting of pH and ionic strength). Therefore, the measured concentrations are to be normalized to the 0h time point ($C_{initial}$; set to 100%). and the deviation from the calculated expected concentration at 0h has to be recorded.

56. In case of investigations of particle agglomeration behaviour by UV-VIS the results are presented in the form of plots, where X-axis represents time (0 to 24 h), and Y-axis represents the absorption measured at a certain time point relative to the initial value of absorption at 0h against the blank solution in the reference beam ($A_{measured}/A_{initial}$)

57. Depending on the results gained according to paragraph 55 or 56, respectively, the investigated nanomaterials can be classified according to figure 2, where option (a) is assigned to low dispersion stability (≤ 10 %), option (b) is assigned to intermediate low (10 % < x < 50 %) and high (50 % ≤ x < 90 %) dispersion stability, respectively, and option (c) is assigned to high dispersion stability (≥ 90 %). Nanomaterials that show a dispersion stability of option (a), ≤ 10 % of the initial concentration (0h measurement) after 6 hours for all conditions of the screening testing can be considered as nanomaterials with low dispersion stability. Nanomaterials that remain at ≥ 90% (c) of the initial concentration (0h measurement) after 6 hours for all conditions of the screening testing can be considered as nanomaterials with high dispersion stability. Nanomaterials that do not fall under these two options (hence (b)) should be considered as nanomaterials with condition-depending dispersion stabilities. Here, dispersion stabilities should be discussed based on the particular test conditions.

58. In case the extended test was performed, conclusions should be made for trends of the phase separation and information on separation characteristics based on the data progress of the hourly measurement point (see scheme in figure 1), e.g. settling due to density or agglomeration, influence of nanomaterial changes during test on dispersion stability, settling behaviour of heterogeneous nanomaterial.

Test report

59. The test report should include the following information:

(a) Information about the tested nanomaterial: brand, name, information on physical- chemical parameters as mentioned in paragraph 23 together with corresponding measuring method

(b) Sonicator brand/type and results of calibration, applied indicated power, amplitude and pulse time, mount of sample and stock dispersion volume

(c) Mass and particle number concentration of the nanomaterial in the test samples based on the information of average particle diameter (from manufacture information or material characterisation)

(d) Deviation (in %) of nanomaterial concentration detected during the 0h measurement ($C_{initial}$) from the expected nanomaterial concentration in the dispersion.

(e) Loss to test vessel wall and deviation of test vessel material, if applicable

(f) Concentration of electrolyte in the investigated samples

(g) Chemical composition of NOM and concentration (as DOC) in the investigated samples

(h) pH of investigated samples after equilibration time and after the completion of experiment.

(i) Sample centrifugation parameters (dimensions of the rotor, radius to the sample surface and the sampling point, rotations per minute, relative centrifugal force and centrifugation time)

(j) Analytical procedure: direct measurement or digestion, measurement principle, instrument type and manufacturer, dilution factors, storage time

(k) Screening test: Table with relative concentration ($C_{measured}$/ $C_{initial}$) ×100 (%) under the tested conditions after 6h plus centrifugation, including coefficient of variation from replicates

(l) Extended test: Plots of relative concentration ($C_{measured}$/ $C_{initial}$) ×100 (%) versus time (0-6 hours) in case of supernatant analysis

(m) Extended test: Table with values of final concentration ($C_{measured}$/ $C_{initial}$) ×100 (%), (6 hours value) in case of supernatant analysis.

(n) In case measurement are performed using UV/VIS: Plots of relative absorption ($A_{measured}$/$A_{initial}$) versus time (0-24 hours).

(o) Interpretation of the agglomeration behaviour of the investigated material as described in paragraphs 57-58 and figure 2.

(p) Amount of dissolved fraction of nanomaterials after 6h in % and mg/l, if applicable.

LITERATURE

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ANNEX 1. DEFINITIONS

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 precipitates of larger than colloidal size (agglomerates) (slightly modified according to 7). In contrast to aggregation where particles held by strong bonds like sinter bridges, agglomeration is a reversible process.

BET – Gas-adsorption method to determine the specific surface area of powders according to the Brunauer–Emmett–Teller theory.

CCC – critical coagulation concentration: the concentration of a specific counter-ion (cation for negatively charged particles, anion for positively charged particles) at which the repulsive forces between particles approach zero and the agglomeration enters a fast, diffusion limited regime. At the CCC the attachment efficiency α becomes 1 and the stability ratio W zero.

Concentrations - Concentrations of the particles and NOM in the samples are given in grams per litre (g/L), miligrams 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 0.45µm filter, a part of the 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.

Experimental Endpoint –Measurement that determines the concentration of particles in the top 0.5 - 1 cm of dispersion after 6 hours of experimental time and a centrifugation based cut-off of 1 µm particle diameter (approximated for spherical, solid particles with the nominal density of the material). The concentration in the supernatant after centrifugation is related to the initial concentration of particles, determined during the first measurement at 0h, and expressed in percentage (%), such as: Experimental Endpoint = $(C_{measured}/C_{initial}) \times 100$. For the UV/VIS spectrophotometer protocol the Experimental Endpoint is $(A_{measured}/A_{initial})$ x100 expressed in percentage (%).

Experimental time – time, required to test dispersion stability of particles is measured in hours (h). The experimental time is 6 hours for sampling in the supernatant and 24h for applying UV/VIS photometry.

IEP – Isoelectric point: the pH at which the zeta potential or particle mobility in response to an electric field is zero. Only in the absence of specifically adsorbing ions which change the surface charge compared to the pristine surface, the IEP equals the PZC.

NOM – Natural organic matter: the organic substances present in surface or ground water. NOM covers humic and non-humic fractions as i.e. 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.

PZC – Point of Zero Charge: the point where the surface charge density is zero in the absence of any specifically adsorbing ions. Its value is given as the negative logarithm of the potential-determining ion. In most cases the potential determining ion is the proton and the value is then given as pH. The PZC is then also termed the PZNPC – point of zero net proton charge.

Size - Size of the particles, aggregates or agglomerates is given in micrometers (µm) or nanometers (nm). The method for particle size determination and the character of the particle size average should be reported.

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.

Zeta Potential – electrokinetic potential: the electric potential at the shear plane of the diffuse double layer. The zeta potential is derived from electro-kinetic phenomena, the movement of the particles in an electric field. The conversion of electrophoretic mobility measurements into zeta potential requires several assumptions about the properties of the double layer. As long as the assumptions are always identical, identical conversions and hence zeta potentials are retrieved. Reporting zeta potentials would require the reporting of the assumptions made to make them comparable.

ANNEX 2

A. ULTRASONIC PROBE EXPERIMENTS: DETERMINATION OF DELIVERED ACOUSTIC ENERGY BY CALORIMETRIC MEASUREMENTS

60. When preparing the dispersion of analysed particles, sonication is normally required to provide a homogenous distribution of particles as a dispersion. Prior to this, the delivered acoustic energy must be determined by calorimetric measurements. The increase of temperature during the application of ultrasonic energy to 500ml of water by probe sonication was measured and the delivered energy then calculated by evaluation of the slope of the temperature curve, using the formula:

$$E = \frac{dT}{dt} m C_p$$

where $\frac{dT}{dt}$ is the change of temperature ($T_{end} - T_{start}$) of the media during the experimental duration ($t_{end} - t_{start}$), C_p is specific heat capacity of the medium (4.184 kJ/g*K for water), m is mass of the medium (g). The value of $\frac{dT}{dt}$ can be directly found from of the equation of the linear trend line data fit presented at Fig. A1. The linear trend line has the general equation of y=kx+b, where k = $\frac{dT}{dt}$ and corresponds to the slope of the line. A Microsoft® Excel test preparation tool is available on the OECD website to assist in calculation of delivered acoustic energy.

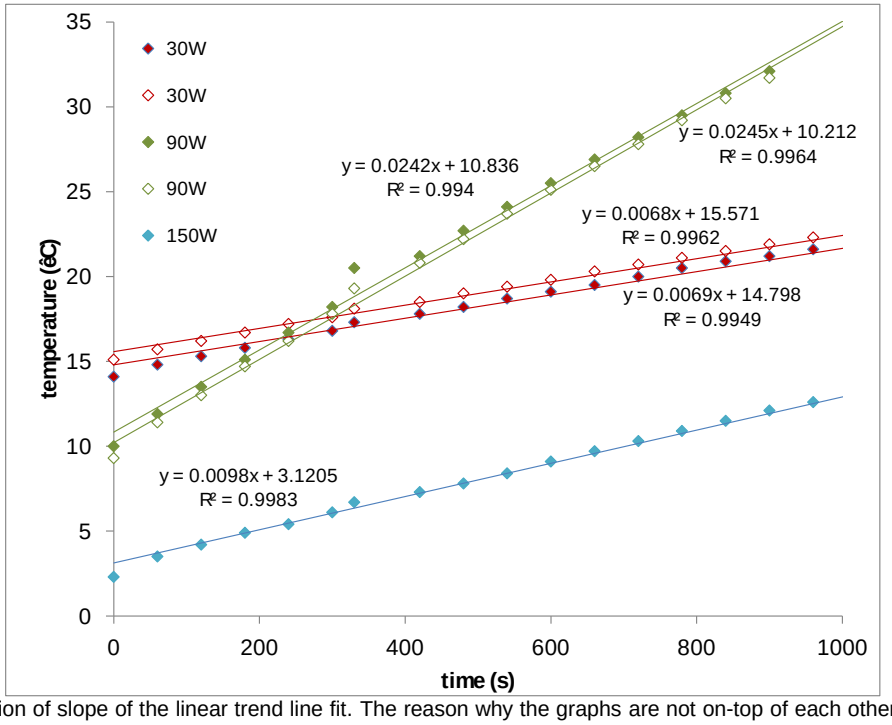

Fig. A1. Determination of slope of the linear trend line fit. The reason why the graphs are not on-top of each other is that the starting temperature was different. But the slopes are identical for both 30W and both 90W curves. And the slope reflects the energy input.

B. TiO_2 (NM-105), Ag (NM-300K), AND MWCNT (NM-400) AS REFERENCE MATERIALS FOR DISPERSION STABILITY INFLUENCED BY pH, IONIC STRENGTH AND NOM

61. In the following test performance and results of NM-105, NM-300K, NM-400 are illustrated for assistance.

Influence of Electrolyte/ DOC on the particle agglomeration behaviour.

62. To study the influence of electrolyte/DOC concentration on the particle agglomeration, samples containing 0, 1 and 10 mM $Ca(NO_3)_2$ with/without 30 mg/l DOC were prepared. 30 mg/l DOC were chosen as worst case scenario, but were later reduced to 10 mg/l because of comparable effects and lower cost to acquire the suggested SRNOM material. Suggested experimental design for investigation of electrolyte/DOC influence on agglomeration behaviour of nanomaterials is presented at Fig. A2.

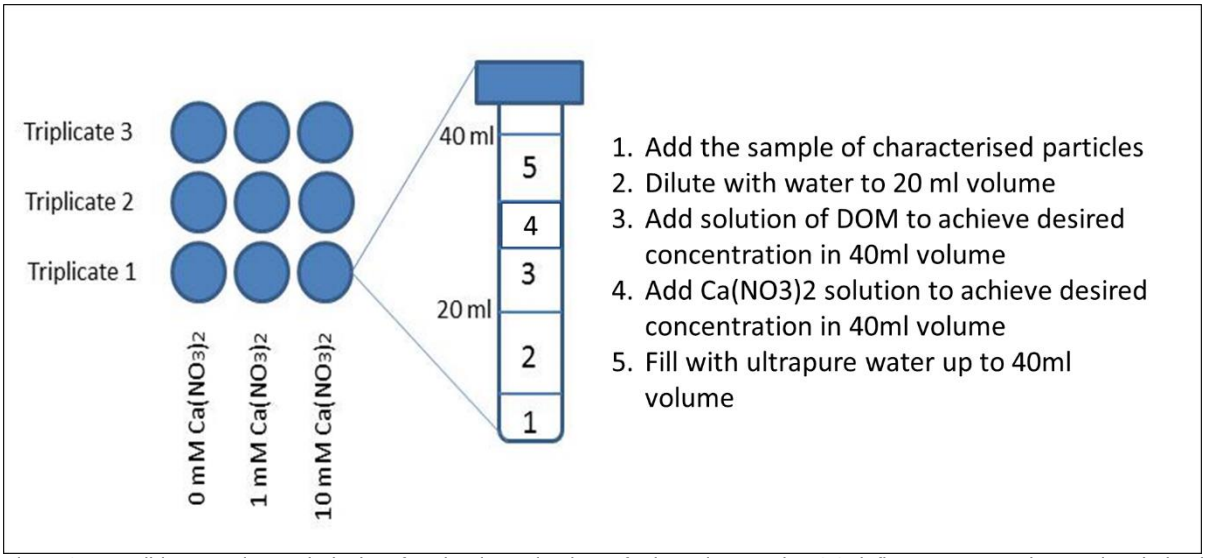

Fig. A2. Possible experimental design for the investigation of electrolyte and DOC influence on agglomeration behaviour of nanoparticles.

63. Presence of $Ca(NO_3)_2$ electrolyte in the dispersions leads to the agglomeration of nanomaterials in the extent proportional to the concentration of electrolyte (Fig. A3a). Presence of DOC however stabilizes the dispersion with electrolyte concentration of 1 mM (Fig. A3b). In this test no pH adjustment or stabilisation was performed.

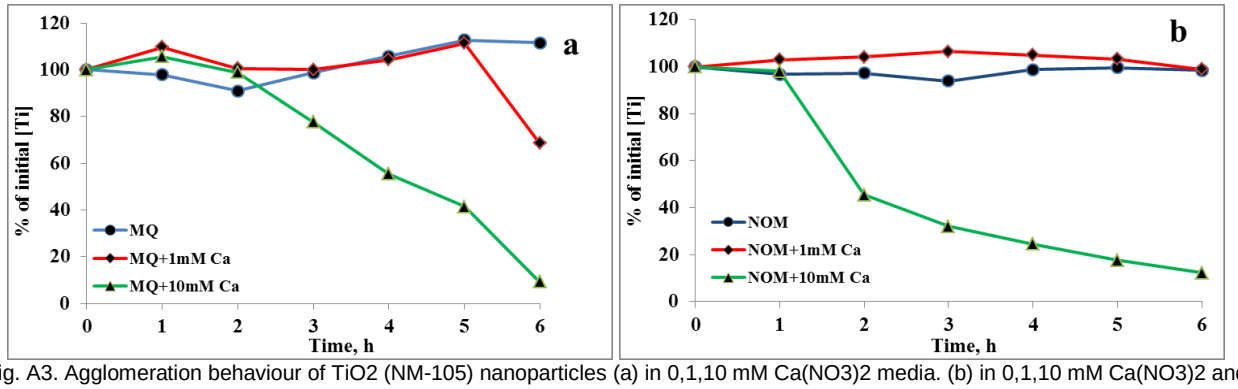

Fig. A3. Agglomeration behaviour of TiO2 (NM-105) nanoparticles (a) in 0,1,10 mM Ca(NO3)2 media. (b) in 0,1,10 mM Ca(NO3)2 and 30 ppm DOC media. Based on obtained data on stability at different Ca concentrations and w/wo NOM, TiO2 is considered as nanomaterial of condition-dependent intermediate stable dispersibility.

64. The deviation of nanomaterial concentration at 0h of the experiments shown Figure A3 are presented in table 1 and 2, and expressed in %. The derivations are calculated as the difference between the expected concentration of nanomaterial, calculated from the dry weight of material in the dispersion and the concentration of nanomaterial detected during the 0h measurement.

Table 1 Deviation of nanomaterial concentration measured at 0h of the experiments presented in Figure A3 (a)

Figure A3 (a)	MQ	MQ + 1 mM Ca	MQ + 10 mM Ca
calculated, mg/L	1.48	1.48	1.48
measured, mg/L	1.25 ± 0.01	1.21 ± 0.12	1.21 ± 0.04
deviation,%	-15.3 ± 0.8	-18.1 ± 7.9	-18.2 ± 2.8

Table 1. Table 2 Deviation of nanomaterial concentration measured at 0h of the experiments presented in Figure A3 (b)

Figure A3 (b)	NOM	NOM + 1 mM Ca	NOM + 10 mM Ca
calculated, mg/L	1.48	1.48	1.48
measured, mg/L	1.60 ± 0.08	1.56 ± 0.09	1.51 ± 0.15
deviation,%	7.9 ± 5.2	5.4 ± 6.4	2.5 ± 9.9

65. Presence of electrolyte or DOC did not influence the stability of Ag (NM-300K) dispersions as can be observed at figure A4. Dispersions remained stable over entire experimental period. In this test no pH adjustment or stabilisation was performed.

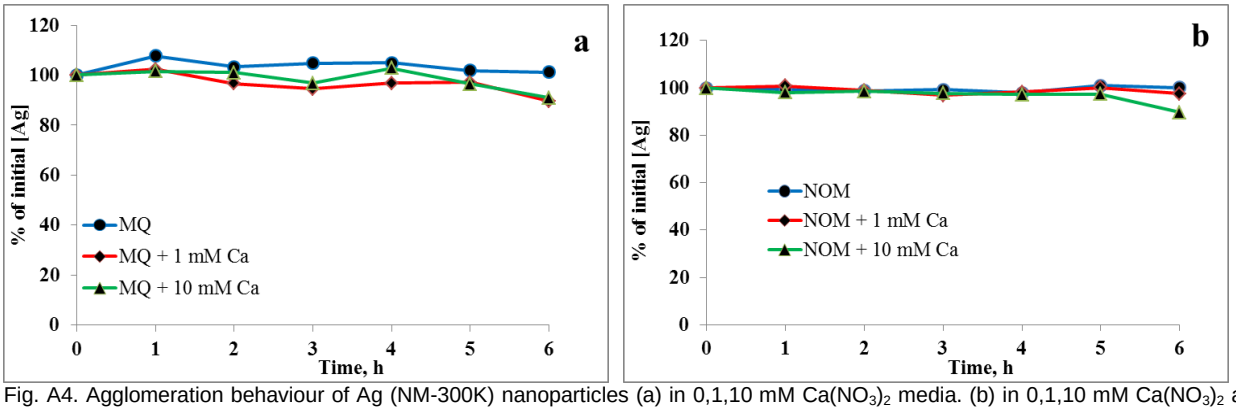

Fig. A4. Agglomeration behaviour of Ag (NM-300K) nanoparticles (a) in 0,1,10 mM $Ca(NO_3)_2$ media. (b) in 0,1,10 mM $Ca(NO_3)_2$ and 30 ppm DOC media. All particles are stable under all conditions (>90% after 6h), therefore NM-300K is considered as nanomaterial of stable dispersion.

66. Deviation of nanomaterial concentration measured at 0h the experiments shown in Figure A4 are presented in table 3 and 4, and expressed in %.

Table 3 Deviation of nanomaterial concentration measured at 0h of the experiments presented in Figure A4 (a)

Figure 4 (a)	MQ	MQ + 1 mM Ca	MQ + 10 mM Ca
calculated, µg/L	1000	1000	1000
measured, µg/L	700 ± 73	693 ± 24	675 ± 18

deviation,%	-30 ± 7	-31 ± 2	-32 ± 2

Table 4 Deviation of nanomaterial concentration measured at 0h of the experiments presented in Figure A4 (b)

Figure 4 (b)	NOM	NOM + 1 mM Ca	NOM + 10 mM Ca
calculated, µg/L	1000	1000	1000
measured, µg/L	760 ± 10	739 ± 8	701 ± 21
deviation,%	-24 ± 1	-26 ± 1	-30 ± 2

Calculating the required amount of DOC.

67. The concentration of DOC was first set to 30 mg/l which corresponds to the 95 % percentile of DOC concentrations found in surface waters (for data see the FOREGS Geochemical Atlas database). Due to the fact that at 30 mg/l DOC, pH 4 or 5 and 10 mmol/L $Ca(NO_3)_2$ there is a risk of NOM agglomeration, the concentration in principal can be reduced, since stabilizing effects can be observed already from 1.5 mg/l for four hours and from 4mg/l for 6h, in case of TiO_2, (Fig. A5). The suggested DOC in the tests was then reduced to 10 mg/l.

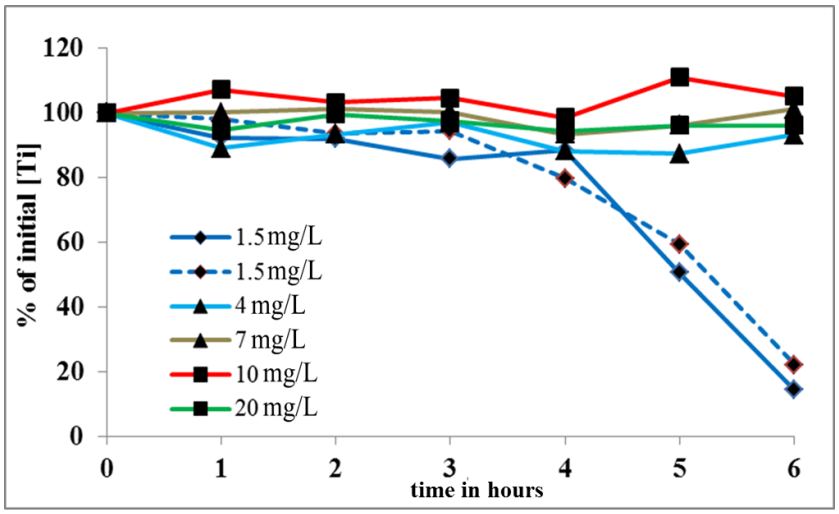

Fig. A5. Dispersion stability of TiO_2 (NM-105) nanomaterials in 1 mM $Ca(NO_3)_2$ and different concentrations of NOM.

68. In principle there are two major effects of the NOM which increase the dispersion stability. One is the surface adsorption of the NOM to the nanomaterials, which increases the magnitude of the negative surface potential by the anionic nature of the de-protonated NOM molecules under the tested pH conditions. The second is the complexation of calcium ions by the NOM and the reduction of calcium activity. For the surface adsorption effect to take place the ratio of NOM molecules to the available surface area of the nanomaterials in the dispersion is more important than a mass concentration in terms of a fixed DOC value. When the same nanomaterial is present in different sizes, the same amount of NOM might have less effect on the material with smaller particles and therefore larger surface area. 10 mg/l of DOC should be sufficient for most nanomaterials to simulate the effect of NOM in surface waters. If smaller DOC concentrations are used, the minimum amount of DOC in the samples of interest should be based on the surface area of the analysed particles in the sample. For each 1 m^2 of nanomaterial surface in the dispersion, 0.004 g of DOC is recommended. This value originates from the assumption that at least 1 molecule of NOM per 1 nm^2 of nanomaterial surface area is needed to provide the successful stabilization of dispersion. 1m^2 can be converted to 1×10^{18} nm^2 that results into same number (1×10^{18}) of NOM molecules needed for the stabilization. Assuming the molecular mass of NOM to be approximately 1500 g/mol and knowing Avogadro's number as 6.02×10^{23} one can calculate the necessary amount of NOM (g) per 1 m^2 that equals to 2.5 mg. This value in turn can be converted into 1.25 mg of DOC per 1 m^2, (2.5 mg

divided by 2) since NOM contains about 50 % of carbon by weight. A certain safety margin being required to account for uncertainties in the assumptions above, the obtained value is increased approximately 3 times, resulting in a value of 0.004 g of DOC per $1m^2$.

69. The use of SRNOM provides the experimenter with well characterised and purified NOM. 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. SRNOM might be substituted with another type of NOM, providing the substituting material has a similar origin/composition and reveals the levels of purity comparable to those of SRNOM. Tables 5 and 6 show the difference between SRNOM and Sigma-Aldrich Humic Acid (HA) in terms of cations and anions content.

Table 5: Elemental analysis of Suwannee River NOM and Sigma-Aldrich Humic Acid (HA)

	Ca	Mg	Na	K	Fe	Ni	Cu	Mn	Co
SRNOM	1.4 +/- 0.6	0.17 +/- 0.01	315 +/- 2.9	5.9 +/- 0.6	8.8 +/-1.28	<0,01	0.05 +/-0.04	<0,01	<0,01
SigmaHA	92.2 +/- 10	6.1 +/- 0.1	698 +/- 4	55.3 +/- 0.3	40.8 +/- 2	0.33 +/-0.03	0.3 +/-0.05	0.03 +/-0.01	0.07 +/-0.01

SRNOM: Suwannee River Natural Organic Matter, HA: humic acid. (Concentrations in mg/L per 1000 mg/L DOC solution)

Table 6: Ion Chromatography analysis of Suwannee River NOM and Sigma-Aldrich Humic Acid

	F^-	Cl^-	NO_2^-	Br^-	NO_3^-	PO_4^{3-}	SO_4^{2-}
SRNOM	n.d.	55.8	n.d.	0.5	35	n.d.	11.3
SigmaHA	2.5	277	n.d.	0.6	20	n.d.	7.8

SRNOM: Suwannee River Natural Organic Matter, HA: humic acid. (Concentrations in mg/L per 1000 mg/L DOC solution)

Influence of pH on the agglomeration behaviour of the particles

70. To study the dispersion stability of tested TiO_2, (NM-105), Ag (NM-300K), and CNT (NM-400) nanomaterials in the media with established pH and without the addition of NOM, $NaHCO_3$ was used as a buffering agent. Experimental design is presented at Fig. A8

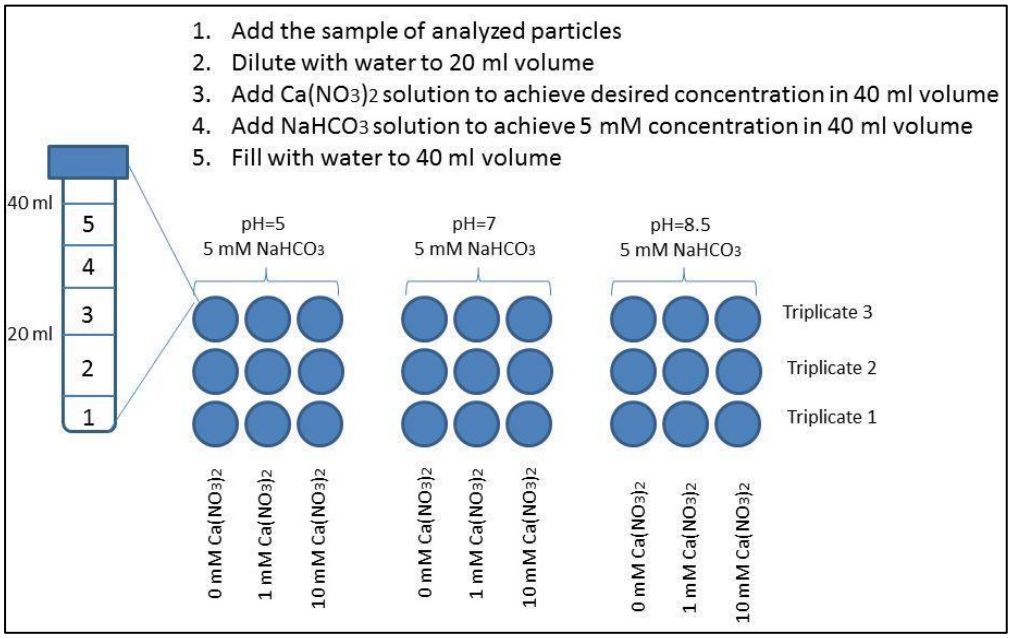

Fig. A8. Possible experimental design for the investigations of nanoparticle agglomeration behaviour at fixed pH.

71. Particle dispersion stability at established pH=8.5, was tested, since this pH was the most unstable pH of all investigated (5/7/8.5). Obtained results for TiO_2 (NM-105) are presented in Fig. A9.

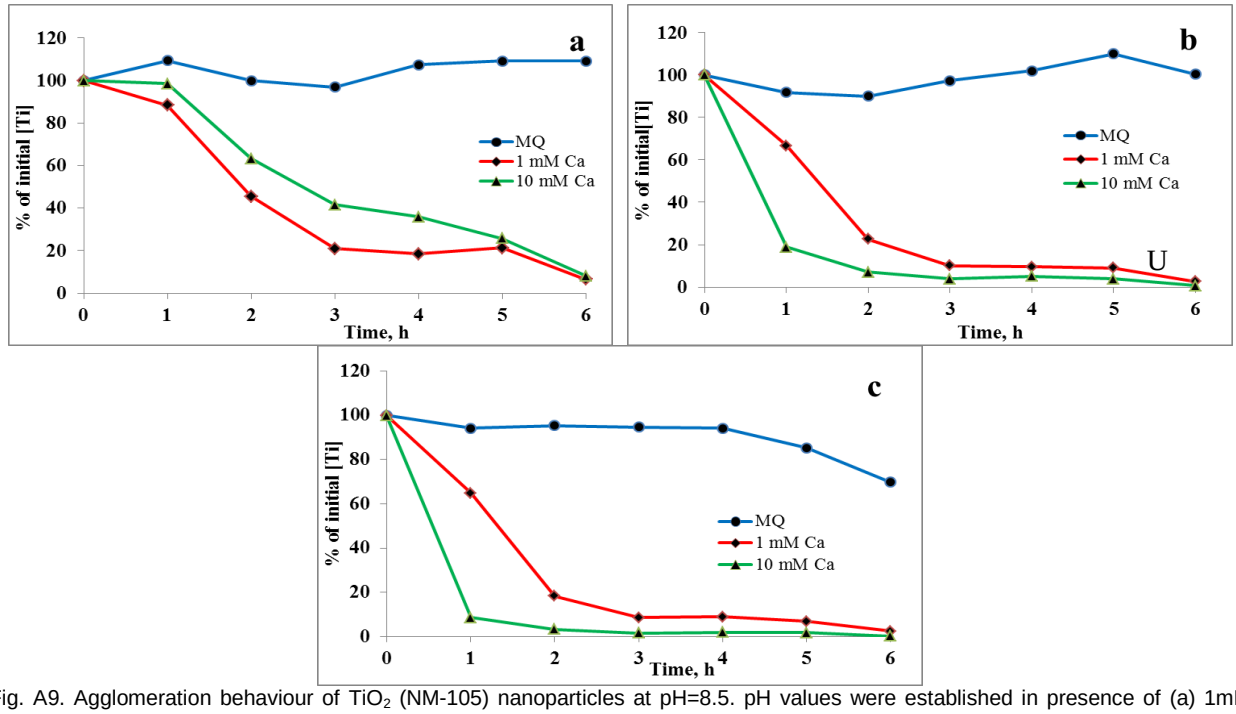

Fig. A9. Agglomeration behaviour of TiO_2 (NM-105) nanoparticles at pH=8.5. pH values were established in presence of (a) 1mM $NaHCO_3$, (b) 5 mM $NaHCO_3$, (c) 10 mM $NaHCO_3$ and the pH values remained stable during all experimental procedures. Based on obtained data on stability at different Ca concentrations, TiO2 is considered nanomaterial of condition-dependent intermediate stable dispersibility.

72. Change in the pH of dispersions (pH=8.5) did not influence the stability of Ag (NM-300K) dispersions as can be observed at figure A10.

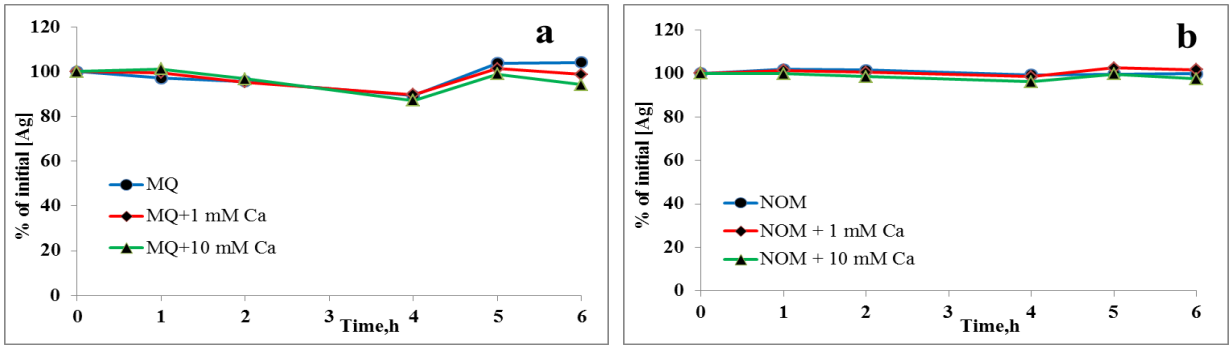

Fig. A10. Agglomeration behaviour of Ag (NM-300K) nanoparticles at pH=8.5. (a)) in 0,1,10 mM $Ca(NO_3)_2$ media. (b) in 0,1,10 mM $Ca(NO_3)_2$ and 30 ppm DOC media. Based on the obtained data on stability under different conditions, Ag is considered as nanomaterial of high dispersion stability.

73. The tested CNTs (NM-400) were not dispersible, what was visible by the sediments in the tubes after sonication. Furthermore, immediate (~10 min) build-up of a deposited fraction made clear that the nanomaterial shows low dispersion stability under given test conditions. Therefore no extended tests have been performed and thus, no graphs are available showing the dispersion stability of unstable CNT.

Influence of particle concentration on their agglomeration behaviour.

74. If there is a need to investigate the influence of particle number concentration on their dispersion stability outside the defined concentration range (paragraph 11, TG), the following routine is recommended. Dispersion stability of TiO_2, (NM-105) nanomaterial was tested in dependence of various particle concentrations: 10^{10}, 10^{11} and 10^{12} particles/L.

75. Experiments revealed that in dispersions with destabilizing Ca-concentrations the agglomeration of dispersions with higher particle number concentrations proceeded faster. (Fig. A11 a, b, and c)

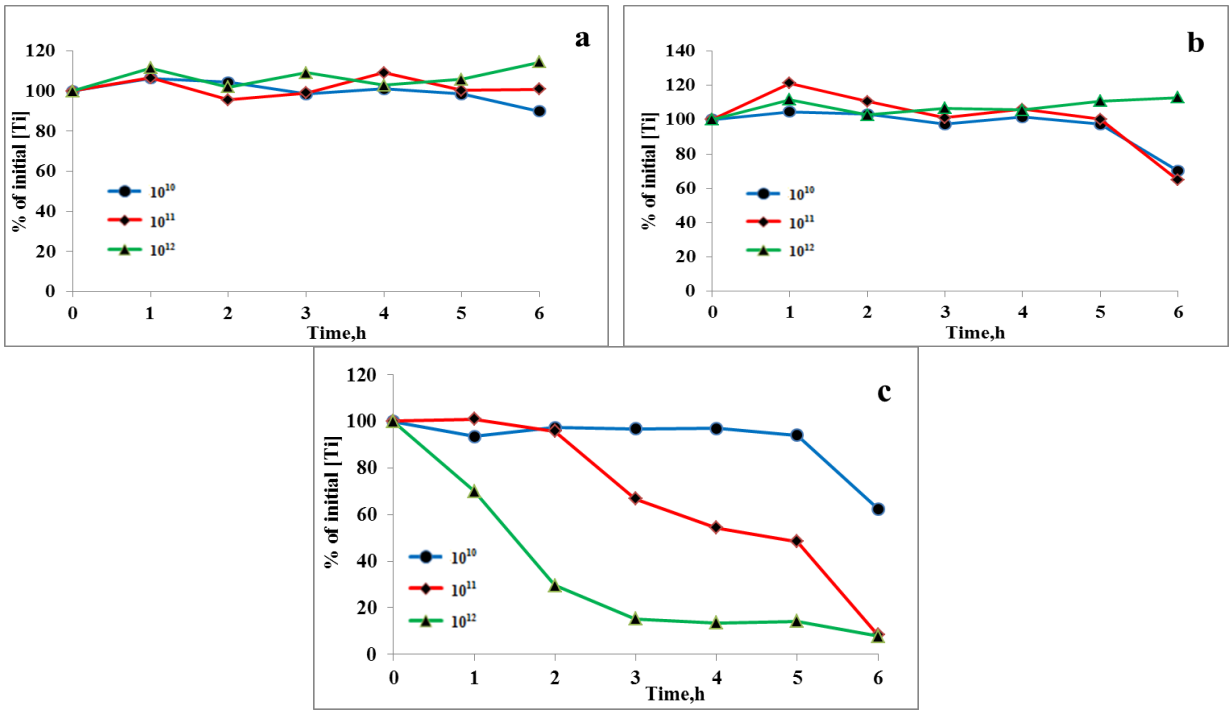

Fig. A11. Dispersion stability of TiO_2 (NM-105) particles in the dispersions of various density: 10^{11}, 10^{12}, and 10^{13} particles /L. (a) in MQ, (b) in 1 mM $Ca(NO_3)_2$ and (c) in 5 mM $Ca(NO_3)_2$.

76. It is therefore recommended to prepare dispersion of a known particle number concentration which should lie in the range of 10^{12} particles/L. The particle number concentration can be calculated by using the average particle size and density. The average particle size may be taken from DLS measurements of the dispersion if existing manufacturer or phys.-chem. characterisation data is not available. The target number concentration range of $0.5x10^{12}$ to $5x10^{12}$ particles/L does not require a very precise size measurement. It is suggested to use the first mode of the intensity weighted size distribution in DLS. Mass based size distributions and their related particle size averages from Field Flow Fractionation-ICPMS or intensity weighted distributions from Field Flow Fractionation-light scattering analysis might as well be used. If number based size distributions are used (i.e. from electron microscopy) those should be converted into mass based distributions and the related particle size average. Material polydispersity, particle shape, morphology of aggregates and variations in particle density from the material density will add error to the number concentration calculation, but as written earlier, it is the objective to meet a relatively broad range of number concentration.

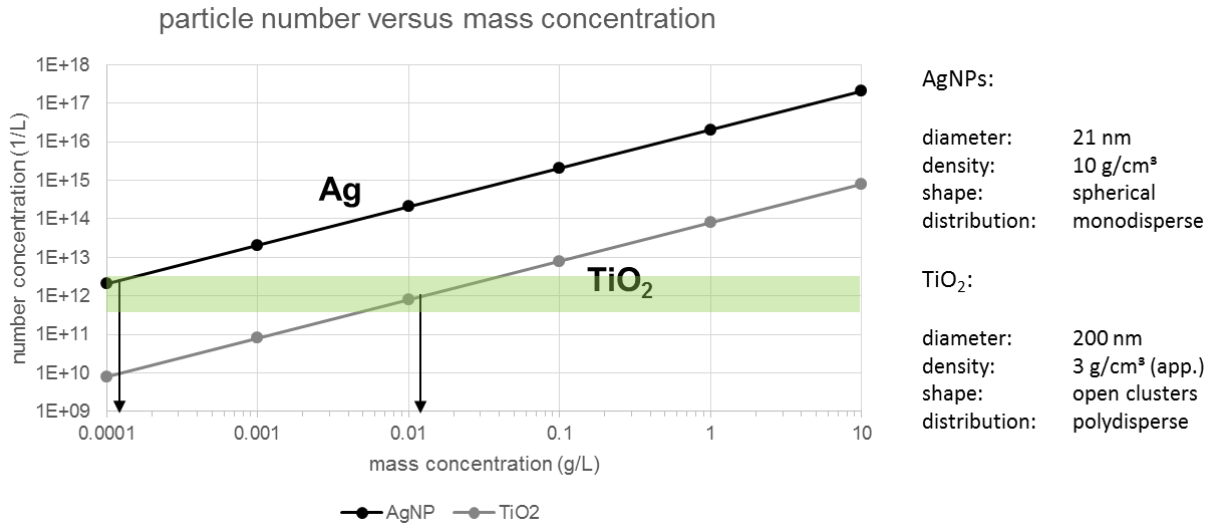

Fig. A12: Example for different mass concentrations needed for different types of nanomaterials to meet the target zone of particle number concentrations.

C. CONTINIOUS MONITORING OF AGGLOMERATION BEHAVIOUR WITH UV-VIS SPECTROPHOTOMETERS

77. Dispersion stability of TiO_2 (NM-105) particles was followed by means of UV-VIS spectroscopy. Obtained results are presented at figures A13a, and b and A14a, and b. They revealed similar character of experimental dependencies towards results obtained by ICP-OES, ICP-MS measurements.

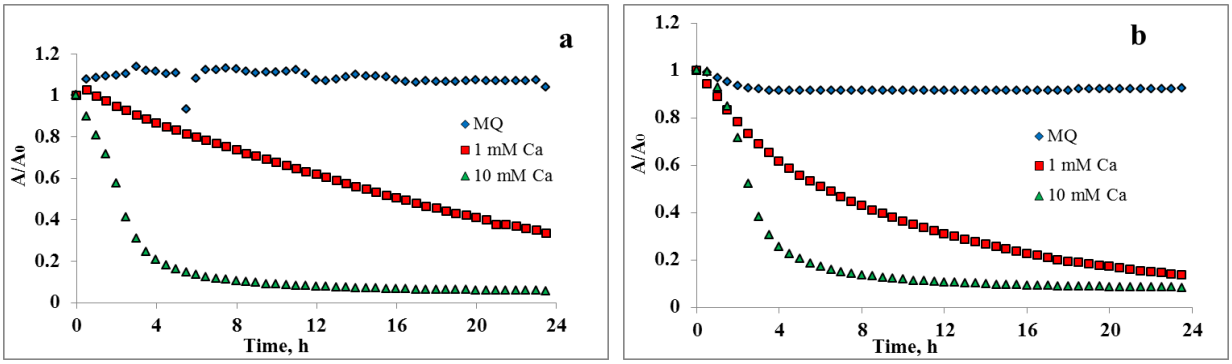

Fig. A13. Dispersion stability of TiO_2 particles in MQ media. (a) at 380 nm wavelength. (b) at 600 nm wavelength.

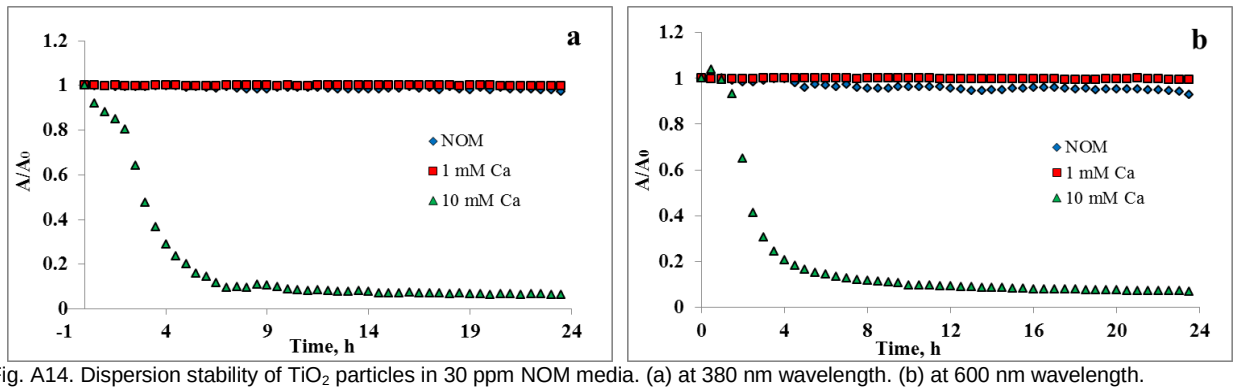

Fig. A14. Dispersion stability of TiO_2 particles in 30 ppm NOM media. (a) at 380 nm wavelength. (b) at 600 nm wavelength.