

NANoREG

Grant Agreement Number 310584

Deliverable D 2.3

Experimental evaluation of OECD methods for analysis of physicochemical MNM properties

Due date of deliverable: 2015/05/01

Actual submission date: 2015/08/9

Author(s) and company:	David González Gálvez, Socorro Vázquez Campos (LEITAT, Partner Nr 20) Heinrich Hofmann (EPFL for FOPH, Partner Nr 40) Carlos Rey-Castro, Josep Galcerán, Calin A. David (UdL, Partner Nr 43) Omar Lozano García, Jorge Mejía Mendoza (UNamur, Partner Nr 22) José Manuel Lloris (AIDICO, Partner Nr 29) Marina Moser and Keld Alstrup Jensen (NRCWE, Partner 4)
Work package/task:	WP2 / Task 2.3
Document status:	draft / <u>final</u>
Confidentiality:	confidential / restricted / <u>public</u>
Key words:	OECD Technical Guidelines, physicochemical characterization, TG 105, TG 106, TG 107, TG 109, TG 110, TG 112, TG 115, TG 117, TG 123, density, granulometry, sorption-desorption, complex-formation, dissociation constant, water solubility, surface tension, N-Octanol-water partition coefficient.

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*This project has received funding from the European Union
Seventh Framework Programme (FP7/2007-2013)
under grant agreement no 310584*



DOCUMENT HISTORY

Version	Date	Reason of change
1	2015/04/07	First complete draft send to NRCWE
2	2015/06/22	First review by NRCWE send to LEITAT
3	2015/07/08	Revised draft by LEITAT send to NRCWE
4	2015/08/10	Revision and comments by NRCWE send to LEITAT and partners
5	2015/09/14	Revised final draft send by LEITAT to NRCWE
5	2015/10/01	Final revision and accept by NRCWE
6	2017/03/07	Project Office harmonized lay-out

Lead beneficiary for this deliverable: Acondicionamiento Tarrasense Asociacion, LEITAT, 20

Owner(s) of this document	
Owner of the content	LEITAT, 20
Co-Owner 1	UNamur, 22
Co-Owner 2	AIDICO, 29
Co-Owner 3	FOPH, 40 (work done by EPFL)
Co-Owner 4	UdL, 43
Co-Owner 5	NRCWE, 4

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Description of task

According to the NANoREG Description of Work, deliverables D2.3 and D2.9 cover the first paragraph of task 2.3, which refers to the study of the applicability of some OECD technical guidelines to nanomaterials characterization: “all procedures for establishment the physicochemical information requested by REACH will be evaluated and revisions validated. The OECD WPNM and RIP-on2 has identified needs to test the applicability of OECD TG 109 (relative density); TG 110 (granulometry); TG 106 (sorption-desorption); TG 105 (water solubility); TG 115 (surface tension); TG 107/117/123 (N-Octanol-water partition coefficient); TG 112 (dissociation constant in water), which may be coupled with TG 108 (complex formation in water). These TG’s needs revision or new TG’s may be established to enable proper description and categorization of particulate NM.

This part of task 2.3 is divided in two sequential deliverables: D2.3 contains the OECD TGs revision based on the literature and expertise and proposes new protocols for physicochemical endpoints derivation, while D2.9 will report the experimental evaluation of these protocols.

1 Summary

The applicability of OECD TG 109 (relative density), TG 110 (granulometry); TG 106 (sorption-desorption), TG 105 (water solubility), TG 115 (surface tension), TG 107/117/123 (N-Octanol-water partition coefficient), TG 112 (dissociation constant in water) and TG 108 (complex formation in water) has been assessed based on expert review of the literature and reports from ECHA, European Commission, OECD, SCENHIR, etc.

The summary of the results are compiled in Table 1 and [section 2.5](#) can be consulted for more information of each TG. There is a series of problems that are common to most endpoints, such as the fact that nanomaterials form colloidal dispersions (not solutions). In this case partition constants cannot be determined or standardized dispersion protocols or dispersion requirements are necessary to perform these tests in a repeatable manner. These problems have to be addressed both in the Technical Guidelines evaluation, but also on a more general level, by changing the list of endpoints to be determined for NMs for regulation purposes.

Table1. Summary of OECD TGs evaluation.

OECD Technical Guideline	Appropriate for NMs	Proposal	Future work
TG 109 (relative density)	Verification necessary	Protocol evaluation.	Gas displacement effect on the true density determination will be evaluated. Evaluation of the measurement of the density of agglomerates
TG 110 (granulometry)	Method A is not applicable to nanomaterials	Completely new protocols (CLS and DLS) are proposed	The proposed protocols will be evaluated and reported in D2.9
	Method B is applicable with actualization	Electronic microscope, which is proposed only for fibres, should be extended to nanomaterials of any morphology.	Work done in Task 2.2.
TG 106 (sorption-desorption)	Not applicable to nanomaterials but may be applied to determine the partition between water and soil of	Development of new detection and quantification methods (e.g. ICP-MS, radiolabelling).	Protocol and detection modification will be evaluated and reported in D2.9

	nanomaterials in some cases		
TG 105 (water solubility)	Not applicable. Requires a different approach. Several points need to be revised	-Kinetic study. -Control of variables such as pH or dissolved gases. -Protocol for dispersibility determination.	Modifications to solubility protocol will be evaluated and reported in D2.9. Relevant SOPs for <i>in vitro</i> , <i>in vivo</i> and environmental dissolution testing will also be presented in Task 2.4 (D2.6, D2.7 and D2.8) Dispersibility protocol will be evaluated and proposed as new protocol to be considered for NM characterization
TG 115 (surface tension)	Modifications necessary.	This protocol is considered relevant only for some NM.	Modifications will be evaluated and the relevancy will be analyzed.
TG 107/117/123 (<i>n</i> -Octanol-water partition coefficient)	Not applicable.	A partition coefficient as defined by OECD is not applicable to nanoparticles. It should be replaced by the determination of hydrophilicity/hydrophobicity.	Evaluation of new methods for testing hydrophilicity/hydrophobicity
TG 112 (dissociation constant in water)	Not considered adequate for its application to NMs in its current form	Develop protocols for other more relevant endpoints such as dissociation of water and hydration, surface acidity and isoelectric point.	Evaluation of dissociation of organic ligands from metallic and metal oxides NMs.
TG 108 (complex formation in water)	Not considered adequate for its application to NMs in its current form, but a similar protocol can be useful to determine NM as vector of toxic metals.	Guideline modification for focusing on the adsorption of trace metals on NMs.	Protocol evaluation with different metals in several conditions. This work will be reported in D2.9.

2 Description of work & main achievements

2.1 Background of the task

The OECD Test Guidelines are a collection of internationally accepted and relevant test methods to determine the safety of chemicals and chemical preparations, including pesticides and industrial chemicals. They cover tests for the physicochemical properties of chemicals, environmental effects, degradation and accumulation in the environment, human health effects, as well as other areas. The OECD Guidelines for the Testing of Chemicals are a unique tool for assessing the potential effects of chemicals on human health and the environment.

Section 1 in the Test Guidelines for the Testing of Chemicals gives guidelines for the characterizing 25 endpoint physical and chemical properties. But there is a doubt whether these test guidelines, established and used for traditional chemicals, are appropriate for characterization and the assessment of the toxicological properties of nanomaterials due to their smaller scale and unique properties in addition to different methods requirement for detection and quantification as compared to molecular and micrometer-size particulate chemicals.

The OECD Working Party on Manufactured Nanomaterials was created in 2006 to efficiently and effectively address the safety challenges of nanomaterials. The WPMN established a project entitled "Manufactured Nanomaterials and Test Guidelines" to review the published Test Guidelines to assess whether or not they are suitable for manufactured nanomaterials. The project might identify the need for new Test Guidelines or amendments to existing Test Guidelines or might develop guidance on how existing Test Guidelines might be applied to nanomaterials.

The OECD WPMN and the REACH Implementation Projects on Nanomaterials (RIP-oN2) have concluded that some OECD Test Guidelines are applicable, in some cases with reservations, while others are inadequate for testing Manufactured Nanomaterials (MN). They have identified the need to test the applicability of OECD TG 109 (relative density); TG 110 (granulometry); TG 106 (sorption-desorption); TG 105 (water solubility); TG 115 (surface tension); TG 107/117/123 (N-Octanol-water partition coefficient); TG 112 (dissociation constant in water), TG 108 (complex formation in water). These TG's need revision or new TG's may be established to enable proper description and categorization of particulate NM.

2.2 Current State of the art of technical guidelines (LEITAT, AIDICO EPFL, UdL, UNamur, NRCWE)

OECD (OECD 2009; OECD 2010a; OECD 2010b) and SCENIHR (SCENIHR 2007) considered that in general there are no significant differences in the applicability of the standard test methods themselves between nanomaterials and conventional substances. However, they underlined a number of considerations in relation to sample preparation and the test system (SCENIHR 2010; OECD 2014). Moreover, the impact of an increased surface area on physicochemical properties should be given thorough consideration when determining the physical hazards.

A range of techniques and analytical procedures have been adapted or developed for the characterization of nanomaterials, including microscopic, spectroscopic, spectrometric and chromatographic techniques. Examples of recent technological advances include photonic, ultrasonic and electrosonic measurement of particle size, tomographic determination of concentration and multiphase flow, optical trapping and atomic force microscopy techniques for measuring inter-particle forces, and in-line imaging techniques combined with image analysis to measure shape factors.

The OECD Test Guidelines selected for review in this deliverable are TG 109 (relative density); TG 110 (granulometry); TG 106 (sorption-desorption); TG 105 (water solubility); TG 115 (surface tension); TG 107/117/123 (N-Octanol-water partition coefficient); TG 112 (dissociation constant in water), TG 108 (complex formation in water).

2.2.1.1 TG 109 (relative density)

Density (g/cm^3) is generally considered as relevant for nanomaterials as for any other chemical by most of the references. NANO SUPPORT (Christensen 2012) points out that it is not always clear whether REACH registrants had provided the specific (relative) density (i.e., the density of a solid 'block' of the material) or the

pour (powder) density. Pour density, also called bulk or apparent density, is given by the mass per volume that a given powder occupies as poured directly into a container without any further compaction. Tap density is a different comparable measure, which describes the mass per volume after powder compaction in a cylinder (50 times repeated 25 mm drop of a cylinder onto a rubber pad) or by the use of a Dry Substance Jolting Volumeter (ISO 787). The differences in the data obtained by these procedures are often important as a powder volume the different is a function of the degree of compaction and is inversely proportional to size. Pour density of a powder such as carbon black is useful for estimating the weight-to-volume relationship for certain applications. The maximum pour (bulk) density of a material that consists of particles is always lower than the tap density, which again is lower than the relative density. In the case of spherical particles the maximum pour density is $\pi(\sqrt{18})$ (≈ 0.74) of the relative density in the case of an ordered array and very narrow size distribution, or 0.64 and 0.54 in the case of random close or random loose packed arrangement, respectively.

EFSA points out that pour density is needed in addition to the specific (relative) density for granular materials (EFSA 2011). It is suggested to include specific density and pour density as information requirements for nanomaterials.

The Scientific Committee on Consumer Safety (SCCS 2012) proposed determination of the apparent (bulk) density of powders and granules using the ratio of the mass to a given volume. For the determination, the substance is put into a receiver of known dimensions and weight. The volume taken by the product includes intermediate and hollow spaces. The apparent density depends on the form of the particles, the composition of the substance and the method of storage. The International Standard ISO 697 and ISO 60 describe the procedure of the determination of apparent density and the tools required. Another alternative method is mentioned in the final report of the project 'Characterisation of nanoscale properties of chemical substances as a basis for regulation under Regulation (EC) 1907/2006 (EC 2006; ISO 2011).

EFSA and SCCS also suggest that information on particle and mass concentrations (number particles/cm³ and number particles/g, respectively) should be provided (EFSA 2011; SCCS 2012). To calculate these concentrations size distribution (granulometry) and true density has to be known

Some studies have shown that the practical density determination in the case of nanoparticles smaller than 100 nm can be significantly impeded by substances adsorbed onto the surface or recondensed liquids (Park et al. 2004). According to Hosokawa, the density of powders consisting of nano-objects can be determined by means of fluid compression and gas displacement pycnometer methods (Hosokawa et al. 2007). Pycnometer methods have been successfully used for measuring the density of nanoscale ceramic powders with primary particle sizes of 15 to 30 nm (Hsiao et al. 2007). The density of porous MWCNT samples was determined using helium pycnometry (Vignes et al. 2009). In the case of hollow nanostructures, closed pores or poorly wetting test media may cause a deformation of the nanostructure by the gas or fluid pressure. Thus, pressure and test medium dependent variations in the determined density may result. Especially mercury pressures of up to one thousand atmospheres are regarded as problematic. In the case of nanoporous materials and porous nano-objects such as nanotubes, the actual pore size and pore infiltrability of the test medium can also affect the measurement result. For this reason, mercury and gas-based methods using e.g., helium may provide different results.

2.2.1.2 TG 110 (granulometry)

Granulometry is fundamentally defined as the measurement or computation of the size-distribution in a collection of grains. The Test Guideline on granulometry is a key endpoint for nanomaterials, especially as particle size and size distribution form an essential part of any definition of nanomaterials and is believed to be an important driver for other properties of the substance. Therefore, it is necessary to have high quality information on this endpoint. Most of the granulometry methods currently used are incapable of distinguishing between primary particles on one hand, and aggregates and agglomerates on the other. However, such analyses have been reported in scientific literature for more than a decade (Xiong & Friedlander 2001; Barone et al. 2012; Eggersdorfer & Pratsinis 2014) and applicable procedures for comprehensive size-analysis of nanomaterials as nano-objects and their aggregates and agglomerates are in advanced stages now (Kanniah et al. 2012; De Temmerman et al. 2014; Murthy et al. 2015).

A recent JRC reference report analyses the possibilities for size measurements in relation to the recommended definition (Christensen 2012). It is concluded that no single method can be applied for all nanomaterials as all methods have limitations and that dedicated guidance development is needed to support implementation in the short term. Several test methods are available for estimating the particle size (distribution) of a nanomaterial/nanoform. However, as, e.g., depicted by the JRC reference report, these methods do not provide the same size (distribution) and have their strengths and weaknesses in relation to different types of nanomaterials. Overall, there is therefore a need for method validations and further

developments as agreement on material-specific reference methods, guidance development, consensus building, etc.

The specific method(s) used to determine nanomaterial size is very important. There are currently very few standard methods for determining size. For example, Transmission Electron Microscopy (TEM) and Atomic Force Microscopy (AFM) measure the size without any organic coating whereas the size determined by Static (SLS) and Dynamic Light Scattering (DLS) and Disc Centrifuge Separation (DCS) includes the organic coating as well as the ionic double layer in the measurement, at least in the case of DLS. Various authors reported that a reported size could deviate considerably depending on the methodology used for characterisation (Park & Grassian 2010; Domingos et al. 2009; Frank et al. 2008). Moreover, the protocols applied can play a role on the sizes measured.

For example, differences in the order of two to three magnitudes were reported, with the methods coming closest to the manufacturer's information being TEM and atomic force microscopy (AFM). Sizing a particulate material needs to be done using different techniques depending on whether the nanoparticles occur as a powder, are dispersed in a liquid, are coated or are embedded in a solid material.

Two methods were suggested to measure primary particle size and particle size distribution: Electron Microscopy (both TEM and SEM) and Dynamic Light Scattering (DLS) (Hankin et al. 2011). Electron Microscopy (EM) is a very powerful technique with a high spatial resolution. The Scanning Electron Microscope (SEM) uses an electron beam to scan the surface of the material, revealing details down to 1-5 nm (using Secondary Electron Imaging technique). The Transmission Electron Microscopy (TEM) normally have even better resolution, down to atomic size. The TEM principle is based on the interaction of a focussed electron beam passing through an ultrathin sample of the material under investigation. TEM and SEM can thus be used to measure size, including primary particle size and size distribution. For example, TEM and SEM can be used to analyse average primary particle size, particle size distribution, as well as to identify the position of nanoparticles in cells, or in some cases to visualize the core-shell structure or the inorganic coating of nanomaterials. However, characterisation of 'soft' matter like liposomes, proteins, polymers (usually used as organic coating of nanomaterials) or even metal colloids by means of EM methods is limited, because materials consisting of carbon and other light elements are relatively weak electron scatters, thus other complementary methods should be applied (CASG Nano/33/2010 RIP-oN 2 Task 4B) if not advanced staining of cryo-electron microscopy procedures can be applied for the purpose.

There are some other drawbacks about the routine use of these techniques to characterise batches of manufactured nanomaterials. The sample is often small, increasing the risk that the sample is not representative of the whole batch. Moreover, the high resolution means that only few nanoparticles (or nanotubes) of the whole sample are detected in every image, reducing the statistical power of the method. To increase the representativeness of the measure, multiple sampling of the batch and thus multiple measurements should be performed, followed by processing of the results with images analysis software. This, on the other hand, conventionally increases the analysis cost and time. However, current developments enable semi-automated to automated analysis, which reduces the analytical and reporting time considerably (De Temmerman et al. 2014; Murthy et al. 2015).

The other suggested method is Dynamic Light Scattering (DLS), which is based on measurement of light backscattered from particles under Brownian motion. DLS in effect measures the hydrodynamic diameter of the particle including all molecules that diffuse together with the particle. DLS is recommended as it, depending on the specific instrument, can measure particle size from less than 1 nm to several micrometers. It is preferentially used to measure the average hydrodynamic size-distribution of an ensemble of colloidal particles. DLS is easy to perform, and it requires a small sample. However, it is important to know that the DLS results have relatively low size-resolution in multi-modal analysis using typical standard equipment (Anderson et al. 2013). Improved size-resolution can be obtained using Multiangle dynamic light scattering (MALS) (Naiim et al. 2015). It should also be mentioned that the calculated hydrodynamic diameter varies with viscosity and the optical parameters of the analysed material and dispersion medium. Therefore these parameters should be known and used for correct sizing and reported to ensure proper interpretation of the results given. Due to such methodological limitations, the hydrodynamic size distribution of an unknown sample cannot be determined fully reliable from DLS measurements alone. Moreover, the DLS size-distribution is a specific measure that gives the equivalent spherical diameter (with a electrical double-layer) and cannot normally resolve shape information. Thus, DLS alone cannot be used to report or assess different dimensions of non-spherical particles. However, some studies have revealed the possibility to use DLS for length determination of Single-walled carbon nanotubes up to 2 μm in size in combination with field flow fractionation (FFF) (Gigault et al. 2010).

A highly promising technique for the size separation of nanoparticles is field flow fractionation (FFF). In this technique a field (i.e., gravitation, centrifugal, magnetic or thermal) is applied to a mixture perpendicular to the mixtures flow direction in order to cause separation due to differing mobilities of the various components

in the field. Separation is based on the diffusion coefficient in an open flow channel and requires optimization for each material and matrix to be analysed.

The main disadvantages of the method are: potential swelling of the membranes used in FFF, interaction of the membrane with the analyte, the need for pre-concentration, additional concentration of sample during equilibration, and increasing possibility of aggregation in the channel.. A more detailed description of this method can be found in RIP-oN2 report, Task 4 (CASG Nano/33/2010 RIP-oN 2 Task 4B) (Hankin et al. 2011). Currently the FFF principle combined with different analytical principles such as MALS, DLS, ICP-MS is in rapid development and shows high potential for complex matrices, but it is not yet ready for generic implementation (Kammer et al. 2011; Dan et al. 2015).

To have a reliable and representative size measurement, it is therefore useful to use more than one measurement technique, increasing the possibility of obtaining the correct primary particle size and particles size distribution of the analyzed sample (Barthel et al. 1998). The requirement of more than one technique to measure particle size and size distribution may be established in a standardised protocol.

2.2.1.3 TG 106 (sorption-desorption)

This property indicates the capacity for a substance to bind to solid surfaces, and thus it is crucial for understanding environmental fate, and determining the most suitable approach for hazard assessment of the sediment and soil compartments.

OECD has concluded that “the three test guidelines relevant for adsorption/desorption screening (OECD TG 106, 108, 121) might be applicable under some circumstances or to some classes of manufactured nanomaterials. It stated that these TGs are applicable to solutions but it is not known how the results might be impacted by the presence of a colloidal suspension, which might be present if the sample manufactured nanomaterial does not completely dissolve” (Hankin et al. 2011). In the case where the NMs are completely dissolved, it is not further a nano-relevant; the TG 106 could be applied as it is. Hence, further work is required to determine this and to modify the TGs, if necessary (OECD 2009; Christensen 2012).

The main problem is that NMs form colloidal dispersions and no solutions, so, as a second phase, NMs never reach the equilibrium and distribution constants cannot be determined. In words of Praetorius et al., “colloidal particles are too large to dissolve; they form their own phase and require an energy input to be suspended throughout another phase. Nanoparticle dispersions can be kinetically stable for a long period of time (typically through electrostatic or steric stabilization) but they will never reach thermodynamic equilibrium and can consequently not be equilibrated with an additional phase” (Praetorius et al. 2014). This means that nanomaterials do not distribute between two phases (liquid-liquid or liquid-solid) following the same rules that organic chemicals do and, moreover, the energy of the system may be reduced if the nanomaterials accumulate in the interphase, reducing the surface of contact (see Figure 1). The fact that nanomaterials do not reach the equilibrium in adsorption-desorption studies with soils was shown even in frontier cases such as with fullerenes (C60) (Forouzanoghar & Kookana 2011).

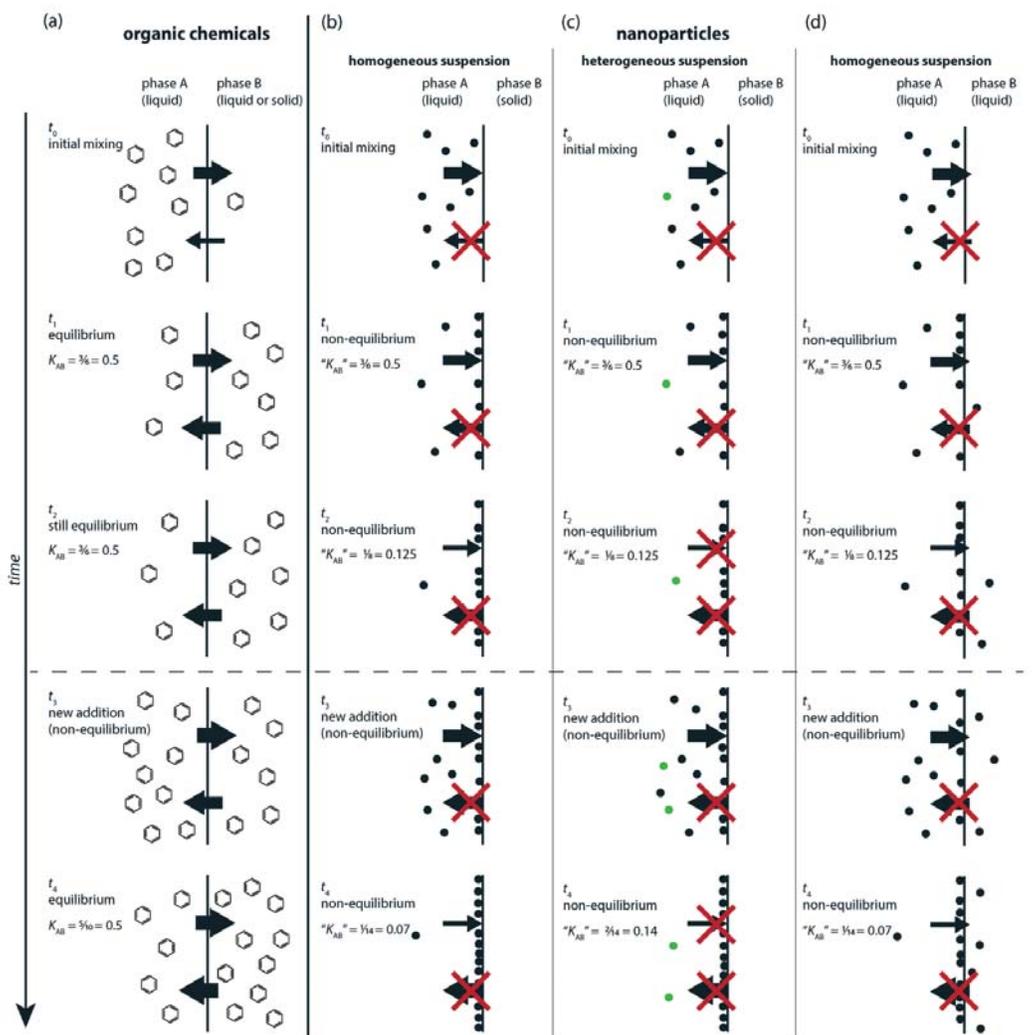


Figure 1. Illustration of the distribution behaviour of dissolved organic chemicals (a) and nanoparticles (b–d) at different time points in a two-phase system. Copied from (Praetorius et al. 2014).

Even though partition coefficients cannot be determined when working with NMs, some authors have approached the problem defining pseudo-partition coefficient. The most common approach is to determine a retention coefficient as it is done following TG 106, but assuming that the equilibrium is not reached and studying the material speciation (Geert Cornelis et al. 2010; Cornelis et al. 2011; Cornelis et al. 2013; Cornelis et al. 2012). The problem of this approach is that the pseudo-constant is totally dependent of multiple factors such as pH, salinity, organic matter, soil composition, soil granulometry, Z-potential or particle size and it becomes practically impossible to predict the nanomaterial fate. So, such a protocol (adapted to the work with nanomaterials) would only be useful to determine NM sorption-desorption in specific cases (relevant for ecotoxicity studies or specific retention studies) and not as a coefficient that helps to predict its environmental behaviour.

Praetorius et al point that a new approach is necessary as it is crucial to use a colloidal perspective (Praetorius et al. 2014). As indicated by Cornelis et al., a new perspective means to have into consideration multitude of factors such as homo- and heteroaggregation, sedimentation or size exclusion (See Figure 2).

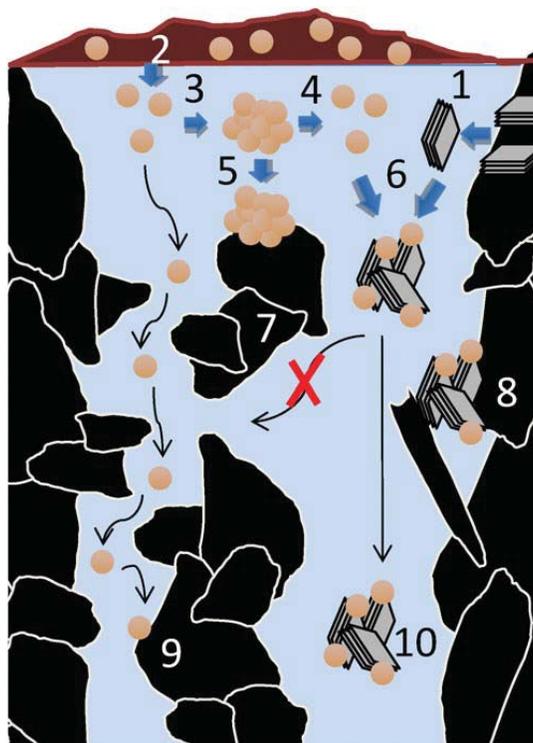


Figure 2. Schematic overview of the main fate-determining parameters of colloids and ENM in solid matrices. 1. Colloid generation. 2. ENM leaching from biosolids. 3. Homoaggregation. 4. Fragmentation. 5. Sedimentation. 6. Heteroaggregation. 7. Size exclusion. 8. Straining. 9. Deposition. 10. Convective transport. Copied from (Cornelis et al. 2014).

In conclusion, nanomaterials adsorption/desorption is a crucial parameter as the ability of nanoforms to bind to surfaces will determine their fate in the environment. Therefore, several physicochemical parameters, such as charge, surface area, porosity and others are parameters that should be considered when characterising nanoforms, since they can help predict the potential of the substances to adsorb or desorb to solid surfaces.

Therefore, in-depth physicochemical characterizations of the nanoforms suspended in environmentally relevant media are necessary to help predict the potential for the substance to partition in different environmental compartments. Further work may be required to establish the relevance of this property for nanomaterials.

2.2.1.4 TG 112 (dissociation constant in water)

OECD indicates that the current OECD test guideline might be applicable under some circumstances or to some classes of manufactured nanomaterials (OECD 2009). This would include specification of test medium and conditions.

All REACH-registrants but one waived this endpoint with the justification that the substance does not contain functional groups capable of dissociation/ionization at relevant pH ranges. Nevertheless, it is possible to make a qualitative prediction on a substance's potential for dissociation regardless of the size of the particle. Note, as discussed in previous sections, that many of the substances assessed can be surface treated. Depending on the treatment, the surface of the particles may have functional groups that are relevant for this endpoint.

OECD have highlighted that surface acidity (related to dissociation constants of surface ionisable sites) is an aspect of surface chemistry that may be particularly relevant, noting that:

- Ionisable sites may influence the surface charge which has been considered significant in toxicological studies; and
- Surface ionisation may also play a major role in colloidal particle stability and may even inhibit migration into hydrophobic phases (e.g., octanol/water partition coefficients).

OECD has concluded that the test guideline relevant to characterising dissociation constant (OECD TG 112) might be applicable under some circumstances or to some classes of manufactured nanomaterials. It stated that this TG is applicable to solutions but it is not known how the results might be impacted by the presence of a colloidal suspension, which might be present if the sample manufactured nanomaterial does not completely dissolve. Hence, further work is required to determine this and to modify the TGs, if necessary (OECD 2009). This test guideline is currently referenced in the REACH Guidance (ECHA 2012), however they indicate that pKa determination cannot be separated from dispersibility and solubility studies.

Anyway, as indicated in section 2.2.1.3, nanomaterials never reach the equilibrium and pKa cannot be determined. As an alternative, some authors have used the isoelectric point, defined as the pH at which the NM dispersion Z-potential is zero (Punnoose et al. 2014; Sahu & Casciano 2009; Powers et al. 2006).

Further work is required to assess the applicability of OECD TG 112 for nanomaterials, and modify it, should this be necessary.

2.2.1.5 TG 105 (water solubility)

Water solubility is addressed by all references as a key property. When referring to organic compounds, solubility indicates the amount of material that can be in a specific medium, but the question complicates when referring to nanomaterials, as we have a colloidal dispersion that, besides, may be dissolved. And both dispersibility and solubility are key properties as they indicate how the material will be environmentally distributed.

First, it is noted by several references that some confusion exists in relation to terminology - also in peer reviewed scientific articles - as it is not always clear whether water solubility or dispersibility is addressed (Christensen 2012; Hankin et al. 2011). It is our understanding that both parameters (water solubility and dispersibility) would be key properties. Water dissolution kinetics is also mentioned by several references as a key parameter. Moreover, dispersion stability is mentioned as a key parameter in RIP-oN2 and VCI (VCI 2008; Hankin et al. 2011). Finally, NANO SUPPORT introduces the term 'ion leaching', (Christensen 2012). Leaching describes selective loss of elements or substances from a matrix into a liquid independent on the mechanisms (e.g., dissolution, de-sorption and matrix diffusion).

A common challenge with the assessment of the water solubility of nanomaterials is the distinction between solubility, dispersion, and ion leaching from the particles. Depending on analytical techniques, a specific challenge is separation of dispersed particles for determination the truly dissolved fraction of a nanomaterial.

It seems relevant to call for scientific consensus building in relation to defining more precisely what should be the information requirement package related to water solubility/dispersibility/ion leaching/dissolution kinetics etc. and when implementing this package, clearly defining the terminology.

The registrants may sometimes be measuring ion leaching from the particles, although this was not always clear, or whether the measured value was actually a dispersion of (fine) nanoparticles. The possible effects of surface treatment on the apparent water solubility and/or dispersion were considered.

Water solubility and ion leaching: It has been recognised that water solubility of and ion leaching from nanomaterials may be higher than for the corresponding bulk form (Vogelsberger et al. 2008). It is recommended that registrants take these factors into account, and one suggestion may be to perform tests on different forms (size distributions) of the substance. As also mentioned in the RIP-oN 2 Final Report (3.5.193) dissolution kinetics may be included as an information requirement under the 'Water solubility' endpoint (Hankin et al. 2011). It should be underlined that dispersibility is a different phenomenon. The issue of dispersion vs. solution may require further development of the existing guidelines on the testing of water solubility and/or the guidance on information requirements for this endpoint.

WP5 partners (which include UdL) worked on the critical evaluation of the alternative analytical methods for solubility testing (Tantra et al. 2015). It assesses potential techniques for the measurement of nanomaterial solubility and evaluates their performance against a set of analytical criteria (based on satisfying the requirements as governed by the cosmetic regulation as well as the need to quantify the concentration of free ions). As indicated, the NM solubility studies result quite difficult due to the presence of colloids in the dispersion, which usually need to be removed from the media, and to the fact that leached ions are in equilibrium between different species (different hydration states and, in complex medium, different compounds and salts). OECD recommends the separation of particulates from the media by centrifugation or filtration, but it has been proven that nanoparticles remain in the dispersion after several centrifugation cycles (Xu et al. 2013) and that can pass through the filters (Tantra et al. 2012; Fabricius et al. 2014). So, other separation techniques are necessary when analyzing nanomaterial solubility, but it results crucial to validate them. Abundant literature can be found in the reported review about dialysis and ultracentrifugation (UF),

field-flow fractionation (FFF), capillary zone electrophoresis (CZE) and ion exchange (cites in (Tantra et al. 2015)).

Detection techniques can be divided in two categories, the ones that measure free ion concentration, such as electrochemical and colorimetric techniques, and the ones that measure the total concentration of dissolved species, such as inductive coupled plasma (ICP) and atomic absorption spectroscopy (AAS). The conclusions of the mentioned review suggest that, at present, no individual technique can fully satisfy all the analytical requirements for NM solubility testing (Tantra et al. 2015). Complementary information may be obtained from the combination of several techniques, e.g. an atomic spectrometry based method in conjunction with an electrochemical (or colorimetric) method. This review also indicates that a research gap remains, related with the need to ensure data reliability.

It is important to mention that other work in WP2 Task 2.3k and 2.4e,f,g,h,i also addresses the methodology to perform dissolution and solubility tests using new systems mimicking specific environmental and biologically relevant conditions or test conditions. This work will be reported in D2.5, D2.7, and D2.8. NRCWE in addition is leading a CEN standard development project on dissolution testing for *in vitro* toxicological purposes using a Sensor Dish Reader method for online pH and O₂ measurement and also developed and used an Atmosphere-Temperature-Controlled Stirred Batch Reactor procedure for NM reactivity and dissolution/solubility testing under highly controlled conditions and measurement of pH titrant volume and redox potential as part of the EU FP7 projects NANODEVICE, ENPRA, HINAMOX and the EAHC project NANOGENOTOX. Draft protocols for these test methods are described in the Appendix of this report and will be further refined and used in task 2.3 and 2.4 of NANoREG. Procedures and data from this type of testing and new standards in preparation should also be considered and to the extent possible also assessed in D2.9.

Further work is required to assess the applicability of OECD TG 105 for nanomaterials, and it is quite obvious that strong modifications will be necessary as the inclusion of a protocol to measure dispersibility.

2.2.1.6 TG 115 (surface tension)

Surface tension is the measure of the force that tends to diminish the area of the interface. Compounds known as surface-active agents (surfactants) reduce the surface tension of a liquid, the interfacial tension between two liquids or between a liquid and a solid. Surfactant molecules typically contain both polar (hydrophilic) and non-polar (lipophilic) moieties. Surface tension measurements require a test material that is stable against hydrolysis during the experiment period and soluble in water at concentrations >1mg/L.

Surface tension is addressed by RIP-oN2 and NANO SUPPORT only and considered as relevant for nanomaterials as for substances in general (Hankin et al. 2011; Christensen 2012). Surface tension is not in general relevant for nanomaterials, except for the special sub-classes of Janus particles which may exhibit domains of differing hydrophilicity. It was noted by the advisory group that this information requirement needs further R&D in relation to its relevance/applicability for (surface modified) nanomaterials.

This test procedure is considered as not applicable or not relevant to the testing of nanoscale materials since they relate to liquid or gaseous substances. For this test guideline applicable to solutions, is not known how the results are influenced by the presence of colloidal suspensions, which are produced by the incomplete dissolution of nano-objects.

This physicochemical test is only necessary if sufficiently high water solubility applies and no information is available on the bulk material. Otherwise the value determined for the bulk material can be used because it involves the examination of dissolved material which is no longer a nanomaterial.

It should be noted that OECD indicates, without further elaboration, that the current OECD guideline is only applicable under some circumstances or to some classes of nanomaterials (OECD 2009).

Very few studies applying surface tension measurements over nanomaterials dispersions can be found in the literature. These studies apply traditional methods as the ones defined in the TG 115 (mostly the ring method), and they show that surface tension depend on several factors, such as concentration (volume fraction), shape, size, viscosity of the dispersant (under the guideline it is always water), aggregation rate and sedimentation rate (Hwang et al. 2008; Khaleduzzaman et al. 2013; Vafaei et al. 2009; Cuenot et al. 2004).

An alternative method to determine the surface tension has been presented (Cuenot et al. 2004). Resonant contact atomic force microscopy (AFM) is used to measure elastic properties on silver and lead nanowires and on polypyrrole nanotubes with an outer diameter ranging between 30 and 250 nm. The increase of the apparent elastic modulus for the smaller diameters is attributed to surface tension effects.

The state-of-the-art analysis indicates that it would be more interesting to pay attention to surface charge as this parameter plays a critical role affecting the dispersion and fate of nanoparticles, the adsorption of ions and the way cells react when exposed to them. Besides, further work is necessary to assess the applicability of TG 115 to nanomaterials.

2.2.1.7 TG 107/117/123 (*n*-Octanol-water partition coefficient)

The octanol-water partition coefficient (K_{OW}) is a key parameter used to determine the hydrophobicity of compounds, which is a fundamental indicator of the fate and transport of contaminants in an aqueous environment (Octanol acts as a surrogate to fatty tissue in living organisms).

The octanol-water partition coefficient (K_{OW}) was used to understand the effects of humic acid (HA) on the dissolution properties of coated, nano-sized zinc oxide (ZnO) (Yang et al. 2009; Bian et al. 2011). The K_{OW} of coated zinc oxide will be determined in both distilled water and water to which HAs have been added. HA concentrations were varied to observe changes related to ZnO solubility in each fraction. Due to the hydrophobic nature of ZnO it can be hypothesized that the NMs will remain within the octanol fraction although over time the HA may increase the solubility of ZnO by changing it to a more hydrophilic nature. Preliminary results show that a more sensitive analytical technique is required for proper NM concentration determination.

In the same way the octanol-water distribution measurements have been applied to the determination of the potential ecological uptake of multi-wall carbon nanotubes. The measurement of the octanol-water partitioning coefficient (K_{OW}) values have been commonly used for sparingly soluble organic contaminants, and previously suggested for use as well with carbonaceous nanomaterials. Substantial differences in apparent distribution coefficients between the two types of CNTs were observed, but these differences did not influence accumulation by either earthworms or oligochaetes, both of which showed minimal nanotube uptake for both types of nanotubes (Petersen et al. 2010). Therefore, the results suggest that traditional distribution behaviour-based K_{OW} approaches are likely not appropriate for predicting CNT bioaccumulation.

Four experimental methods are described to measure $\log K_{OW}$, of which three are direct measurement methods (Shake Flask Method, Generator Column Method, and Slow-Stirring Method), and one indirect measurement method (Reverse Phase HPLC Method). Highly accurate measurements of $\log K_{OW} > \sim 5$ are complicated by the fact that small amounts of octanol are entrained in the aqueous phase, leading to a potential underestimation of the measured $\log K_{OW}$ values. All of the direct methods for measuring $\log K_{OW}$ require quantifying the test material in either octanol or water and preferably in both matrices.

Partition coefficient *n*-octanol/water is addressed by all references. However, as e.g. discussed by RIP-oN2 (Hankin et al. 2011), the use of this parameter may not be as straightforward as for organic chemicals. Under REACH, the endpoint is waived for inorganic substances. However, this approach may be challenged in the case that the nanomaterials are surface modified with organic groups.

In the guidance recommendations for this test guideline, RIP-oN2 (Hankin et al. 2011) points out that current OECD methods for determining this property would likely need to be modified (based on OECD, 2009) and that it can be difficult to interpret the results due to the difficulty in distinguishing between solubility and dispersibility given the small size of the particles.

The view that further R&D is needed was supported by the advisory group, which in addition pointed out that it may be more relevant to develop/agree upon a methodology which more generally could express the hydrophilicity/hydrophobicity of the nanomaterial.

All of the registration dossiers of NM examined refer to their equivalent bulk inorganic substance, so, the waiver must be considered to apply. It is important to note that in cases where a substance is surface treated with organic groups (e.g. hydrophobic or hydrophilic surface treatment), the relevance and applicability of this waiver should be questioned, and registrants should more carefully examine the hydrophobicity/hydrophilicity of the substance and their effect over its biodistribution.

Also the determination of octanol-water partition coefficient was evaluated as not applicable to nanomaterials because no thermodynamic equilibrium distribution of a second phase in two different solvents exists, and the need to find new methods for the determination of hydrophilicity/hydrophobicity of nanomaterials, was also discussed.

2.2.1.8 TG 108 (*complex formation in water*)

Complex formation in water is a key parameter used to determine the capacity of a substance to bind a metal and modify its biological and environmental fate and, so, its ability to get into the food chain. This becomes especially relevant when referring to toxic metallic cations, as referred in TG 108: cadmium, copper, cobalt, chromium, lead and zinc. According to RIP-oN 2, TG 108 is also relevant for

sorption/desorption capacity determination, but its applicability may be impacted due to the presence of colloids in the medium (Hankin et al. 2011).

A search in January 2015 in the Web of Science of the terms "nanoparticle*", "isotherm" and either "heavy metal", "Zn", "Cd", "Pb" or "Cu", excluding proceedings papers, yielded a total of 541 records. The time evolution of such publications is shown below (Figure 1).

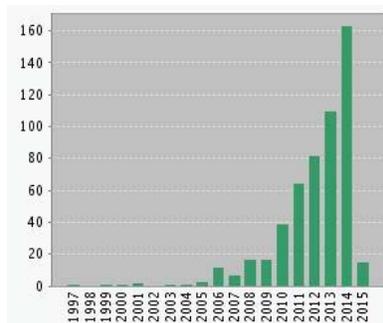


Figure 3: time evolution of relevant scientific literature regarding adsorption of trace metals on NMs (source: *Web of Science*)

This evolution indicates the growing interest of the topic of metal adsorption on NMs. However, some of these publications are not specifically focused on nanoregulation, but are driven by the interest in metal removal using NMs for remediation purposes (Heidari et al. 2009; Huang & Chen 2009; Zhou et al. 2009; Lu et al. 2006). Recent reviews dealing with metal removal via nanoparticles (which also quote to more specific reviews) are (Sanchez et al. 2011; Mohmood et al. 2013; Trujillo-Reyes et al. 2014).

The relevance of pH (Sheng et al. 2010; Chen et al. 2010) and ionic strength (salinity) of the medium has been highlighted, although some reports indicate a much lesser impact of the latter parameter (Tan et al. 2007). However, the study of other factors (crystal structure, nanoparticle size, temperature change, etc.) is very preliminary or practically inexistent (Gao et al. 2004; Xie & Gao 2009).

Some works have also considered the concomitant presence of humic or fulvic substances (Mahdavi et al. 2013; Sun et al. 2012; Liang et al. 2011; Sheng et al. 2010). These kinds of organic matter are ubiquitous in natural waters and they are believed to form organic coatings (usually referred to as "eco-corona") around the NMs to drastically modify its adsorptive, stability and transport properties in the water compartments (Lynch et al. 2014).

The majority of publications reported a procedure for quantifying the amount of adsorbed metal ions consisting in mixing the metal ions with the nanoparticles, followed by a technique for NM removal, such as **(ultra)centrifugation or (nano)filtration, and subsequent elemental analysis of the supernatant/filtrate** (e.g. by ICP-MS). The adsorbed amount of metal was then indirectly derived from a mass balance (Ha et al. 2009; Liang et al. 2011; Sheng et al. 2010; Mahdavi et al. 2013). This methodology has several drawbacks such as loss of dissolved metal due to adsorption in the filters (G Cornelis et al. 2010), or possible overestimation of the non-adsorbed metal concentration due to persistence of a significant amount of NM particles in the supernatant/filtrate after the solid-liquid separation step. A lesser amount of articles reported the use of voltametric methods (Kumar et al. 2014; Rotureau 2014; Duval et al. 2013; Domingos et al. 2008; Vale et al. 2014). It has to be reminded that classical polarography (a subclass of voltammetry) has almost been abandoned in favour of more recent voltammetric methods (as mentioned above). The use of modern electrochemical techniques (including potentiometry with ion selective electrodes) has the advantage of their **relatively low sensitivity to interferences associated with the colloidal nature of the dispersions**, which often make unnecessary the use of solid-liquid separation steps.

Regarding the physicochemical interpretation of results, most of the articles consider different isotherm models (mostly Langmuir and Freundlich equations) for the description of the trace metal bound at equilibrium, whereas the examples reporting kinetic adsorption data are much scarcer (Lu et al. 2006; Mahdavi et al. 2013). Typical kinetic models are those of first order (Engates & Shipley 2011) and second order (Contreras et al. 2012; Recillas et al. 2011; Hu & Shipley 2012; Liang et al. 2011; Lu et al. 2006; Mahdavi et al. 2013).

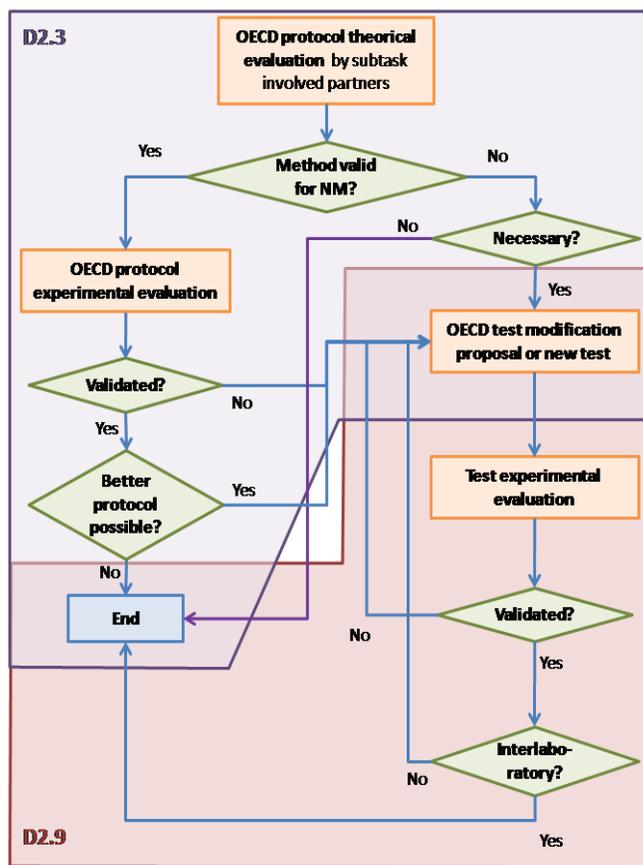
So, it is agreed by the experts that the endpoint is relevant and there is sufficient literature with different approaches leading with the problem of working with colloidal dispersions.

2.3 Suitability and proposed modifications to the OECD Technical Guidelines

Deliverables 2.3 and 2.9 report a continuum of work developed in subtasks a-h of Task 2.3, from OECD TG analysis to final validation of modified protocols. The strategy that has been followed is summarized in the decision tree (Figure 1).

In a first step, each OECD method is evaluated; these evaluations are based on compilation of literature results and on the expertise of the partners in this specific task (LEITAT, UdL, EPFL and UNamur). Where relevant, the most recent specific advice and guidance from OECD's Working Party on Manufactured Nanomaterials (OECD 2014), REACH Implementation Projects on Nanomaterials (Hankin et al. 2011), European Chemicals Agency (ECHA 2012), etc. is taken into account. From this evaluation, it will be decided if the method is applicable to nanomaterials (all nanomaterials or only some type), if small changes are necessary or if a complete new method is necessary.

When the OECD method is considered as not applicable/not appropriate, a new protocol is elaborated by the partners and the variables to be tested in the future. This protocol modifications and evaluations are reported in this deliverable (D2.3) case by case. The experimental evaluation and further validation of these protocols will be reported in D2.9.



Yes / No could be applicable only to some NM

Figure 4. Decision tree used in Task 2.3 subtask a-h to validate OECD TG for nanomaterials.

2.3.1 OECD TG 109 Density of Liquids and Solids (EPFL, LEITAT)

The OECD Guideline 109 is valid for 4 types of densities:

- Density
- Relative density (D20/4, density at 20 degrees Centigrade relative to water at 4 degrees Centigrade) may be used to compare different chemicals and is not relevant for nanomaterials
- Pour density (powders)
- Tap density (powders)
- Agglomerate density

The evaluation is focused on the methods useful for the determination of the densities of solids, especially nanosized powders will be discussed.

2.3.1.1 True density

The OECD Guideline describes three different methods. Two of them are clearly not applicable to nanomaterials:

- **Hydrostatic balance:** This method needs large sample volumes, and the materials must show an excellent wettability with the liquid. As the pores between nanosized particles, especially in agglomerates, are very small, the contact angle have to be in the range of <10 to guarantee a filling of the accessible pores. This is not the case for most nanomaterials.
- **Pycnometer:** not useful for fine powder because the pycnometer could not be filled in a controlled manner, a least not to 100 % with solid (without any pores).

The only applicable method is the **Air comparison pycnometer**. This method is also often used in research and in industrial quality control for powders. Concerning nanosized powders, own first tests as well as published results, show two reasons for a deviation of the results from the true density:

- The hydroxyl layer at the surface of the particles is getting more important with decreasing size. Measurements with Zirconia have shown a decreasing density, especially for diameter < 15 nm with decreasing size, even the crystal phase was not changing (Opalinska et al. 2015). The effect was related to hydroxyl group at the surface. This means the powder needs to undergo a controlled thermal treatment and storage before measurement to reach values which correspond to the true density of the material.¹
- The gas displacement process has also an influence. Measurements are done in series (for example 100 measurement with the same sample in the closed pycnometer. When measuring very fine powders we have observed a systematic shift of the results with increasing number of cycles, meaning the evacuation of the pycnometer chamber is different (air-helium exchange) (Park et al. 2004).

Our impression is that the protocol for true density determination has to be slightly adapted when used with nanomaterials. Additionally, we would like to take attention to the fact that a relatively large amount of powder has to be used for the measurement: sample volumes between 2 and 50 cm³, which correspond in the case of nanomaterial to 0.2 to 5 g (relative pour density of 10%). Handling such amount of **dry powders** needs often a processing following nano-safety guidelines. When decreasing sample volume, the uncertainty of the results is increasing. An evaluation of the minimum amount of nanomaterials for a valuable and reliable measurement has to be determined.

The OECD guideline gives no reference to a standard for air comparison pycnometers.

2.3.1.2 Pour and Tap density

According to the OECD guideline 109, the poured and tapped density of powders should be measured following the guideline of CIPAC (www.cipac.org): MT 186 BULK DENSITY

Definition:

The 'pour density' is the apparent density of a bed of material formed in a container of standard dimensions when a specified amount of the material is introduced without settling. The 'tap density' is the density after the material is vibrated or tapped under standard conditions.

Method:

A known weight of a solid material is placed in a glass measuring cylinder and its volume measured (to determine the 'pour density'). The cylinder is then raised and allowed to fall vertically through a distance of 25 mm on to a rubber pad (50 times) or this is carried out using a Dry Substance Jolting Volumeter ISO 787 (Note 1). The volume is measured again (to determine the 'tap density').

¹ "The density of very fine ZrO₂ (11 nm) is low at 5.22 g/cm³ compared to 5.68 g/cm³ for the monoclinic phase of bulk zirconia. Therefore, the growth of the particles causes an increase in the density making the density inversely proportional to the specific surface area. This observation is related to the presence of hydroxyl groups on the nanomaterial surface." (Opalinska et al. 2015)

In Europe, we can find the EUROPEAN STANDARD EN 725-9 (March 2006), Advanced technical ceramics - Methods of test for ceramic powders:

- Part 8 *Determination of tapped bulk density*
- Part 9 *Determination of un-tapped bulk density*

A detailed description of the protocol is:

Part 9: Determination of un-tapped bulk density

- 4.1 *Stainless steel cylindrical container of volume approximately 100 cm³ and a diameter to height ratio in the range 0.9 to 1.1. The exact volume of the container shall be measured to the nearest 0.2 cm³.*
- 4.2 *Sieve, with an aperture size of 710 µm as specified in ISO 565 and with a typical diameter of 80 mm.*
- 4.3 *Balance with a resolution of 0.01 g.*
- 4.4 *Tray, to collect the powder during the filling of the container.*
- 4.5 *Suitable assembly for allowing the powder to flow and fill the container (4.1) evenly, including a fixing system for the sieve (4.2) with a cone in its lower part to allow the sieve and the cone to be set to a predetermined height above the top level of the container, and a system to regulate the vibration of the sieve.*

An application of this procedure to nanopowder is, in principle, possible but would be difficult to carry out because the flowability of nanopowder may be too poor and the necessary amount is very large so that special safety procedure is necessary to handle the large amount of dry nanopowders (Nanosafety lab).

2.3.1.3 **Agglomerate density**

For most in vitro tests the density of the agglomerates is one of the most important parameter. The density of agglomerates determines substantially the transport properties in in-vitro tests and has to be included into the OECD guideline for density measurement. In a recent work of Glen DeLoid et al., an easy and cheap method to measure the agglomerate density was reported (DeLoid et al. 2014).

2.3.2 **OECD TG 110 Granulometry (UNamur, LEITAT, NRCWE)**

2.3.2.1 **OECD TG evaluation**

This report refers to parts of document OECD TG 110 where we deem there is a lack of applicability to NP measurement. To each of these parts a proposal to improve the document in the context of NP measurement is done.

In page 3, section 2A, it is mentioned in the 2nd paragraph that “Method A, which determines the effective hydrodynamic radius, R_s , will be used for both fibrous and non-fibrous particulates without prior inspection. It is useful only in the range $2 \mu\text{m} < R_s < 100 \mu\text{m}$ ”. This sentence is clearly out of scope with the current capabilities and definition of a nanoparticle, for which a standard convention is taken that anything below 100 nm in at least one dimension constitute a NP (SCENIHR 2010). Also current nanomaterial granulometry techniques allow for measurement down to 5 nm, like CLS (Centrifugal Liquid Separation) or Electron Microscopy techniques (ISO 2007a). Therefore, the following amendment to this sentence is proposed: “Method A, which determines the effective hydrodynamic radius, R_s , will be used for both fibrous and non-fibrous particulates without prior inspection. It is useful only in the range $5 \text{ nm} < R_s < 100 \mu\text{m}$ ”.

In page 3, section 2A subsection Definitions and units, the Stokes equation is shown as: $v = 2_g R_s^2 (d_1 - d_2) / 9\eta$. This equation applies as well to NP particle size distribution (PSD), the equation should not have g as a subscript but rather as a regular character. Hence, the Stokes equation should read as $v = 2gR_s^2 (d_1 - d_2) / 9\eta$.

In page 4, section 2A subsection Reference substances, it is stated that “Five reference substances of defined particle size covering the overall range 0.35 to 650 µm (excepting the 50 to 200 µm region) have been certified with respect to the cumulative mass distribution of particles versus equivalent settling rate diameter or equivalent volume diameter”. Currently there are smaller reference materials, more suitable as

NP reference materials, developed from different substances such as latex, PVC or silica which are currently available (Kestens & Roebben 2014) as well as Au (see NIST RM8011, RM8012, RM8013). Therefore, the following amendment to this sentence is proposed: “There are substances of defined particle size covering the overall range 0.010 μm to 650 μm which have been certified with respect to the cumulative number and mass distribution of particles versus equivalent settling rate diameter or equivalent volume diameter and hydrodynamic diameters”.

In page 4, section 2A subsection Reference materials, in Calibration materials it is stated that “Method A: A binary or ternary mixture of latex spheres ($2\mu\text{m} \leq d \leq 100 \mu\text{m}$) is suggested”. Under the arguments of the previous paragraph the following amendment is proposed: “Method A: A binary or ternary mixture of latex spheres and or Au particles ($0.010 \mu\text{m} \leq d \leq 100 \mu\text{m}$) is suggested”

In page 4, section 2A, sub-section Reference materials, in Evaluation materials, it is stated that “Method A: A ternary mixture of 2 μm , 50 μm and 100 μm latex spheres (which provides a discrete calibrated distribution) plus a sample of crushed quartz (continuous distribution)”. Under the arguments of 2 paragraphs above the following amendment is proposed: “Method A: A binary or ternary mixture in the range of the measured particles of a used substance for reference materials (i.e. latex, PVC, silica, and Au) plus a sample of crushed quartz (continuous distribution)”.

In pages 4-5, section 2A subsection Principle of the test methods, there is a description of 3 principles particle PSD measurement:

Sedimentation: this principle, while the simplest of all, is not suitable in practice for NP measurement due to extreme long times that would take NPs to sediment, down to a certain size and density.

Centrifugation: a variation of sedimentation principle where instead of using gravity as the acting force a centrifugal force is applied. This principle is suitable for NP measurement, especially when speeds $> 18000 \text{ g}$ (Relative Centrifugal Force) are achieved.

Coulter counter: A principle based on the change of electrical resistance between electrolyte solutions caused by passing particles through a single or multiple microchannels. This principle is compatible with the principle of sedimentation and currently there are other solutions based on pressure and adaptable microchannels, also called pores, which makes this principle compatible with NPs due to the shortening of measurement times. The performance regarding size-resolution of this type of instrument in the form of tunable resistive pulse sensor (TRPS) may be relatively high as demonstrated in a test with DLS, PTA (Particle Tracking Analysis), TEM and DCS as demonstrated in a test for a mixtures of 220, 330 and 410 nm-size latex particles (Anderson et al. 2013). In addition to these 3 principles used for PSD, there is the principle of Brownian motion for which there are a few techniques available for measuring NPs such as Dynamic Light Scattering (DLS) and Nanoparticle Tracking Analysis (NTA). This principle should be added to the section Principle of test methods. In addition it should be noted that not all techniques based on the above mentioned principles are compatible with the EC nanomaterial definition requiring the reported values in number percentage of objects with minimum diameter between 1 and 100 nm in number percentage and not weight percentage.

In page 5, section 2A subsection Principle of the test methods, there is the statement: “The sample should also be subjected to a simple light microscopic examination to determine the approximate nature of the particles (e.g. plates, needles, etc.)”. This claim certainly does not apply for NP due to their very small dimensions. Therefore, Electron Microscopy and/or Atomic Force Microscopy are options sought for observing the nature of the particles in the nanometer range or alternatively e.g., mass-spectrometric, Raman or UV-VIS analysis for specific compounds such as the carbonaceous materials. The following amendment is proposed: “The sample should also be subjected to a simple light microscopic examination to determine the approximate nature of the particles (e.g. plates, needles, etc.) in case of microparticles $> 2 \mu\text{m}$, otherwise electron microscopy or atomic force microscopy under low suitable concentration conditions or chemical/spectroscopic measurements for e.g., fullerene and graphene materials should be used to elucidate their identity, size and nature”.

In page 7, section 2A subsection Quality criteria, in Sensitivity it is stated that “In the general case (Method A) particles as small as 2 μm and as large as 200 μm must be measurable”. Given the proposed extension of document TG 110, the following amendment is proposed: “In the general case (Method A) particles as small as 0.005 μm and as large as 200 μm must be measurable with at least one of the proposed principles”. However, for nanomaterial definition, a precision of a lower upper size-range for the identification of a nanomaterial may be warranted.

In page 9, section 2B subsection Test conditions and apparatus, it is stated: “Method A: Ambient conditions. Measuring apparatus for all methods are readily available. Pipettes and sedimentation balances are used for the sedimentation methods”. This statement is clearly incomplete even at the time TG 110 was released. The

following amendment is proposed “Method A: Ambient conditions. Measuring apparatus for all methods are readily available. Pipettes, balances, sedimentation balances, syringes, beakers and other instruments may be required for each method”.

In page 10, section 3, it is stated that: “Method A: Data should be obtained for 3 size ranges: > 200 µm, < 2 µm and the region 2 to 200 µm. Only in the latter range should the distribution curve be prepared. It should have sufficient µm increments to resolve the curve (subpopulations). A histogram presentation is required plus a statement on the weight per cent of material > 200 µm and < 2 µm”. Given the inclusion of NP PSD, this statement should be modified to report data in the region of interest, additionally, given the definition of nanomaterials by the EC, the reported data should be in number. The following amendment is proposed: “Method A: Data should be obtained for 3 size ranges: above the region of interest, below the region of interest and in the region of interest. The region of interest is defined as the size range where the main particle population is expected to be observed. A histogram or a plot is required plus a statement of the weight per cent of material above and below the region of interest. Data should be reported in weight percent and for nanoparticles in number percent”.

2.3.2.2 OECD TG modifications

Taking into account that document TG 110 was released 33 years ago, it should be assumed that the analytical technology has advanced and new options for granulometry as well as simplification and automation of the measurement processes. Indeed, this has been the case for granulometry which now is able to measure nanometer sized particles. This point has also been recognized in other reports (Hankin et al. 2011), concluding that Method A is not applicable to nanomaterials (OECD 2009). In fact one of the most important aspects, often overlooked, is the sample preparation methodologies for which there are several relatively recent ISO documents covering relevant aspects for specific case scenarios (ISO 2010a; ISO 2010b; ISO 2007b; ISO 2000; ISO 2005).

In the context of NP granulometry measurement, it has been established that no individual technique can satisfy a meaningful characterization of nanomaterials (Hankin et al. 2011). So a combination of methods for primary particle size measurement and methods for hydrodynamic size measurement is necessary. For the first case, electron microscopy techniques (method B) are the more indicated and traditionally used; this protocol revision is currently being done in WP2 T2.2. In the case of granulometry of NM dispersions, there are at least additional 4 available techniques based on robust detection technologies:

- 1) Centrifugal Liquid Sedimentation (CLS): Based on the centrifugation principle and the density difference between particle and fluid, samples from a few µm down to 5 nm can be measured in a reasonable amount of time. Samples can be unstable under dispersion conditions and have multiple populations. Number statistics can be obtained. The limitation of CLS is the measurement time, which can increase up to impractical times for low density samples and/or very small samples like the 5 nm range. There are specific ISO documents describing the principle and the technique using a laser source as the probing device (photocentrifuge method), respectively (Merkus 2009; ISO 2001; ISO 2007a). Another alternative is to use an X-ray source, however the resolution is greatly limited by the capacity of small diameter particles to absorb X-rays, limiting this alternative to diameters of about 20-30 nm. Another technique from the same principle is ultracentrifugation, achieving higher speeds hence able to measure faster samples than CLS.
- 2) Dynamic Light Scattering (DLS): Based on the Brownian motion principle DLS can measure, relatively fast, sample with a lowest resolution of approximately 1 nm. Limitations include: the lack of number statistics, multiple populations will give a bias to the largest diameter population, if the sample sediments, then bias will occur in the form of unreasonable large diameters (Hankin et al. 2011).
- 3) Nanoparticle Tracking Analysis (NTA): Based on the Brownian motion principle NTA tracks the light scattering of particles through a series of images which then a software integrates to provide NP size and motion path. Number statistics are possible. Limitations include: reduced amount of measured sample in a small focal plane, multiple populations could bias statistics towards the largest diameter population, tracking capability is restricted to a minimum of 10-20 nm particles with small absorption coefficients. Dilutions, of three or more orders of magnitude of the samples are needed.
- 4) Coulter counter: Based on the electrical resistance variation this technique can also provide number statistics and PSD. Its main limitation is in term of performance versus the above 3 techniques, hindered by the fact that microchannels can get clogged in case of big diameter samples or multiple population samples.

It should be noted that each technique requires proper calibration standards for the desired zones of interest. Moreover, it is necessary to use a common dispersion protocol. The intention behind the NANoREG sonication protocol is to have a comparable sonication energy proportioned to all samples. In the case of

granulometry we propose to modify the protocol in order for the sample to receive the energy with more deagglomeration efficiency.

Given the need to adapt existing methodologies for PSD for NP measurement in order to address regulatory, scientific, and industrial needs, UNamur has been actively participating in RRs at European level and leading the development of an SOP for the photocentrifuge method of CLS. It is in the NANoREG framework that UNamur proposes the revision of this existing SOP (prepared under the FP7 project QualityNano) and, given the set of limitations set for the centrifugation principle in terms of diameters and observation techniques, provides a generalized protocol for NP measurement. The existing SOP is included in the annex.

LEITAT proposes to use NANoREG DLS protocol for Benchmark data determination as SOP for hydrodynamic size determination, but with the modified sonication protocol considering optimal dispersibility criterion for physico-chemical characterization.

2.3.3 OECD TG 106 sorption-desorption (EPFL, LEITAT)

This Guideline is aimed estimating the adsorption/desorption behaviour of a substance on soils. The goal is to obtain a sorption value, which can be used to predict partitioning of the substance between water and soil under a variety of environmental conditions; to this end, equilibrium adsorption coefficients for a chemical on various soils are determined as a function of soil characteristics. The principle of the method is based in the addition of non-labelled or radiolabelled substances at known concentrations in 0.01 M CaCl₂ to soil samples of known dry weight, which have been pre-equilibrated in 0.01 M CaCl₂. The mixture is agitated for an appropriate time. The soil suspensions are then separated by centrifugation and, if so wished, filtration and, finally, the aqueous phase is analyzed. In the case of radiolabelled test substances, the concentration of the substance adsorbed at the soil could be measured. The test is applicable to any chemical substance that an analytical method with sufficient accuracy is available for.

Following characteristics of the substance have to be known before carrying out the test:

- a) solubility in water [OECD Guideline 105];
- b) vapour pressure [OECD Guideline 104] or/and Henry's law constant (not considered important for nanomaterials);
- c) abiotic hydrolysis as a function of pH [OECD Guideline 111];
- d) octanol/water partition coefficient [OECD Guidelines 107, 117 and 123];
- e) ready biodegradability [OECD Guideline 301] or aerobic and anaerobic transformation in soil;
- f) pK_a of ionisable substances;
- g) direct photolysis in water (i.e. UV-Vis absorption spectrum in water, quantum yield) and photodegradation on soil.

Application of OECD TG 106 to nanomaterials presents the following problems that have to be discussed before any experimental validation:

- The necessary characteristics listed above, especially solubility in water [OECD TG 105] and *n*-octanol/water partition coefficient [OECD TG 107, 117 and 123], are still under development and adaption for nanoparticles. It is still not clear if these endpoints will be used or new (more representative) ones will be proposed and developed.
- Adsorption of nanomaterials on soil (organic and inorganic) is based on colloidal interaction (particle/particle and particle/plate), therefore the adhesion forces are strongly determined by the properties of the local environment (pH, salt concentration, presence of organic molecules in solution or in the soil, etc.)
- Centrifugation for separation is not possible because the soil could include particles from similar sizes and the nanomaterial will co-sediment with the soil
- Mechanical capture of particles in the soil, the roughness of the soil particles and porosity will influence the results substantially. Physicochemical properties are well defined and established, but mechanical capture would be statistical.
- As colloidal stability of the nanomaterials is strongly influenced by the overall as well local condition (pH, salt concentration, presence of dissolved organic molecules), a change in the size distribution could occur during the test period.
- Test condition in the OECD guideline 106 (paragraph 43) also has to be discussed in detail: **For analytical reasons all colloidal particles have to be isolated from the solvent which are not from the test substance.** This is impossible, colloidal particles are the target.

- Chemical analysis of elements which are only present in the test particles could be a solution to determine the adsorption coefficient. Unfortunately, this solution is only applicable to very few nanomaterials compositions because most of the soils are composed with elements which are also present in the nanomaterials of interest (TiO₂, SiO₂, Fe₂O₃, Carbon...). Radiolabelling would be an expensive and not always available alternative.
- The term “equilibrium” in relation to adsorption of nanomaterials is not useful. Nanomaterials are a second phase and, for this reason, cannot reach the equilibrium by definition (Praetorius et al. 2014).

2.3.4 OECD TG 105 (water solubility) (UdL, EPFL, LEITAT, NRCWE)

2.3.4.1 On the definitions and measurands in TG 105

It has been pointed out that some ambiguity in the definition of NM solubility persists, concerning the fundamental distinction between “dispersion” and “solution” (OECD 2012).

According to the IUPAC definition (IUPAC 1997) solubility is the analytical composition of a saturated solution, expressed as the proportion of a designated solute in a designated solvent. Solubility may be expressed in units of concentration, molality, mole fraction, mole ratio, etc. ISO/TR 13014:2012 guidance also gives a similar definition “... solubility is the maximum mass or concentration of the solute that can be dissolved in a unit mass or volume of the solvent at specified (or standard) temperature and pressure, unit [kg/kg] or [kg/m³] or mole/mole”. According to ECHA, “it is important to recognise that solubility and dispersibility are two distinct phenomena. Solubility is the **degree to which a material (the solute) can be dissolved in another material (the solvent) such that a single, homogeneous, temporally stable phase (a solution down to the molecular level) results**, and is relevant to solids, liquids and gases. **Dispersibility is the degree to which a particulate material can be uniformly distributed in another material (the dispersing medium or continuous phase)**” (ECHA 2012). Historically, the term “dissolved” meant the component of a liquid sample that had passed through a 0.45µm (or similar) filter. However, as (colloidal) dispersions of nanoparticles might also pass through such filters, it is recommended that use of the term “dissolved” should be restricted to the formation of true solutions, and where both liquid and particulates are present the term “dispersed” should be used (ECHA 2012). Nevertheless, it is also recognized that in the case of NMs, it can be difficult to distinguish between when a substance is dispersed and when it is dissolved due to its small particle size, as discrimination between molecular species and particle may be difficult in some cases (e.g. fullerenes). Material-specific requirements for separation may be needed for generalization of this TG.

The elaboration of guidelines for the determination of NM dispersibility is the object of a separate report within task 2.3.

There is also some confusion between the terms “dissolution” and “solubility”, which are often used interchangeably. It must be pointed out that **solubility is assumed to correspond to an equilibrium situation, whereas dissolution is considered a kinetic process**. In this respect, SCENHIR opinion states that “One of the unknown processes relevant to the environmental exposure assessment of nanomaterials is the extent/rate of dissolution of nanomaterials in water. It is unlikely that the standard OECD methods for measuring solubility of nanomaterials in water can provide the required information and it is recommended to revise these methods to accommodate the measurement of the rate of dissolution of nanomaterials in the natural environment” (SCENHIR 2009).

In some inorganic NMs, dissolution leads to the release of ionic species (such as Zn²⁺ ions, in the case of ZnO) up to an equilibrium situation governed, at least in principle, by a solubility product constant. The release of ions into solution, however, may also be a result of hydrochemical reactivity of the particles as a result of, e.g., redox reactions (Ag⁰, Fe⁰, etc.), or due to leaching of impurities (such as traces of metal catalysts in the case of CNTs (Hsieh et al. 2012)) as well as the presence of incompletely reduced metal salt precursors (as in the case of Ag and Au NMs synthesized by chemical reduction methods (Ma et al. 2012)). For all these reasons, RIP-oN2 report uses for this physicochemical endpoint the expression “**Solubility & release of metal ions into solution**”. The cases of redox reaction and presence of impurities are some examples of situations where the dissolution process, even if it leads to an “equilibrium” (or steady state) situation, it probably cannot be described strictly as “solubility” from a thermodynamic point of view (i.e., determined by a solubility product constant, as in the case of ionic salts). The cases of redox reactivity are often kinetically very slow or completely hindered by metastable situations (like surface passivation of Ag⁰ particles due to formation of insoluble, non-permeable chloride or sulphide coatings (Levard et al. 2012)). RIP-oN2 acknowledges, in particular, the case of Ag NMs, as “that there is no information available on the kinetics of dissolution in dependence of nanosilver particle properties (...) and properties of the medium (like pH, dissolved organic carbon, silver-complexing ions) and recommended the inclusion of ‘Dissolution

Kinetics' as a sub-information requirement under the existing REACH Information Requirement for 'Solubility' (Hankin et al. 2011).

The current definition of solubility states that measurement should only be focused on the total amount of dissolved nanomaterial, without taking into account the differentiation of the resulting dissolved species. However, it may be argued that regulation of environmental risk assessment of conventional (not "nano") metal compounds (such as ECHA guidance) has recognized the need to measure speciation (i.e. the distribution of an element amongst dissolved species) (ECHA 2012). In fact, a significant number of nanotoxicology studies have suggested that it is the free ions that are correlated to NM toxicity (Ivask et al. 2012; Pettitt & Lead 2013). The important role that free ions play in governing toxicity supports the recommendation that the **measurement of solubility would also report the free ion concentrations** as additional information, where relevant.

As a conclusion, it is pointed out that a combination of terms and/or measurands may be needed to describe NM solubility.

2.3.4.2 Key aspects in TG 105 concerning applicability to NMs

It is beyond the scope of this report to include an extensive review of the factors that may be potentially relevant for the measurement of dissolution kinetics and equilibrium solubility of NMs. But, among the most significant, we can indicate (Misra et al. 2012):

- a) pH (Levard et al. 2012).
- b) temperature (Maurer et al. 2014).
- c) presence of dissolved ligands, synthetic stabilizing agents (surfactants, capping agents, surface coatings, etc.) and natural organic matter such as humic substances or proteins (Nel et al. 2009).
- d) nanomaterial particle size and surface tension, as described by the Ostwald-Freundlich equation (Johnson 2012; David et al. 2012), and concomitant kinetic processes such as Ostwald ripening (Vogelsberger et al. 2008).
- e) mass of the particles exposed, aggregate/agglomerate state and surface area, which may influence the rate of dissolution (He et al. 2013).
- f) oxygen concentration and redox potential of the liquid dispersion (Liu & Hurt 2010).
- g) hydrodynamic conditions (e.g., stagnant water vs. mechanical agitation) determining the mass transport mechanisms, which affect dissolution kinetics, except in those cases where surface chemical reaction is the rate-limiting step (Batchelor-McAuley et al. 2014).
- h) presence of salts in the test medium, such as chlorides, phosphates, carbonates, etc. which can lead to concomitant formation of solid precipitates (Mu et al. 2014).

The following part of this report refers to sections of document OECD TG 105 that have been identified as requiring revision due to NM-specific features. Where relevant, a recommendation is included in each case.

Section 2. Initial considerations: The guideline addresses the "determination of the solubility in water of **essentially pure substances which are stable in water and not volatile.**"

The requirement of purity (from a chemical point of view) does not hold for a wide number of NMs, including coated, core-shell, composite materials, nano-alloys, etc. In those cases, differential solubilization of the various chemical components of the NM is envisaged, and, therefore, the analytical determination procedure and data reporting should acknowledge this. In those pertinent cases, speciation information regarding the composition of the liquid phase is advisable.

The requirement of water stability is also compromised in many cases due to hydrochemical reactivity. It has been noted that solubility, hydrolytic stability and dissociation constant in water are inter-related physicochemical properties, so that assessment of one of them requires necessarily knowledge of the other two, and thus an integrated testing strategy has been proposed for the combined testing of these endpoints (Hankin et al. 2011).

Section 3. Initial considerations: The flask method, which is indicated for the least soluble materials (with solubility below 0.01 g/L), has several important potential drawbacks for its application to the solubility testing of NMs, as commented below. Moreover, there is very little information in specialized literature about the use of elution methods in this context. Therefore, at this stage, we recommend to assess the applicability of the flask method only.

Sections 4-5. Definitions and units: See discussion in section “On the definitions and measurands in TG 105” above. Solubility in mol/L should also be accepted, as it is more convenient for comparison purposes.

Section 6. Reference substances: Due to reasons explained below, it would be advisable to have reference NMs for the assessment of the efficiency of the solid/liquid separation step and, ideally, for the accuracy of the analytical method used (Tantra et al. 2015). It is acknowledged, however, that no such reference materials are available at the moment.

Section 7. Test conditions: A temperature of 25° C is recommended, for comparison with thermodynamic data of bulk material analogues (see section 3.5. Theoretical modelling using speciation software, in ref. (Tantra et al. 2015)). An uncertainty of $\pm 0.1^\circ$ C should be easily achievable with modern thermostating equipment. The relevance of temperature is particularly important if kinetic dissolution data are to be recorded.

Section 8. Preliminary test: The visual check can be affected by the possibility that apparently transparent solutions may still contain particles, due to small size of NMs and potentially low scattering and/or absorption in the visible light range. Instead, the use of centrifugation or light scattering count methods (e.g., derived count rate measurement available in commercial DLS equipments) is recommended in order to check the presence of remaining particles. However, the scattering count rate may not be sensitive enough at very low particle concentrations.

Additionally, an approximate value for the solubility of some NMs can be obtained from theoretical calculations of the bulk material analogues, whenever the relevant thermodynamic information is available and particle-size effects on solubility are not too important (see Figure 5 and ref. (Tantra et al. 2015) section 3.5. *Theoretical modelling using speciation software*).

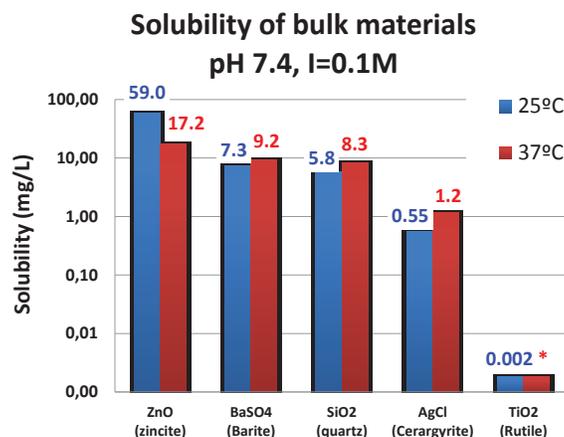


Figure 5: Equilibrium solubility of bulk analogues of different core NMs, as predicted by Visual MINTEQ v.3.0 (using the standard thermodynamic database) in a 0.1M NaCl medium at pH 7.4 and 25°C or 37°C. AgCl is included under the assumption that this phase may control the dissolution of Ag in chloride media (and in absence of sulphides). The * in TiO₂ indicates that the value at 37°C is uncertain, as the dissolution enthalpy is not available. Note the logarithmic scale in the vertical axis. **Main dissolved species and fraction of the total dissolved concentration** (from left to right): Zn²⁺ (93%), Ba²⁺ (97%), H₄SiO₄ (100%), AgCl₂⁻ (80%, Ag⁺ being less than 0.1%), Ti(OH)₄ (100%).

Section 9-16. The column elution method: this method is recommended for scarcely soluble substances that are able to form a stable coating on the inert supporting material used as stationary phase. The supporting material is coated with the test substance by dissolving the latter in a volatile solvent, mixing it with the inert material and evaporating the solvent (section 13). This method, however, might not be applicable to many NMs (in particular, most inorganic, not functionalized NMs) that will not be dispersed in low dielectric constant solvents. Also, the assumption that the tested NM will stick to the supporting material is questionable. Therefore, a relative large fraction of NM may be expected to percolate. The method describes the use of just a “small plug of glasswool” to filter out particles (section 10), but this is clearly not adequate in the case of particles of sizes in the nanometer range. Therefore, more demanding filtration procedures should be required (such as ultrafiltration), which would render the column elution method unpractical due to clogging and/or overpressure. As a conclusion, **the assessment of this method for NMs is not recommended at this stage**. Alternatively, the use of columns loaded with chelating and/or ion exchange resins for the retention of the dissolved ions present in NM dispersions could be an option, and

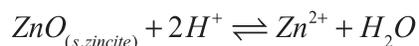
there are some recent examples in literature (Merdzan et al. 2014; Hadioui et al. 2013). However, this alternative still deserves further testing and validation.

Section 17. Notes: Instead of just double distilled water as eluent, higher quality water should be recommended, such as Nanopure Diamond UV and Millipore MilliQ-filtered water (following the guidelines of NANoREG dispersion protocols). The presence of particles and elemental impurities in the test water media should be specifically checked and water samples used for blind control analysis.

Section 19. Notes: See “Considerations on the assessment of particle removal” below.

Section 20. Notes: The use of indicator strips for the measurement of pH is not considered precise enough. Note, for instance, the case of solubility of ZnO-based NMs, where 0.5 units of pH leads to a change of one order of magnitude in the concentration of Zn^{2+} (David et al. 2012). Instead, potentiometric measurement of pH with conveniently calibrated glass electrodes is recommended.

Section 21. Principle of Flask method: saturation is supposed to be achieved by a heating and cooling cycle. Although most substances increase their solubility as temperature rises, it is not always true. A paradigmatic example of significant relevance for NMs is the case of ZnO, whose value of the standard enthalpy change of the dissolution reaction:



has a negative value of -61.7 kJ/mol at 25°C (Zhang & Muhammed 2001), which means that **ZnO solubility decreases as temperature increases**, according to the van't Hoff equation. This behaviour has been experimentally confirmed by UdL with ZnO NMs.

Moreover, in the case of NMs releasing metal ions as a result of hydrochemical reactivity (redox reactions) and/or metal leaching, the dissolution process may not be reversible with regard to temperature. Therefore, the use of **temperature changes to ensure attainment of saturation is discouraged**.

Section 22. Apparatus: it should include equipment for the preparation of NM dispersions (ultrasound probe, etc.). Specific details on the instrumentation for the solid-liquid separation step should be listed. Specific details on water quality are also required (see comment on section 17 above).

Section 23. Procedure: the guideline does not address the possible influence of dissolved gases (CO_2 , O_2) on dissolution due to their effect in redox processes, presence of soluble metal complexes and/or concomitant solid phases (such as bicarbonates or carbonates). The use of degassed solvents, as well as proper inert or known/measured realistic atmosphere conditions in the vessels is advisable. Also the value of the redox potential of the testing solutions should be checked and reported.

One of the most critical points of TG 105 concerns time and the expression “equilibrium” (no further change of the result with dissolution time).

Vogelsberger *et al.* (Schmidt & Vogelsberger 2006; Vogelsberger et al. 2008) have shown that, for thermodynamic and kinetic reasons (including nucleation, particle growth, Ostwald ripening and other phenomena), the solubility of a substance in form of a nanoparticle is time-dependent with a maximum of solubility around 24 to 100 h (see Figure 6, *left*). The figure shows clearly that for titanium dioxide the solubility at the beginning could be up to 3 times higher than in “equilibrium”. The results of Vogelsberger were successfully repeated in EPFL lab, by measuring the solubility of Al_2O_3 and Cr_2O_3 up to 200 h.

It has also been reported [17] that ZnO NM dissolution may reach an “equilibrium” situation within a shorter time scale (several minutes to 24h), as shown in Figure 6, *right*). This presumably happens whenever particle growth and ripening processes are relatively slow. In these situations, solubility reflects a contribution from the particle size, as described by the Ostwald-Freundlich equation (Hunter 2001; Bian et al. 2011; Mudunkotuwa et al. 2012):

$$\frac{S}{S_0} = \exp\left[\frac{2\gamma\bar{V}}{RT\bar{r}}\right]$$

where S is the solubility in the NM dispersion, S_0 is the bulk solubility, γ is the surface energy, \bar{V} is the molar volume, R is the gas constant, T is the temperature and \bar{r} the radius of the primary particle. Equivalent expressions have been reported in terms of specific surface area of the solid (Stumm & Morgan 1996).

It is interestingly to note that *in vitro* toxicity experiments are often carried out during 12 and 24 h. Therefore the **dissolution time plays an important role** and, especially regarding toxicity testing, it has to be discussed which dissolution time is relevant or if the dissolved concentration as function of time has to be determined.

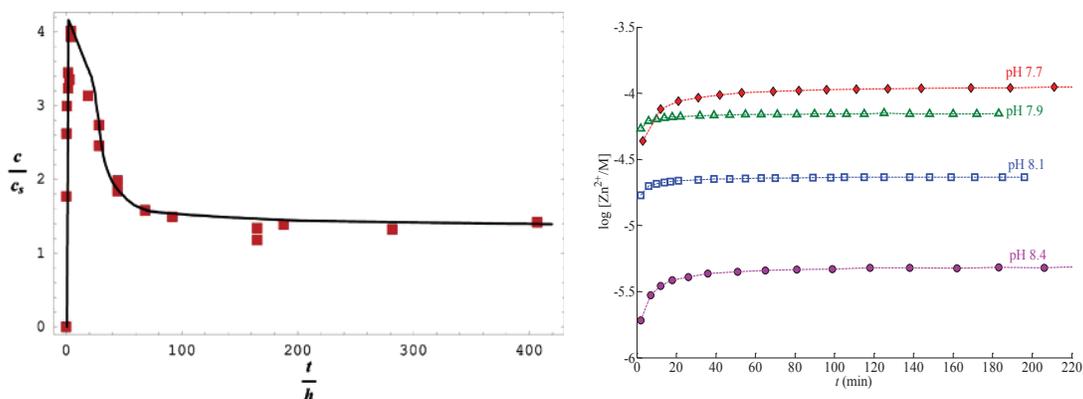


Figure 6: (left) Comparison of experiment and theory for the dissolution kinetics of a self-made titanium dioxide sample (Schmidt & Vogelsberger 2006); (right) Evolution of the free Zn^{2+} concentration (as measured with AGNES) vs. time elapsed after the addition of stock solution of commercial ZnO NPs to a buffer solution. Filled/open markers: 71 and 20 nm primary diameter NPs, respectively (David et al. 2012).

Kinetics in the short time regime (i.e., whenever Ostwald ripening processes are negligible) has been described in the literature using e.g., the Noyes-Whitney equation (Peng et al. 2011):

$$S_t = S(1 - e^{-kt})$$

where S_t is the dissolved concentration measured at time t , S is the solubility at equilibrium, and k is a dissolution rate constant. The rate constant is dependent, among other factors, on the specific surface area of the material and mass transport mechanism. Therefore, proper account of the aggregation/agglomeration state of the NM sample, stirring rate, etc. is recommended if kinetic data are going to be recorded.

Finally, considerations on the assessment of particle removal and suitable analytical method apply (see below).

Section 25. Procedure: the same comments on section 20 above apply here.

Section 26. Analytical determination: see “State of the art on Analytical Methods” below.

Section 30. Data and reporting: the test report description should be updated to incorporate additional information regarding NM specific features, as discussed above. Also adequate reference to testing results of other physical chemistry endpoints (e.g. dissociation constant in water, surface reactivity, etc.) should be reported.

2.3.4.3 Considerations on the assessment of particle removal

The method used for particle removal is not clearly stated in the current TG, although section 23 (Flask method) suggests the use of centrifugation (with no details on the exact conditions). It is our opinion that the specific details of the procedure for solid-liquid separation are of utmost importance, and probably deserve the development and assessment of specific guidelines.

A preliminary theoretical assessment of the fraction of particles remaining in the supernatant after centrifugation may be achieved by solving the Mason-Weaver equation (Elimelech 1995) describing the sedimentation and diffusion of particles under a centrifugal field, on the basis of the relative centrifugal force, time of centrifugation and dimensions of the centrifuge tubes used. In this case, the terminal velocity should be calculated for the primary particle dimensions (smallest size fraction of the NM). UdL has developed computer models, which are available on request.

The criterion recommended in the TG for the assessment of presence of particles in the liquid phase is considered weak, because the observation of the Tyndall effect (Mie scattering for particles of 0.2 to 2 wavelengths of light (200 nm -1300 nm) and Rayleigh scattering for particles < 200 nm) is difficult especially at low concentration of colloidal particles, or in the case of nanoparticles with a diameter < 100 nm. A technical solution might be that dynamic light scattering (i.e., by measuring the derived count rate and comparing it with that of the pure solvent) or another particle diameter measuring method is used to check if particles are still in the solution. It must be pointed out that, in general, these methods have relatively high

limits of detection, which represents an important point of concern. The presence of nanoparticles in the final solution may lead to false results, especially in the case of low solubility. On the other hand, the extent to which possible remaining particles in the liquid sample may interfere on the specific analytical method used (atomic spectrometry, voltammetry, etc.) should be assessed.

2.3.5 OECD TG 115 (surface tension) (LEITAT, EPFL)

Surface tension is the elastic tendency of liquids, which makes them acquire the least surface area possible. OECD TG 115 was developed for measuring the surface tension of aqueous solutions, so, a revision is necessary in order to study its performance with nanomaterial dispersions (Saidur et al. 2011). It is important to remark that nanomaterial dispersions are a two-phase metastable system and, so, the surface tension between a two-phase system and air would be measured, thing that contradicts the Guideline. Besides, according to RIP-oN2, the effect of nanomaterials over water surface tension will be low, except in the specific cases where the nanomaterial may act as a surfactant, nanoparticles also known as Janus particles (Ngai & Bon 2014; Hankin et al. 2011).

As defined for aqueous solutions, the existing guideline gives a crucial importance to the solubility of the tested compound, a very important parameter that defines the concentration at which the test has to be performed. The effect of the nanomaterials over the dispersion surface tension will depend completely on the dispersion stability and on the tendency of the nanomaterials to accumulate in the water surface. In the case of nanomaterial dispersions, both solubility and dispersibility play an important role and have to be known.

The three most important factors that may affect the surface tension of a nanofluid are (Khaleduzzaman et al. 2013):

- Dispersibility
- Volume fraction
- Temperature

Temperature can be set the same as in the current guideline, so that dispersibility and volume fraction remain as the only variable parameters. However, its influence will be studied in Deliverable 2.9 work.

Dispersibility depends on several factors, mainly: concentration (volume fraction), shape, size, viscosity of the dispersant (under the guideline it is always water), aggregation rate and sedimentation rate (Hwang et al. 2008). However, the amount that can be dispersed and its stability can vary considerably depending on the dispersion principle (e.g., electrostatic, steric, depletion or blocking/polymeric stabilization). Moreover, dispersibility will show if the nanomaterials accumulate between the water and gas phase, which would alter the value of the measured surface tension.

In the current guideline the preparation of the sample is provided by the following rule:

- *The concentration should be 90% of the saturation solubility.*
- *If this concentration is higher than 1 g/l, a concentration of 1g/l should be used in the test.*

As this rule is not applicable for nanomaterials, a study should be conducted to determine which would be the right concentration to carry out the test, according to nanomaterials dispersibility in water.

Another aspect, which is not considered in the guideline, is how the solution is prepared (agitation and temperature) as it would not affect the measure as long as the solute is completely dissolved. However, this could be a critical point in the preparation of nanomaterial dispersions. A reproducible and reliable sonication/disaggregation protocol has to be established to test if dispersibility has an impact on the surface tension of nanomaterial dispersions.

The viscosity of the dispersion should not be a problem as in the current guideline the highest value accepted is 200 mPa·s and the dispersant used is distilled water, whose dynamic viscosity is 1 mPa·s at 20°C. This is a parameter that should be taken in consideration when deciding the concentration of the samples to make sure viscosity will not exceed 200 mPa·s in any case (Rudyak 2013).

Volume fraction is the parameter that it is most commonly used for measuring concentration in nanomaterial dispersions in surface tension studies. Khaleduzzaman and co-workers showed that concentration has significant effects over surface tension of nanomaterial dispersions (Khaleduzzaman et al. 2013). Nevertheless, they obtained contradictory results in the relations of surface tension versus nanomaterial concentration. These results were explained by the findings in another work (Vafaei et al. 2009), which had shown, with Bi₂Te₃, that electrostatic interactions may occur at low NM concentration. Then, surface tension decreases until a saturation phenomena is reached; but the concentration reaches a point at which van der Waals interaction energies become dominant and lead to higher surface energy and, so, to an increase of

surface tension. At the moment, there is no model able to precisely predict the enhancement or detracting of surface tension with respect to volume fractions.

Within Deliverable 2.9, both the effect of dispersibility and volume fraction will be studied. The work will be done basically with NANoREG core materials, but probably a separate study with Janus particles will be necessary. This type of particles is currently not included among the NANoREG test nanomaterials.

The Guidelines describe three methods for the surface tension determination: plate method, stirrup method and ring method, all of them fully described in the ISO Standard 304-1985 (ISO 1985). Based on our expertise, these methods are fully applicable and do not need any revision apart from the dispersion-solution pre-requirement.

2.3.6 OECD TG 107/117/123 (octanol-water partition coefficient) (EPFL, LEITAT)

The partition coefficient (P) is defined as the ratio of the equilibrium concentrations of a dissolved substance in a two-phase system consisting of two largely immiscible solvents. In the case of *n*-octanol and water, several reasons make these Guidelines not applicable for nanoparticles:

- In contrast to dissolved molecules forming a solution, nanoparticles form colloidal dispersions,² which are per definition multiphase systems and thermodynamically unstable. Colloidal particles form a second phase and require an energy input to be suspended throughout another phase. Nanoparticle dispersions can be kinetically stable for a long period of time (typically through electrostatic or steric stabilization) but they will never reach thermodynamic equilibrium (See Figure 3). Colloidal suspensions do not fulfil the basic requirement for a test of partition coefficient.
- The results of a test under the OECD guidelines will depend strongly on the sample preparation and the energy added during the shaking/dispersion process (the distribution is a result of a kinetic and not of an equilibrium reaction). The colloidal stability is also based on kinetic process and will influence strongly the behaviour in one or the other liquid (agglomeration, sedimentation). Therefore only particles which show a colloidal stability in both liquids are of interest for a test.
- As a three-phase thermodynamically unstable system, it has been experimentally observed that the nanomaterials tend to accumulate in the liquids interface or on the surface of the less dense liquid, as it is anticipated by the energy theory.

Our theoretical analysis concludes that a partition coefficient as defined by OECD is not applicable to nanoparticles and alternative endpoints are necessary. In words of Praetorius and co-workers: «It is critical to ensure that defined fate descriptors have a well-founded physical or chemical meaning and are not simply artefacts of an experimental design» (Praetorius et al. 2014).

2.3.7 OECD TG 112 (dissociation constants in water) (EPFL, UdL, LEITAT)

OECD TG 112 deals, in theory, with any dissociation constant of a substance, but it is basically used for pKa determination, as solubility by dissociation is determined according to OECD TG 105. Another dissociation reaction constant that would be determined under this guideline, though not described in the text is the dissociation constant of organometallic compounds.

For most of the nanomaterials (oxides, salts) dissociation and solubility is similar. For dissociation determination of oxides, the solubility of the material at different pH is needed. As mentioned in the assessment of OECD TG 105, "Solubility in water" existing methods for solubility measurements are not adapted to nanomaterials. Evidently, the same remarks are valid for the determination of the dissociation constants.

The acidity of the nanomaterial can be referred as in any other chemical compound, but, as in the case of macromolecules such as proteins, the number of acid/basic sites is enormous and other endpoints are more appropriate: surface acidity, surface redox, isoelectric point, etc.

² A colloidal dispersion is a system of particles in the size range of approximately 1 nm to 1 µm dispersed in a continuous phase of a different composition or state. Unlike a solution, a dispersion is composed of more than one phase and is only uniform on a macroscopic scale, but not on a microscopic scale (IUPAC 1997).

It is far more important to determine the capacity of a nanomaterial to dissociate water. Water adsorption and further dissociation of water molecules in contact with the material change at the surface and, so, becomes relevant in nanomaterials due to their high surface vs. volume ratio.

The surface properties and sorption behaviour of metal oxides in aqueous media play an important role in the transport of ions in the environment (Lefèvre et al. 2002). Several types of surface complexation models have been developed (1-pK, 2-pK, multisite complexation model, etc.) in order to calculate the thermodynamic equilibrium constants of the complexes formed between surface functional groups and adsorbed ions. The models assume that equilibrium is achieved at the oxide/water (or electrolyte) interface, which usually means that the hydroxylation of the surface by dissociative adsorption of water molecules is complete. This is the main reason for the “hydration” procedure, which is applied to oxides after synthesis at high temperatures. Another fact is the possibility of transformations leading to oxi-hydroxides or other phases at the surface, reactions which need a few weeks, but the kinetics of this reaction and its consequences on the surface properties have not been thoroughly studied. This complex formation will be longer developed in the OECD TG 108 evaluation.

Another dissociation reaction that would be in the scope of this guideline is the organic coating dissociation from the surface in the case of inorganic nanomaterials coated by organic ligands, i.e. Ag@BSA. As in other guidelines, solubility and dispersibility would need to be determined and a dispersion protocol clearly defined. A method for organic compound in water quantification (HPLC, GC, titration) is necessary.

2.3.8 OECD TG 108 (complex formation in water) (UdL, LEITAT)

2.3.8.1 Considerations on definitions and relevance of the physicochemistry endpoint

Document OECD TG 108 deals with the thermodynamic description (via the determination of stability constants) of the complexation between a **dissolved** ligand (the tested chemical substance, which has to be “soluble” enough) and a metal. In page 1, the category of metal is focused: «This is of outstanding importance in the case of cadmium, copper, cobalt, chromium, lead and zinc». The interest for such complexation stems from the concern that the availability of the heavy metal might be enhanced: «the ability of a new chemical to form soluble metal complexes may increase the availability to food chains» (page 1).

Since the publication of OECD TG 108, there have been many improvements in the polarographic and other electroanalytical techniques in order to determine a stability constant (e.g. SSCP (Town & van Leeuwen 2003; Pinheiro & van Leeuwen 2004), AGNES (Galceran et al. 2004), etc.), while the classical DeFord-Hume method (indicated in the OECD TG 108 report) has been shown to be restricted only to substances that form complexes with a limited adsorption onto the electrode (DeFord & Hume 1951).

In just a few studies (Domingos et al. 2008; Goveia et al. 2011), the binding of heavy metals to nanomaterials has been described as a **complexation process**, i.e.: a result of chemical reactions among the metal ions and a well-defined chemical species to form entities (“complexes”) having a precise stoichiometry. The stability constant of these complexes relates the amount of free sites on the surface of nanomaterials, the amount of occupied sites (bound metal) and the concentration of free metal in solution. Within this formalism, the main “nano” specific feature (for applying dynamic techniques such as SSCP) is the extremely low diffusion coefficient of the metal-nanoparticle “complex”.

However, from a physicochemical point of view, a most accurate description of the interaction phenomenon among trace metals and solids particles (such as those of NMs) should be based on the concept of **adsorption**, i.e.: a process involving the adhesion (through covalent bonds, electrostatic interactions, etc.) of metal ions on the solid surface of NMs. In fact, there is an equivalence between the different approaches (complexation/adsorption) that has been detailed in (Garcés et al. 2002).

Whatever the physical interpretation of this phenomenon is, the central idea is that the formation of new entities (via association of the heavy metal to the NM surface) modifies the availability of a heavy metal in environmental aqueous compartments. It is well known that the huge surface areas resulting from the fine division of the material render surface phenomena specifically relevant (either as a benefit or a potential risk) in NMs. An increased adsorption of heavy metals on nanomaterials (in comparison to the bulk material) has been reported in the literature (Engates & Shipley 2011; Liang et al. 2011; Chen et al. 2010). As a result, the heavy metals could be accumulated on the NMs, transported with them and delivered to organisms in a kind of **“Trojan horse” effect** (Limbach et al. 2007; Grassian 2008; Vale et al. 2014; Goveia et al. 2011). Thus, from a regulatory point of view, there are sound reasons to maintain the focus on risk assessment of NMs concerning their role as “vectors” of heavy metals, even though the key physicochemical phenomenon should be termed adsorption (of trace metals onto NMs) rather than complexation. In the following, we adhere to the terminology of adsorption without any loss of generality.

2.3.8.2 State of the art on adsorption of trace metals on NMs

2.3.8.3 Key aspects in TG 108 concerning applicability to NMs

The following summarizes some of the main aspects of concern regarding the applications of this guideline to NMs, as well as relevant recommendations.

Section 1. Introductory information: The previous knowledge on NM water solubility is particularly relevant (see report on TG 105), since the concomitant release of metal ions may interfere with the test. However, the method is stated to be «applicable only if the water solubility is higher than 10^{-5} M». This is not pertinent to NMs, as in this case the physicochemical endpoint does not refer to soluble metal complexes anymore, but to metal adsorption on solid surfaces. Besides, NM water dispersibility is also crucial for the test as the aggregation state determines available surface (and pores) and, so, real adsorption capacity. This makes necessary to have a robust, reliable and reproducible dispersion protocol. Dispersion protocol effect over the guideline performance will be studied in Deliverable 2.9.

Section 2. Method: The guideline is based on the consensus method of classical polarography, i.e., the direct measurement of the shift in the half-wave potentials of electroactive metal species using dropping-mercury electrodes. We recommend to substitute this method by more modern voltammetric techniques, such as AGNES or SSCP, that allow reaching lower detection limits and a higher robustness with regard to interferences due to electrodic adsorption (which is particularly relevant in the presence of surfactant coatings or stabilizing agents in NM dispersions). The Appendix section of this report contains a summary of a preliminary protocol developed by UdL for the measurement of Zn^{2+} ions in NM dispersions using AGNES (see section 7.2). Further testing of this procedure will be carried out for Deliverable 2.9.

However, due to the large number of publications addressing the use of solid-liquid separation steps and subsequent elemental analysis (e.g. ICP-MS), the consideration of similar methods should also be taken into account for the eventual development of a new Test Guideline for NMs (especially for trace metals such as Cr, Ni, Co, etc. which are not suitable for AGNES).

Section 2. Definitions and units: Interpretation of results in terms of a complexation reaction among well-defined simple chemical species, and determination of a complex formation constant using the current method, based on the DeFord-Hume formalism and the assumption of equal diffusion coefficients for metal ions and complexes, is not considered valid for NM dispersions. It is recommended to use instead a method based on the measurement of the decrease in the concentration of dissolved metal ion, in combination with a mass balance, for the calculation of the amount of metal adsorbed on the NM. The results should be interpreted by means of an adequate adsorption isotherm model, and have into consideration total surface and available/exposed surface. Taking into account that the large-coverage regime is unlikely to be reached in environmentally realistic scenarios, the revised protocol could focus on the low-coverage regime (i.e. Henry's adsorption constant).

Section 2. Reference substances: The current reference substances are obviously not adequate. The assessment and validation of one or more NMs as candidate reference materials is strongly recommended.

Section 2.B. Description of the test procedure: Guidance on the development of a revised experimental protocol can be found in regulatory literature related with the testing of adsorption of chemicals on solids, e.g. in EPA's *Technical Resource Document on Batch-Type Procedures for Estimating Soil Adsorption of Chemicals* (EPA/530/SW-87/006-F, April 1992).

Section 3. Data and reporting: Interpretation of results should be based on convenient adsorption isotherm equations (and, eventually, kinetic models). See also EPA/530/SW-87/006-F document.

2.4 Summary and Conclusions

We are confronted with two different types of endpoints in the analysis of OECD guidelines:

- Physical endpoints (density and granulometry), which need a verification or an actualization of methods in order to be applied to nanomaterials.
- Physicochemical endpoints (partition constants, reaction constants, solubility...), which need a different point of view when working with nanomaterials as they are thermodynamically unstable systems that do not reach the equilibrium and, in the best cases, they can be in a pseudo-equilibrium state.

Conclusions for each guideline are detailed below, though there is one conclusion that is valid for all the guidelines that have been analyzed: solubility is an endpoint that usually has to be known before performing the determination of another physicochemical endpoint; when working with nanomaterials dispersions are

prepared and not solutions, so dispersibility needs to be introduced as a determinant endpoint. We propose a protocol for dispersibility determination, which will be studied and developed in Deliverable 2.9.

2.4.1 OECD TG 109 Density of Liquids and Solids

The true density (also called relative density and specific gravity) measurement **needs a verification** of the drying process of the powders before measurement and of the influence of the evacuation step (adsorbed air which is not replaced by He). A safety assessment of the measurement process is necessary because larger amount of nanopowders are needed.

Poured and tapped density could be determined by the proposed methods and standards, but a change to standards developed for ceramic powder gives a more adapted measurement procedure. Anyway, the flowability of nanopowders and the large amount of dry powders that are needed for the measurement lead to a more complex handling and, so, nano-safety aspects have to be taken in consideration.

2.4.2 OECD TG 110 Granulometry

We conclude that granulometry of NM needs a two-ways approach. Method B described in the OECD TG 110 document would be useful for primary size determination after the necessary revisions (work done in T2.2).

The Method A of OECD TG 110 document requires several revisions to extend the proposed principles into NP granulometry. Text changes have been proposed to address the applicability of NP granulometry to the protocols. It is proposed to include a revised protocol for the CLS photocentrifuge method and another one for the DLS method. In our opinion, similarly expressed by several other documents, the OECD TG 110 document should be revised and updated in terms of the new options for particle measurement, including nanoparticles, for which the method A could be complemented with the CLS and DLS SOPs which we propose to develop. These methods measure the granulometry of a dispersion, so a predispersion protocol needs to be included and dispersibility and solubility determination would be crucial for this end-point determination.

2.4.3 OECD TG 106 sorption-desorption

The OECD guideline 106 is nowadays **not applicable to nanomaterials** as it needs OECD TG 105, which has to be adapted to NM, and an alternative for OECD TG 107/117/123.

Anyway, we consider that **the Guideline may be applied to determine the partition between water and soil of nanomaterials in some cases.** The protocol would need some modifications, principally regarding the water/soil separation step. Additionally methods for characterisation of adsorbed nanomaterials have to be developed. At the moment it seems that eventually radiolabelling and direct metal detection in the soil and/or water could be a solution for some materials.

These protocol and detection modifications will be studied and developed within deliverable 2.9.

2.4.4 OECD TG 105 water solubility

The **OECD TG 105 document requires several revisions** to extend the proposed principles to NM solubility. There is a fundamental **ambiguity in the definition of NM solubility as opposed to dispersibility.** The former is determined by the hydrochemical reactivity of the NMs and the release of ionic species, and describes a fraction of material that is transformed to completely different species with non-nano characteristics. On the other hand, dispersibility is related with the amount of NM remaining in water (while keeping its particulate nature) under certain conditions, which is more in agreement with the original aim of the TG for conventional molecular substances, but in the case of NMs it is usually an ill-defined property (from the point of view of thermodynamics). By keeping the first interpretation of NM solubility in mind, UdL has identified several aspects of the protocol that would require specific modifications due to the distinct features of NMs as compared with conventional chemicals.

The original protocol is addressed to essentially pure substances that are inert in water, which may not necessarily be the case for NMs. For instance, it has been noted that a more **strict control of pH** would be necessary for materials such as ZnO or Ag, whose dissolution behaviour is strongly dependent on this variable. The **presence of dissolved gases** (CO₂, O₂) is also an important factor that should be considered in the protocols (due to its relevance for redox processes and precipitation of concomitant solid phases). Either control and monitoring of atmospheric composition, pH and redox potentials/oxygen concentrations as proposed in new Task 2.3 and 2.4 SOPs may improve data quality considerably. The effect of particle size on solubility is another important issue that is not currently contemplated in the protocol, and comparison with solubility of bulk material analogues depends on the timescale considered for equilibration due to, e.g., Ostwald ripening processes. In some cases, the dissolution kinetics may show a maximum of solubility after

24 to 100 h. Moreover, the fact that the dissolution of NMs (like Ag) can be, in some situations, kinetically slow or hindered by metastable states is not accounted for. **Determination of kinetic dissolution data is therefore recommended.**

It is also explicitly assumed that solubility increases with **temperature**, which is not the case in materials such as ZnO. Other details have been identified as potential key aspects for the successful development of a revised protocol adapted to NMs, in particular the **assessment of remaining solids in the liquid phase after solid/liquid separation step** (in the case of the flask method) or the retention efficiency of NMs in the column and possible clogging problems related with the very small size range of the materials (in the case of the column method). **It is proposed to disregard the column method, due to practical considerations** (difficulties in the filtration of small particles).

Further testing of some of the recommended considerations will be carried out for Deliverable 2.9.

2.4.5 OECD TG TG 115 surface tension

The guideline is **not considered adequate for its application to NMs in its current form**. To allow an application to NMs, a method for calculating an adequate concentration for the samples that should be measured should be developed. It should take the viscosity range of the guideline, the dispersibility and the effects of the volume fraction into consideration.

There are not many models for predicting the viscosity of an aqueous dispersion of nanomaterials, but as the range of the measure is quite wide, this should not be a problem.

Dispersibility will depend on the method used to disperse the nanomaterials in the distilled water, so that the process could be modified for those which do not disperse well, avoiding changes to the concentration for some particular nanomaterial dispersions. The effects of dispersibility and concentration (volume fraction) on the surface tension have already to be accurately studied.

Although some critic parameters have been identified, we think this Guideline can be adapted to be used for nanomaterials.

2.4.6 OECD TG 107/117/123 N-Octanol-water partition coefficient

The OECD Guidelines are not applicable to nanomaterial suspension. The guideline has to be replaced by a determination of the colloidal stability of the nanomaterials in different liquids (or another parameter to measure their affinity to different matrices). This would be more useful and reliable for an estimation of the behaviour of nanomaterials in the environment.

These conclusions are concordant with the last OECD revisions: «The group concluded that the octanol-water partition coefficient should not be assessed for nanomaterials. The new test methods that need to be designed instead, considering other kinetic and surface chemical effects, are not yet sufficiently developed for standardisation or for becoming OECD TGs» (OECD 2014).

2.4.7 OECD TG 112 dissociation constant in water

The guideline is **not considered adequate for its application to NMs in its current form**. With some modifications, it could be interesting in specific cases, such as organic coating dissociation from inorganic nanomaterials.

For regulative purposes, it would be more important to develop methods and standards to determine the surface properties like dissociation of water and hydration, surface acidity and isoelectric point. In combination with OECD Guideline 105, an important characterisation of nanoparticles could be performed.

2.4.8 OECD TG 108 complex formation in water

The polarographic method described in OECD TG 108 ("Complex formation ability in water") is **not considered adequate for its application to NMs in its current form**. However, the assessment of the capacity of NMs to act as vector of toxic metals is of great interest from a regulatory point of view, which justifies the relevance of this endpoint. Therefore, a revision of this guideline is recommended to focus on the **adsorption of trace metals on NMs**. Notice that the testing metal of choice should not be a constituent of the NM, or else TG 105 (solubility and release of metals) should apply. Thermodynamic information to be reported in the revised OECD TG 108 (given by the description of the isotherm) could be complemented with kinetic data. For the ecotoxicity risk assessment of NMs as vectors of toxic metals, complementary experiments could be suggested in synthetic aqueous media (sea, estuarine and freshwater) with added humic acid up to representative environmental concentrations. A judicious selection of pH, ionic strengths and temperature should be exercised.

3 Deviations from the work plan

As no OECD Guideline has been found directly applicable to nanomaterials (from the ones that have been analyzed) all the experimental work will be reported in Deliverable 2.9.

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5 Annexes

5.1 SOP for Dispersibility determination

The dispersibility of a nanopowder is of crucial relevance for other endpoints determination and standardized protocols, at the same level that solubility is determinant for traditional chemicals. Dispersibility results crucial for the determination of endpoints such as octanol-water partition coefficient, complex formation in water or sorption-desorption and, so, affecting fate, bioaccumulation, biodistribution, dosimetry, etcetera.

5.1.1 Definitions

Dispersion (Hackley & Ferraris 2001)

In general, a two-phase system in which discontinuities of any kind (solid, liquid, gas) are dispersed in a *continuous phase* of a different composition or state; more specifically in the field of material science and nanomaterials, the term dispersion is used to describe a *suspension* of solid *particles* in a liquid medium. In the following we will discuss only “suspensions”. The term suspension is used for all type and size of particles as dispersed phase.

The term *sol* in which the dispersed phase is <1 micrometer (colloid) and the *dispersion* medium is a liquid is not used. Additionally the term is also used in in the case of a sol-gel process where the sol is a liquid with dissolved molecules.

Stability of a Suspension (colloidal stability)

Suspensions that do not aggregate at a significant rate are said to be colloidal stable. Colloidal stability is a form of kinetic stability, and it is therefore considered a metastable thermodynamic state. A suspension may exist for an appreciable length of time and therefore exhibit kinetic stability. A suspension that has sufficient kinetic stability to prevent the occurrence of significant aggregation as measured over a relevant time frame. Stability may be ascertained by suitable experimental means, such as particle size, turbidity or sedimentation measurements as function of time.

Dispersibility

The dispersibility of a powder could be defined by the particle size (mean and distribution) in a defined liquid after the particles has underwent a dispersion process. The dispersion process has to be done by an ultrasound treatment.

5.1.2 Existing methods

Although the term “Dispersibility” is not well defined, good dispersibility means the rapid formation of a dispersion. The characteristic of a good dispersible powder is a high degree of dispersion (particle size in the dispersion is as near as possible to the primary particle size) and a high rate (low energy input) at which the powder can be dispersed. Several methods were developed for the determination of the dispersibility, but mostly for particles in the size range of micro-meter and larger. Also the existing standards are developed for such powders, like Standard Test Method for Percent Dispersibility (ASTM 2008). This test method is used to determine the percent dispersibility of dry pesticide formulations and is not applicable for our needs. Similar for ISO 8780-1 «Pigments and extenders — Methods of dispersion for assessment of dispersion characteristics», which describes the dispersibility of pigments larger than 1 µm (ISO 1990).

5.1.3 Determination of the “Dispersibility of Nanopowders”

The dispersibility could be expressed as the relative size D_{BET}/D as function of the applied dispersion energy; where D is D_{50} measured by DLS, D_{BET} the particle size calculated from the specific surface.

For the measurement of the dispersibility, the procedure of ENPRA «The ENPRA dispersion protocol for NANoREG», Version 1.0; Date: 11 July, 2014, including the calibration process as described in NANoREG SOP for probe-sonicator calibration of delivered acoustic power and de-agglomeration efficiency for *in vitro* and *in vivo* toxicological testing gives a very useful base. The size and size distribution measurement has to be carried out following the «NANoREG SOP for measurement of hydrodynamic Size-Distribution and Dispersion Stability by Dynamic Light Scattering (DLS)». Combining these methods and carrying out the powder dispersion at different amount of energy inputs will allow a determination of the powder behaviour during the formation of the dispersion process. In contrast to the dispersion method of NANoREG, the aim of this procedure is not an optimal or reproducible dispersion but the characterisation of the powder behaviour during the dispersion process.

The use of DBET is justified by the observation that the particle size calculated from the result of the measurement of the specific surface is not influenced by the degree of agglomeration and the value corresponds therefore to the primary particle size.

Following results are expected:

- Dispersibility is $\ll 1$ if no change of the mean particle size during ultrasonic treatment is observed
- Dispersibility is 1 if particle size after ultrasonic treatment is D_{BET}

It is also interesting to determine the slope of the change of the dispersibility with the energy applied (or specific energy in the case different volume and concentration are used) by ultrasound treatment (dD/dE) and the Energy input at the maximum of the dispersibility.

It is evident that parameters like type of solvent, pH (Zeta potential), concentration of surfactants and ionic strengths have to be known and must be mentioned in the measuring protocol.

5.2 SOP for CLS photocentrifuge method

TITLE QUALITYNANO DCS SOP – PARTICLE SIZE DISTRIBUTION FOR SILICA NANOPARTICLES

Authors: O. Lozano, J. Mejia, S. Lucas; University of Namur (UNAMUR)
M. Monopoli; University College Dublin (UCD)
S. Mullens, I. Nelissen; Flemish Institute for Technological Research (VITO)
S. Pattel, S. Lawson, University of Leeds (ULEEDS)

DCS RR Leader: S. Lucas

Version: 1.2

1 OBJECTIVE AND SCOPE

1.1 Objective

To develop a Standard Operating Procedure (SOP) for Differential Centrifugal Sedimentation (DCS) on the determination of the particle size distribution (PSD) of dispersed Silica nanoparticles.

1.2 Scope

This SOP was developed for an Inter-Laboratory Comparison (ILC) of silica nanoparticle dispersions measured by DCS under the framework of the EC FP7 project QualityNano, in WP2 – NA2: Nanomaterials Hub & Round Robin assessment. This SOP applies only for disk centrifuge systems from CPS Instruments. Silica nanoparticle dispersions will be provided from the QualityNano consortium.

2 ABBREVIATIONS

DCS : Differential Centrifugal Sedimentation
EC : European Commission
Nanomaterial : A material with at least one dimension in the range of 1 to 100 nm
NM(s) : Nanomaterials(s)
NP(s) : Nanoparticle(s)
PSD : Particle Size Distribution
ILC : Inter-Laboratory Comparison
SOP : Standard Operating Procedure

3 PRINCIPLE

Differential Centrifugal Sedimentation (DCS), also known as Centrifugal Liquid Sedimentation (CLS), is a centrifugal method based on the settling rate of particles in a liquid under a centrifugal field. At low Reynolds numbers the particle size in relation to the settling velocity is dictated by Stokes' law. The Stokes diameter D

is determined as a function of time t:

$$D = \sqrt{\frac{18\eta \ln(R_f / R_0)}{(\rho_p - \rho_f)\omega^2 t}} \quad (\text{Eq. 1})$$

where η is the fluid viscosity, R_f the measurement radius, R_0 the starting radius, ρ_p the particle density, ρ_f the fluid density, and ω the rotational speed. All these parameters are constants for a specific measurement.

4 METHOD

This is an overview of the measurement method: A spinning disk is set at a specific velocity ω . A sucrose gradient is prepared to stabilize the sedimentation. Dodecane is added after the gradient as a buffer layer to prevent streaming, ensuring the injected (nano)particle dispersions a smooth transition into the gradient and laminar flow. A procedure is selected to measure the (nano)particles. A certified calibration standard is used to determine the correct diameter-time relation. Finally, the sample is injected and measured. The concentration of particles in the injected volume should ideally be < 0.25 % (m/v). The measured size range is variable and depends on the speed of the disk and the used gradient, ranging from 5 nm to 40 μm . Other factors, like the difference in particle-fluid density, can affect the minimum measurable size.

5 EQUIPMENT

A. DCS system (from CPS Instruments Inc.)

B. Syringes and needles (Syringes: 1mL Injekt-F sterile for sample and dodecane injection, BD 3mL syringe for sucrose injection. Needles: BN2015 20 ga for both syringes. A 50 mL syringe and a flexible plastic tube are needed for taking out the sucrose gradient when cleaning the spinning disk.)

C. Deionized water.

D. Sucrose (CAS Number 57-50-1).

E. Dodecane (CAS Number 112-40-3).

F. Calibration standard (Certified PVC microparticles provided by CPS Instruments).

G. Graduated containers, i.e. glass beakers or falkon tubes.

H. Digital weight balance.

I. Thermometer.

J. Soft tissues (e.g., Tork premium soft tissue).

K. Silica NP dispersion, nominal diameter: 100 nm, concentration: 1 $\text{mg}\cdot\text{mL}^{-1}$.

L. Quality Control, PVC dispersion.

6 SAFETY PRECAUTIONS

6.1 Operator

Always operate and make preparations with a lab coat and gloves. Work on a chemical hood or equivalent ventilation protection environment for any dispersion preparation using a powder NM (dry) or volatile substances.

6.2 Instrument

The DCS system must not be used/cleaned with acetone or chlorinated solvents.

7 STANDARD OPERATING PROCEDURE

7.1 Preparing the sucrose solution

8 % and 24 % (m/m) sucrose solutions will be prepared. Two graduated containers properly identified as 8 % sucrose and 24 % sucrose are required. Preparation:

1. 8 % sucrose: Place the container on the microbalance, making sure it is offset at zero with the container on it. Then add 2 g of sucrose followed by deionized water until a weight of 25 g is obtained.
2. 24 % sucrose: Place the container on the microbalance, making sure it is offset at zero with the container on it. Then add 6 g of sucrose followed by deionized water until a weight of 25 g is obtained.

Agitate each container gently until all the sucrose is dissolved.

7.2 Starting the DCS equipment, injection of gradient and dodecane

The equipment should be ON, indicated by the red light ON in the right side of the front panel. If the laser has been off, turn it on (black button on the back of the instrument) and the red light ON (right side of the front panel) should appear. No measurement should be performed until the laser has been ON for at least 1 hour.

Place the plastic cap (attached to the cap screwdriver) into the disk, make sure it is well pressed to the disk. The cap has a small hole marker which should be aligned with a similar hole marker made on the disk. Ensure the disk is clean, if not use a soft tissue and water or ethanol to clean the transparent side of the disk (front and back). Be careful not to touch the laser diode. Close the CPS lid.

In the computer run the CPSV95 executable file (or the name of the executable file that controls the CPS equipment). In the Set Point Control section select the option 'Manual' and set the rotation speed to 18000 rpm.

The sucrose gradient is injected with a series of sucrose mixtures:

Order	Sucrose 24 %	Sucrose 8 %	Total volume
	mL		
1	1.6	0	1.6
2	1.4	0.2	1.6
3	1.2	0.4	1.6
4	1.0	0.6	1.6
5	0.8	0.8	1.6
6	0.6	1.0	1.6
7	0.4	1.2	1.6
8	0.2	1.4	1.6
9	0	1.6	1.6

Table S1. Sucrose mixtures (24 and 8 %) and order for building the sucrose gradient in the disk.

Use a BD 3mL syringe to inject the sucrose mixtures. With each mixture in the syringe, shake the syringe horizontally at least 10 times to ensure a proper mixture. As a first step, inject the first gradient volume (no. 1) to the disk, and then press START to begin the disk rotation. Afterwards, when the disk reaches the set rotation speed (18000 rpm) inject the subsequent gradient volumes (no. 2-9). The sucrose gradient is built by injecting progressively to the disk (i.e. starting with 1.6 mL from 24 % sucrose). Each time a total volume of 1.6 mL is injected.

Use a new BD 3mL syringe to inject 0.5 mL of dodecane. Allow 30 minutes for the dodecane and gradient to stabilize.

7.3 Defining a sample procedure

The procedure contains the physical parameters of the particle, the calibration to be used and the gradient. To define a procedure click on the button 'Procedure Definition' from the main menu, opening a window as shown in Figure 1. In 'New Name' enter the name 'SIO2_QNANO'. For the windows 'Sample parameters', 'Calibration Standard Parameters', and 'Fluid Parameters', use the values shown in Table 2. Left unchanged the parameters from the window 'Presentation Parameters'. Click on 'Save and Exit'.

Note: The physical sample parameters (density, refraction index and absorption) are values either taken from the literature (bulk material) or provided by the material supplier.

Procedure	SiO ₂
Sample Parameters	
Maximum diameter (µm)	10.0
Minimum diameter (µm)	0.03
Particle density (g/mL)	2.31
Particle refraction index	1.46
Particle absorption (K)	0.001
Non-sphericity factor	1
Calibration Standard Parameters	

Peak diameter (µm)	0.226 *
Half Height Peak Width (µm)	0.1 *
Particle density (g/mL)	1.385 *
Fluid Parameters	
Fluid density (g/mL)	1.064
Fluid refractive index	1.349
Fluid viscosity (cps)	1.3
Presentation parameters	
Display Mode	Weight
Display Curves	Differential
Y-axis Scaling/Normalization	Height
Show Grids	Horizontal and Vertical
Peak Detection Sensitivity	05 Height 10 Window
Display Operating Data	Yes
Distribution Table	None
X-axis Scale	Log

Table S2. Parameters to be input for Silica measurement. * Calibration Standard Parameters should correspond to the calibration standard used at each lab.

In 'New Name' enter the name 'Silica NPs'. Click on 'Save and Exit'.

The screenshot shows the 'Procedure Definition' window with the following sections and values:

- General Info:** Current Procedure: SIO2_QNANO, New Name: SIO2_QNANO, Date: 2/17/2014, Time: 11:47:02 AM.
- Buttons:** Save and Exit, Exit Without Saving.
- Options:** Make this the default procedure. Estimated Runtime: 1,851.9 Min. Maximum Recommended Disc Speed: 1767 RPM.
- Sample Parameters:**
 - Maximum Diameter: 10.0 microns
 - Minimum Diameter: 0.03 microns
 - Particle Density: 2.31 g/ml
 - Particle Refractive Index: 1.46
 - Particle Absorption: 0.001 K
 - Non-Sphericity Factor: 1
- Calibration Standard Parameters:**
 - Peak Diameter: 0.226 microns
 - Half Height Peak Width: 0.1 microns
 - Particle Density: 1.385 g/ml
- Fluid Parameters:**
 - Fluid Density: 1.064 g/ml
 - Fluid Refractive Index: 1.349
 - Fluid Viscosity: 1.3 cps
- Presentation Parameters:**
 - Display Mode: Weight, Surface, Number, Absorption
 - Display Curves: Differential, Integral, Both
 - Y-Axis Scaling / Normalization: Height, Area, Fixed, Manual
 - Show Grids: Horizontal, Vertical
 - Peak Detection Sensitivity: 05 Height, 10 Window
 - Display Operating Data: Yes, No
 - Distribution Table: None, Fine, Coarse, Custom
 - X-axis Scale: Log, Linear

Figure S1. Procedure definition window. It contains the parameters used for Silica measurements. Note that the parameters for the 'Calibration Standard Parameters' subsection depend on the specific calibration standard being used at each laboratory.

7.4 Measurements

7.4.1 Selecting the procedure

Click on the button 'Choose Procedure'. Select the procedure corresponding to the material and then click on the button 'Change to Selected Procedure'.

7.4.2 Measurements

Notes: 1) All volumes of silica are 0.2 mL, all volumes of the calibration standard are 0.1 mL.

2) All measured peaks should have their intensity higher than 10 % in the blue line (see Figure 2).

1. Click on the button 'Operate Analyzer' from the main menu.
2. Type on the 'Sample' line: **Silica s1**. Click ENTER.
 - a. The software will ask for the calibration standard: Take 0.1 mL of the calibration standard with a syringe (with needle). Place the syringe in the orifice for sample injection. At the same time press the 'space bar' on the keyboard and inject the calibration standard.
 - b. When the measurement is completed, take 0.2 mL of Silica with a syringe (with needle). Place the syringe in the orifice for sample injection. At the same time press the 'space bar' on the keyboard and inject the calibration standard.
3. Type on the 'Sample' line: **Silica s2**. Click ENTER.
 - a. The software will ask for the calibration standard: Take 0.1 mL of the calibration standard with a syringe (with needle). Place the syringe in the orifice for sample injection. At the same time press the 'space bar' on the keyboard and inject the calibration standard.
 - b. When the measurement is completed, take 0.2 mL of Silica with a syringe (with needle). Place the syringe in the orifice for sample injection. At the same time press the 'space bar' on the keyboard and inject the calibration standard.
4. Type on the 'Sample' line: **Silica s3**. Click ENTER.
 - a. The software will ask for the calibration standard: Take 0.1 mL of the calibration standard with a syringe (with needle). Place the syringe in the orifice for sample injection. At the same time press the 'space bar' on the keyboard and inject the calibration standard.
 - b. When the measurement is completed, take 0.2 mL of Silica with a syringe (with needle). Place the syringe in the orifice for sample injection. At the same time press the 'space bar' on the keyboard and inject the calibration standard.

Repeat this procedure on 3 consecutive days, performing all the measurements by the same operator.

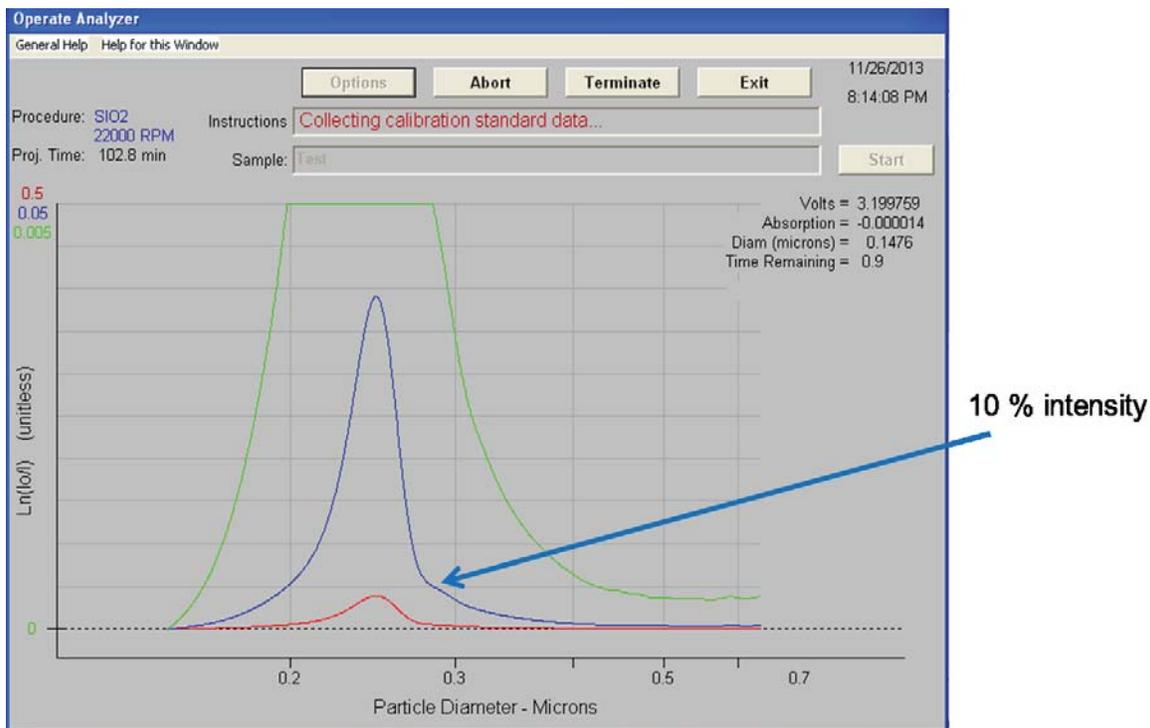


Figure S2. Curve of the calibration standard. A good calibration is obtained when the blue curve is above the 10 % intensity level (first line from bottom-up, see arrow).

7.5 Cleaning the disk after use

This procedure initiates after the disk is stopped (Go to the main menu and press STOP). After the disk stops rotating, open the lid. With the 20 mL syringe insert the attached plastic tube and collect part of the sucrose gradient. Throw it in a container (i.e. glass or plastic beaker). Open the cap of the disk, clean it carefully with the soft tissue (e.g., Tork premium soft tissue). Continue with the syringe collecting the rest of the sucrose gradient. Add a few millilitres of deionized water or ethanol, rotate a little bit the disk, and extract the water with the syringe. Use soft tissue to clean and dry the inside of the disk.

Make special attention to the extreme end of the disc (where the rubber seal is placed) because the measured particles sediment there and it may take a few cleaning cycles with the soft tissue and deionized water/ethanol until most of the particles are removed.

7.6 Determining the Particle Size Distribution

From the main menu click on the 'Retrieve Distribution' button and then click on the 'Choose Procedure' button. Select the procedure under which the measurements were done, and click on the 'OK' button. This will make the software go to the window of the procedure containing all the files measured under that procedure. Select the filename of the measured sample and click on the 'View Files' button.

Select the display weight mode. The PSD is determined from this mode (see references). A two-column table is automatically displayed with columns named Peaks and Half-Width.

7.7 Reporting the data

The Peaks and Half-Width of the each sample should be reported in an excel file, clearly identifying sample name and parameters. The highest peak should be reported as the major peak, and any other peaks as minor peaks. If a peak appears only in some measurements, report that as an anomaly. In all cases determined peak and half-width should be reported.

This SOP uses in part references from:

1. IRMM Test protocol for the characterization of the particle size of colloidal silica candidate reference materials with Centrifugal Liquid Sedimentation (CLS)
2. JRC Interlaboratory comparison of methods for the measurement of particle size, effective particle density and zeta potential of silica nanoparticles in an aqueous solution

3. ISO 13318-1 Determination of particle size distribution by centrifugal sedimentation methods – Part 1: General principles and guidelines
4. ISO 13318-2 Determination of particle size distribution by centrifugal sedimentation methods – Part 2: Photocentrifuge method

5.3 SOP for AGNES measurements of trace metal ions in NM dispersions

TITLE

1.1.1.1.1.1. PRELIMINARY PROTOCOL FOR THE

Authors: Calin A. David, Josep Galceran, Carlos Rey-Castro, Jaume Puy, Encarnació Companys, José Salvador, and Josep Monné (University of Lleida, Spain)

Date: 17th September 2013

Version: 1.0

Notes:

The present protocol has been developed for the measurement of Zn²⁺ ions in dispersions of ZnO NMs, and it has been tested in several recent publications (Galceran et al. 2014; Adam et al. 2014; David et al. 2012; Mu et al. 2014). The extension of this procedure for the measurement of Cd²⁺ and Pb²⁺ ions in dispersions of other NMs is currently ongoing. It must be pointed out that AGNES is suitable for the measurement of a limited number of metal ions only (mainly: Zn²⁺, Pb²⁺, Cd²⁺, Cu²⁺, In³⁺, Tl⁺, Sn²⁺).

Summary:

AGNES is an electroanalytical technique consisting of two stages (Galceran et al. 2014; Galceran et al. 2004). The goal of the first stage is to achieve a special situation of equilibrium. The simplest potential program for the first stage consists in a deposition potential E_1 applied for a long enough time (t_1) to reduce the analyte metal into the mercury electrode until Nernstian equilibrium conditions. This E_1 prescribes a preconcentration factor or gain $Y=Y_1$ between $[Zn^0]$ in the amalgam and $[Zn^{2+}]$ in the solution. At the end of this first stage, a situation of no concentration gradient at either side of the electrode surface has also to be attained. The goal of the second stage is to quantify the reduced metal accumulated along the first stage. A simple implementation of the second stage is the application of a reoxidation potential E_2 under diffusion limited conditions. The free metal ion concentration in solution can be obtained from the faradaic current I , which results from the subtraction of a suitable blank to the measured current at a fixed time t_2 , using

$$[Zn^{2+}] = \frac{I}{Y\eta} \quad (1)$$

The proportionality factor η can be obtained from a calibration.

AGNES can be performed using standard non-expensive polarographic equipment that is widely available. UdL has developed this AGNES protocol with an Eco Chemie Autolab PGSTAT12 potentiostat attached to a Metrohm 663VA stand. The working electrode was a Metrohm multimode mercury drop electrode (selected drop radius around $r_0 = 1.41 \times 10^{-4}$ m). The auxiliary electrode was a glassy carbon electrode and the reference electrode was Ag/AgCl/3 mol L⁻¹ KCl, encased in a jacket containing 0.1 mol L⁻¹ KCl or KNO₃. A blanket of previously water-saturated N₂ flows continuously through the voltammetric cell of the stand to avoid O₂ interferences. The flow of N₂ was regulated and decreased in order to avoid evaporation in long term experiments.

Concentrated stock suspensions of the nanoparticle powders use previously boiled MilliQ water without any additive or background electrolyte and are sonicated for 30 min (Branson 3210, 100 W) to yield 1 to 4g/L ZnO concentrations. The preparation and conservation of stock solutions is carried out under nitrogen atmosphere to avoid CO₂ fixation. The solubility experiments are carried out by adding a suitable aliquot of this concentrated NP stock to a voltammetric cell containing the previously deoxygenated and buffered test medium under N₂ atmosphere. The final particle concentration in the experiments can be 50-100 mg ZnO/L, depending on pH. The test media can be prepared with KCl or KNO₃ and Tris (tris(hydroxymethyl)aminomethane) or MOPS (3-(N-morpholino) propanesulfonic acid) pH buffers. Thermodynamic properties discard any influence of the buffer on the values of $[Zn^{2+}]$ measured. The total ionic strength can be fixed at 0.1 M (or any other value), with a suitable buffer concentration. HCl and KOH can be used to adjust the pH. To obtain a homogeneous dispersion in each experiment, the ZnO-NP stock dispersion is sonicated for 10 min just before the addition to the buffer solution. Subsequent to the

thermodynamic or kinetic experiment, the diluted ZnO nanoparticle solutions can be standardized (i.e. via determination of total zinc) by applying AGNES after acidification of the solution.

Due to its large influence on ZnO solubility, the pH of the dispersion has to be continuously controlled during the experiments using a combination glass electrode (Orion 9103) inserted in the voltammetric cell, and attached to a Thermo Orion 720A ion analyzer. The glass electrode has to be previously calibrated using commercial pH standards. It is recommended to use a glass jacketed cell thermostated at 25.0 ± 0.1 °C in all measurements.

Some ZnO dispersions may induce adsorption phenomena that lead to a falling of the Hg drop (at pH>8.8). These phenomena can be avoided using lower total ZnO NP concentrations (50 mg/L), occasional cleaning of the capillary and shorter deposition times [2].

The application of AGNES requires the selection of the appropriate parameters (gains and times). For Y_1 ranging from 0.01 to 10, equilibrium can be reached in a deposition time t_1 less than 100 s (the general rule of thumb is $t_1 = 7 \times Y_1$) using $Y_2 = 10^{-8}$. In order to remove other contributions different from the faradaic one, one can subtract the "shifted" blank, which consists of applying (to the solution containing the metal) a potential program shifted to a range in which there is no analyte deposition. A discussion on possible strategies to avoid interferences due to dissolved surfactants derived from stabilizing NM coatings can be found in (Galceran et al. 2014).

This protocol has been validated by comparison with ICP-OES measurements and thermodynamic speciation calculations (Visual Minteq).

Forthcoming protocol developments:

Ongoing work is aimed at developing this SOP for the measurement of other metal ions (Cd^{2+} , Pb^{2+}) in dispersions of different NMs, and its validation by comparison with other techniques such as ISE potentiometry, ICP-MS (after ultrafiltration or centrifugation) and DGT. Special attention will be devoted to the hypothetical influence of the presence of organic coatings on the NMs.

5.4 Protocol for Sensor Dish Reader (SDR) analyses of MN hydrochemical reactivity and biodurability

Source:

Jensen KA, Kembouche Y., and Nielsen SH, 2013. Deliverable 4.7: Hydrochemical reactivity, solubility, and biodurability of NANOGENOTOX nanomaterials. Edited by Jensen K.A. and Thieret N., March 2013, 60 pp. http://www.nanogenotox.eu/files/PDF/Deliverables/d4.7_hydrochemical_reactivity_and_biodurability.doc#_Toc351405986

5.4.1 Chemicals and Equipment

- Gamble's solution = GS. (see Table B.1)
- Revco RCO3000T-9-VBC Cell Incubator (5% CO₂; 37C)
- Vortex system (IKA VIBRAMIX-VXR)
- Sensor Dish Reader 305 and 315 (PreSens Precision Sensing GmbH, Germany)
- 1 Oxydish[®] OD24 for O₂ measurements (PreSens Precision Sensing GmbH, Germany)
- 1 Hydrodish[®] HD24 for pH measurements (PreSens Precision Sensing GmbH, Germany)
- Software: SDR_v38.exe (methods and data-logging)
- Computer for the SDR_v38.exe software
- 20,000 RCF Centrifuge (Ole Dich)

The specific equipments listed above are not mandatory, but similar equipment is required

For all preparations, verified elementally clean Nanopure water is used as the source of water. Elemental water analysis is needed to ensure purity of water.

Specifications of Nanopure Diamond UV water:

Purification system is designed to 4 stages of de-ionization combined with UV light-treatment:

Resistivity: ≤ 18.2 MΩ-cm at 25°C

Pyrogens: < 0.001 EU/ml

Total Organic Carbon: < 3.0 ppb

Other: nuclease-free (RNase and DNase).

Filter: 0.2 μm filter (γ-irradiated Barnstead D3750 Hollow fibre filter)

5.4.2 Solubility and hydrochemical reactivity screening using the SDR system

The SDR system can only be used for monitoring the pH reactivity and O₂ variation in NPs dispersions with a pH range between 5 and 9. Real-time monitoring is done using the SDR incubation dishes mounted with sensors for online pH and O₂ concentration measurements. Relevant test conditions are maintained by placing the whole system in a cell incubation oven with a well-controlled atmosphere (37°C and 5% CO₂ air supply).

5.4.3 Protocol

The measurements are made in order to measure the chemical reactivity of the particles in hydrous media (e.g., NM dispersion media, synthetic lung lining fluid, and cell media). The end-points are pH and oxygen concentration (O₂). The tests are run as duplicate exposures in three doses (0.08, 0.16 and 0.32 mg/ml) for each substance. The experiments run 24 hours in 24-well trays with internal sensors.

1) Dispense proportionate volumes of media (GS)[£] in SDR pH (white sensor) and O₂ (orange-red sensor) plates

- Use well A1 for the highest dose 0.32mg/ml
- Level A: 1.872 mL; series B: 1.936 ml; range C: 1.968 and series D: 2 ml media(GS)
- The last well is always 2 ml of pure media control

2) Place the SDR plates on the respective readers (carefully ensure that the SDR plates are placed on the correct readers). Check that they are in the right position and make three point measurements using the program "single measurement" command. - This is done to confirm that the sensors are working and also establish a general 0-reference.

- Press 3 times the "single measurement" with Tab enabled reader 305
- Press 3 times on "single measurement" with Tab enabled reader 315).

3) Dispense the appropriate doses into the wells of pH and O₂ plates

- Start with the highest dose of the first test material in well A1
- Row 1: 0.128 mL; Row 2: 0.064 mL; range 3: 0.032 ml.

4) Place the pH and O₂ SDR plates on SDS read and check that they are in the right position

5) Start measurement by pressing the "Start Measurement"

6) After 1 hour the sample rate changed to 15 min

Set a measurement on pause by pressing "Start / Stop"

- Change the sample rate to 15 min
- Accept that this change sættings for all readers - click "yes"
Start the measurement again

7) After a total of 24 hours, end the measurement.

- stop data recording
- stop the vortes shaker
- taken the SDR plates out for sampling of the liquids filtration and centrifugation

£ Gamble's solution adjusted to pH 7.4 .

5.4.4 *Filtration and centrifugation*

The samples are filtered and centrifuged for subsequent chemical analysis .

1) The sample for the highest dose and the reference medium is sampled by syringe and filtered through a 0.2µm CAMECA filter syringe. To obtain a duplicate, the high-dose sample from both the pH and O₂ is sampled (allows 4 mL for each material in duplicate).

- The sample is taken by inserting the tip of the syringe down to the bottom of the well and then the liquid is pumped in and out until the sample is well suspended.
- Then all the liquid is sampled (there may be a little trace of liquid left in the edge of the well, which cannot be sampled)

2) Filter the sample through a 0.2µm CAMECA filter and lead the filtrate into an acid-washed collected tube. This should produce almost 3.5 ml of liquid per sample.

3) Sample 2 x 1.5 ml of each filtered sample by pipette and enter it into 2 ml acid-washed Eppendorf tubes.

4) Centrifuge the filtered samples in the Eppendorf tubes for 60 minutes at 20,000 G using an Ole Dich centrifuge set to slow de-acceleration and 25°C.

- Place the particle samples in the outer ring, which is yield highest RCF.

- Place the media controls in the inner ring, which yield lower RCF.

5) After centrifugation, sample the upper 1.25 ml of the fluid from each pair of the centrifuge tube by pipette, and pool these into a 5-10 ml acid washed centrifuge tube. This solution is used for chemical analysis. The pipetted volume is added an additional 1 ml of 2% ultra-pure HNO₃ in nanopure water to prevent reprecipitation.

6) The liquid samples are stored in darkness at room temperature until analysed.

5.4.5 *Test mediums (modified from reference):*

Examples of test mediums used for the 24-hour reactivity and dissolution studies could be 0.05% BSA as defined in the generic NANOGENOTOX dispersion report, the EURIMA low-Ca Gambles solution tabulated below (Table S1), and Caco 2 cell medium.

The Gamble's solution (Table S1) is the low-Ca solution given by Sebastian et al. (2005). The chemicals are dissolved in 2 L Nanopure water (Nanopure Diamond UV) and placed in an ultrasonic bath for 15 min. Thereafter, the solution is filtered through a 0.45µm sterile filter. The final solution is added 6ml of 4 % formaldehyde per 2 L Gamble's solution to avoid growth and placed in darkness in the refrigerator.

Table S1: Composition of the EURIMA low-Ca Gamble's solution

Compounds	Concentration [mg/ml]
NaCl	6600
NaHCO ₃	2703
CaCl ₂	22
Na ₂ HPO ₄ ·12H ₂ O	358
Na ₂ SO ₄	79
MgCl ₂ ·6H ₂ O	212
H ₂ NCH ₂ CO ₂ H (glycerine)	118
Na ₂ -citrate·2H ₂ O	153
Na ₃ -citrate·2H ₂ O	180
Na-pyrovate	172
Na-Lactate	175
HCl (1:1) [€]	1.1 – 1.5

[€]Used to adjust pH to 7.4 prior to testing

5.4.6 *Comments on use and applicability (modified from reference)*

The method is currently under establishment. There is no previous similar method. Therefore comparison and validation of measurement data are based on comparisons with conventional potentiometric measurement techniques for pH and redox potential.

Test conditions are currently mimicking in vitro testing, but in principle other conditions can be tested as well as well as different durations of testing. Longer test durations may be required to investigate potential alteration of low to very-low soluble NM.

Improved procedures for particle filtration before analysis of the solvent needs to be considered.

5.5 Protocol for hydrochemical reactivity and biodurability testing using an Atmosphere-Temperature-pH-controlled Stirred Batch-Reactor

Source:

Jensen K.A., Clausen P.A., Birkedal R., Kembouche Y., Christiansen E., Levin M., Koponen I.K., Jacobsen N.R., Wallin, H., de Temmerman P.-J., Mast, J., Guiot, C., Spalla, O., Motzkus, C., Shivachev, B., Rousset, D., Bau, S., and Witschger, O., 2011. – Deliverable 2: Standard operating procedures for characterization of the selected manufactured nanomaterials types. Edited by Jensen K.A. and Thieret N, June 2011, 80 pp. http://www.nanogenotox.eu/files/PDF/Deliverables/nanogenotox%20deliverable%20wp4_%20sops%20report.pdf

5.5.1 *General description*

This method has been developed to investigate the short-term biodurability and hydrochemical reactivity (acidity and redox activity and particle agglomeration/dissolution/precipitation dynamics) of NM and dissolved chemicals when incubated with synthetic lung-lining fluids under highly controlled pH-levels and atmospheric composition. The pH-reactivity is quantified by the amount of titrant required to maintain pH 7.4 in the slight CO₂-enriched test atmosphere using a titrator for pH-control. The redox activity is measured by online using a redox potential electrode and is used as an integral measure for oxidative/reductive capacity of the particle. Finally, the average zeta-size is monitored on-line using flow-cell dynamic light scattering. This enables analysis of agglomeration/deagglomeration as well as dissolution/precipitation phenomena. The system can also be used for longer term tests, which requires a small modification of the instrumental SOPs.

5.5.2 *Chemicals and Equipment*

- Synthetic lung-lining fluid: Low-Ca Gambles solution = gs. (see Table 8.1 for composition)
- Atmosphere: Bubbled 3.75% CO₂ og 96.25% HEPA-filtered cleaned air
- Initial pH 7.4 adjustment agents: 7.7 M HCl (Analytical grade)
- pH-titrant: 1 M NaOH (Analytical grade)

- pH 7.4 calibration pH-buffer (e.g, IUPAC pH 7.413)
- pH 4.0 calibration pH-buffer (e.g, IUPAC pH 4.005)
- Redox buffers (e.g, Hamilton (DURACELL) +271 and +574 mV)
- Thermostatic water-bath (Gant W28) for controlling reactor temperature at: 37.4°C
- Gas-mixing and humidifier control unit
- pH electrode: PHC2401 (Radiometer Inc.)
- E_h electrode: MC3051 PT-9 (Radiometer Inc.)
- Two temperature electrodes: T201 (Radiometer Inc.)
- Titrator: TIM856 (Radiometer Inc.) installed with TIM85 vs. 1.6 or higher
- Multimeter for E_h monitoring: PHM240 (Radiometer Inc.)
- Reactor: 80 ml water-flow Pyrex glass reactor
- Aluminum foil for light-impermeable wrapping of the water-flow glass reactor
- Magnets for stirring
- Peristaltic pump (Titramaster)
- DLS: Malvern Zetasizer Nano ZS installed with Zetasizer software 5.1 or higher (Malvern Inc.)
- One computer to control the titramaster and log results from pH-measurements and titrant dosing
- One computer to log redox data sampled by the PHM240 and operate the Malvern DLS

The specific equipments listed above are not mandatory, but similar equipment is required

5.5.3 Procedure

5.5.3.1 Test set-up

The test setup is illustrated in Figure S4. The flow-through test reactor is placed in a titrator and connected to the Gant W28 thermostatic bath. A Teflon tube is connected to a flow-controller where CO₂ air is mixed with purified and HEPA-filtered air and humidified by bubbling through Nanopure Diamond UV water prior to leading the test atmosphere into the flow-through reactor. The titrant is added to a titrant vessel and syringes and tubes are connected with the dosage tube placed in the test reactor (depth during test: 1 cm above the magnetic stirrer). A pH-meter is fixed in position in a suitable hole in the reactor cap and connected to the computer used to control the TIM856 titrator. The redox electrode is fixed in position in a suitable hole in the reactor cap and connected to a multimeter (PHM240). The printer port is connected to a computer with a RS232 data cable to record data using the terminal program. A temperature electrode is coupled each of the pH and E_h electrodes to enable correction for temperature fluctuations. Two latex tubes are placed in the reactor. The first is lead through the peristaltic pump and connected to the Malvern flow cuvette. The second tube is connected to the outlet of the flow cuvette and lead back to the reactor to complete sample circulation. At start-up, the test reactor is wrapped in aluminum foil to inhibit possible artefacts caused by photocatalytic reactions.

5.5.3.2 Preparing Gamblés solution

The chemicals are dissolved in 2 L Nanopure water (Nanopure Diamond UV) and placed in an ultrasonic bath for 15 min. Thereafter, the solution is filtered through a 0.45µm sterile filter. The final solution is added 6ml of 4 % formaldehyde per 2 L Gamblé's solution to avoid growth and placed in darkness in the refrigerator.

5.5.3.3 Specifications of Nanopure Diamond UV water:

The water purification system is designed to 4 stages of de-ionization combined with UV light-treatment.:

Resistivity: ≤ 18.2 MΩ-cm at 25°C

Pyrogens: < 0.001 EU/ml

Total Organic Carbon: < 3.0 ppb

Other: nuclease-free (RNase andDNase).

Filter: 0.2 µm filter (γ-irradiated Barnstead D3750 Hollow fibre filter)

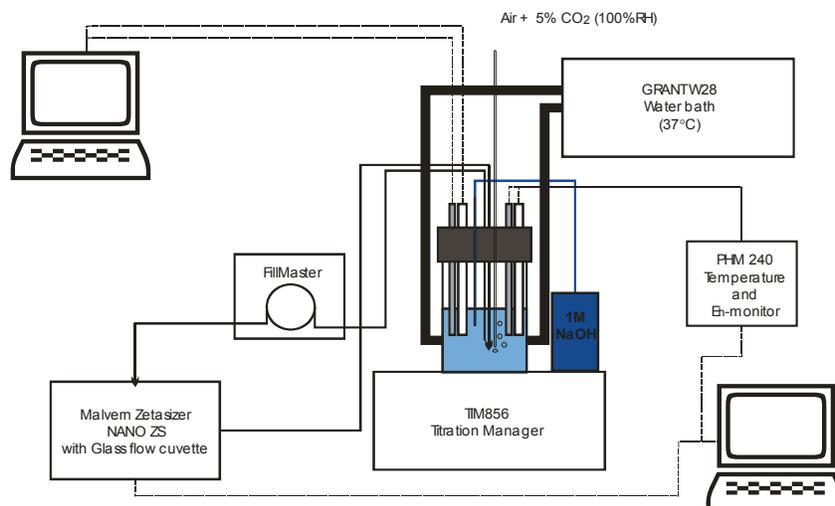


Figure S4. Principal set-up of the pH and atmosphere-controlled stirred batch reactor hydrochemical reactivity and biodurability tester

5.5.3.4 Performing a 24-hour test

- 1) Turn on the water-bath and adjust the temperature to 37.4°C
- 2) Turn on computers, DLS, Titramaster, Fillmaster and PHM240.
- 3) Connect the DLS flow-reactor to the latex tubes and circulate at least 10 ml Gambles solution through the tubes and flow-reactor for about 5-10 minutes using a continuous circulation program in the fillmaster.
- 4) Check that the titrator reagents are correct and that the syringe and tubings are free of air-bubbles. If air-bubbles are present, check for leaks and condition the system when it is verified that the system is tight.
- 5) In Titramaster, choose and export the suitable “application” for online control of pH, temperature to the TIM856
- 6) Choose method for logging redox potential and temperature at 5 min interval on PHM240
- 7) Start the Zetasizer software and program it for online measurement each 30 min using appropriate intensities and absorption for medium and particulate phase as well as dielectric constant and viscosity if the Gambles solution in the Measurement set-up. If properties of the Gambles solution are unknown, use the parameters for water. The measurement temperature is 37°C and the equilibration time will always be 2 minutes.
- 8) Add 80ml low-Ca Gambles solution to the reaction vessel: $V_{gs}=80\text{ml}$).
- 9) Weigh out NM to achieve the target concentration in a 20 ml scintillation vial.
- 10) Withdraw 10ml from the V_{gs} and add this to the scintillation vial
- 11) Disperse the NM by shaking, ultrasound or ultrasonic probe depending on protocol.
In NANOGENOTOX, dispersions will be made following the conditions in the general NANOGENOTOX dispersion protocol, which is 16 min at 10% amplitude and 400 W in a ice-water using the 400 Watt Branson Sonifier S-450D (Branson Ultrasonics Corp., Danbury, CT, USA) equipped with a standard 13 mm disruptor horn (Model number: 101-147-037).
- 12) Start the terminal program on computer 2, to log the redox potential and associated temperature
- 13) Calibrate the redox electrode using one or two redox reference solutions (e.g., Hamilton (DURACELL +271 and +574 mV). Use 5 min test logging each 30 seconds to verify stability.
- 14) Calibrate the pH electrode using at least two standard such IUPAC pH 4.005 and 7.413 following the calibration SOP in the TIM85 leading to sensitivity above 98.5%.
- 15) Install the batch reactor with the 70 ml of V_{gs} in the TIM856 and mount all electrodes, temperature sensors, and tubes and add the magnetic stirrer.

- 15) Start magnetic stirring at 400 rpm using the interface of the TIM856
- 16) Adjust the pH of the Gambles solution to pH 7.4 using 7.7 M HCl.
- 17) Place the Teflon tube leading test atmosphere into the reactor into the Gambles solution and position it 1 cm above the magnetic stirrer and initiate bubbling with 150 ml/min CO₂ adjusted air (96.25 vol% air and 3.75% CO₂).
- 18) Empty the DLS flow-cuvette for Gambles solution "cleaning fluid"
- 19) Start the titrator application in TIM85 and redox monitoring each 5 minutes using the PHM240:
- 20) After 1 min, slowly add the test material dispersed in 10 ml Gambles solution into the reactor using a pipette.
- 21) Start the peristaltic pump and circulate 5 ml during 5 minutes
- 22) Make consecutively six DLS measurement manually, using automatic optimization of sub-runs and measurement time and note the settings for the subsequent periodic analysis
- 23) Program the peristaltic pump to circulate 5 ml per 5 minutes through the flow-cuvette every 30 minutes starting 20 minutes after the start of the experiment
- 24) Program the DLS to make a single size analysis every 30 minutes starting 27 minutes after start of the experiment. This allows stability and thermal calibration to establish in the DLS flow-cell.
- 25) Check the acidity of the dispersion. If the pH exceed 7.4 and continues to rise, abort the test and change titrant to 1 M HCl and start a new test from the beginning. If the reaction follows expectations, continue the experiment for 24 hours
- 26) At the end of the experiment (24-hour reaction)
- 27) End TIM85 application and save printouts and data
- 28) Immediately sample 10 µl with pipette and add this to a TEM grid (Lacey Carbon or Holey Carbon 200mesh Cu TEM grids).
- 29) Immediately withdraw 2 x 20 ml for ultracentrifugation: (Sorvall rotor SA-600, 16000 rpm i 20min, without de-acceleration; 20C).
- 30) Sample 20 ml from the reactor for measurement of viscosity at 37°C using the Vibracell Viscometer. This value may be used to correct the DLS measurements
- 31) Immediately withdraw 2 x 15 ml supernatant for elemental chemical analysis ICP-MS/AAS and filter it through a 0.45 µm filter or better.
- 32) Sample the centrifugation pellet for X-ray diffractometry.

5.5.4 *Data treatment*

All data data are synchronized and collected into one excel sheet.

The following data are collected:

- Time 1
- pH
- Temperature 1
- Added total volume of titrant
- Added differential volume of titrant
- Time 2
- Eh
- Temperature 2
- Zeta-size as well as size-distribution data
- Total concentration of leached/dissolved constituent elements
- Initial and final information on crystallinity and crystalline phases
- Information from electron microscopy

Monitoring E_h data are corrected for temperature deviations from the calibration temperature

E_h results may be used as monitored or re-calculated to determine the theoretical electron contraction in the test fluids according to the equation

$$E_h = -2,3RT/F \cdot \log[e^-]$$

The pH and Eh data can be plotted to evaluate the potential influence of the test NM on the local physiological conditions and the potential oxidative stress in the local region. Released elemental concentrations can be used to determine the biodurability of the test material.

5.5.5 *Comments on use and applicability (modified from reference)*

The method is currently under establishment and reference can mainly be made to man-made vitrous fibers and asbestos, which may be considered useful benchmark materials. However, similar results may not be obtained as compared to historical tests due to highly improved test conditions.

Longer test durations may be required to investigate potential alteration of low to very-low soluble NM.

Improved procedures for particle filtration before analysis of the solvent needs to be considered.

Online DLS analysis have proven to be not suitable due to accumulation of particles in the flow cuvette and generally agglomeration in the test chamber prevents analysis giving useful data.