PATROLS
Advanced Tools for NanoSafety Testing

This project has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement No 760813

PATROLS Standard Operating Procedures (SOP)

In situ detection of particle size distribution and concentration of ions released in ecotox media

This is a SOP used by members of PATROLS only

Adapted from the NanoImpactNet SOP, Clift *et al* (Deliverable 5.4 under the European Commission's 7th Framework Programme, Grant Agreement 218539).

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Document History:

Version	Approval Date	Description of the change	Author(s) of change
1.0	13/04/2021	Initial Document	Jorge Mejia
1.1	15/04/2021	Improved draft	Ilaria Zanoni, Anna Costa

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1 Introduction:

DOMAIN: Material characterization

The aim of the work was to support the study of NPs fate in water ecosystems, by characterizing how intrinsic properties change in environmental relevant media. Three main endpoints were addressed: static dissolution, agglomeration degree and distribution along the water-column, evaluated in acellular *in-vitro* systems. In agreement with OECD guidelines, Elendt M7 was applied for fresh water compartments, mimicking the life condition for *Daphnia magna* speciem[1,2,3]. Egg Water medium was used for the sea water ecosystem, usually applied in *in-vivo* test on *Zebrafish (Danio rerio)* species. The final aim of this work was that to provide physicochemical data that can be used to improve eco-tox dose-response model and derive more accurate reference values (PNEC) for scientific and regulatory purposes.

The correlation between NPs agglomeration vs time of exposure was investigated, assessing particle size distribution by Centrifugal Liquid Sedimentation technique (CLS)In order to validate the methodology, we also estimated PSD across the water column by DLS and measured the concentration at half water column height by ICP-OES. Overall the results allow to predict the sedimentation behaviour in a typical in vivo ecotox model.

1.1 Scope and limits of the protocol

The objective of this SOP is to detect the particle size distribution of metallic and oxide (nano)materials and the concentration of ions released in ecotox relevant media. This SOP evaluates the fate (timewise) of the particle size distribution during an ecotox related test, in relevant media such as: Elendt M7 and / or Egg water.

One challenge, for the techniques described in this SOP, is the complexity of the medium used to disperse the (nano)materials, which is constituted of a matrix of elements and nutriments. Some relevant experience from the user is required.

The use of such a complex medium requires the use of relevant standards for size and the use of adequate densities values for the (nano)material.

1.2 Validation state of protocol

Level of advancement towards standardization	**Level reached** (please mark only one with "X")
Stage 1: Internal laboratory method under development	
Stage 2: Validated internal laboratory method	X
Stage 3: Interlaboratory tested method	
Stage 4: Method validated by Round Robin testing	
Standardisation plans	
Is the method considered for standardisation (OECD SPSF or similar)?	N
Has the method been submitted for standardisation (to OECD, CEN, ISO,…) in its own right or as part of another standardisation project?	N
Is the method included in an existing standard (or ongoing standardisation work)	N

2 Terms and Definitions:

Agglomerate

Collection of weakly or medium strongly bound *particles* where the resulting external surface area is similar to the sum of the surface areas of the individual components.

Note 1 to entry: The forces holding an agglomerate together are weak forces, for example van der Waals forces or simple physical entanglement.

Note 2 to entry: Agglomerates are also termed secondary particles and the original source particles are termed primary particles.

[SOURCE: ISO 26824:2013, 1.2]

Aggregate

Particle comprising strongly bonded or fused particles where the resulting external surface area is significantly smaller than the sum of surface areas of the individual components.

Note 1 to entry: The forces holding an aggregate together are strong forces, for example covalent or ionic bonds, or those resulting from sintering or complex physical entanglement, or otherwise combined former primary particles.

Note 2 to entry: Aggregates are also termed secondary particles and the original source particles are termed primary particles.

[SOURCE: ISO 26824:2013, 1.3, modified — Note 1 adapted.]

Nanoscale

Length range approximately from 1 nm to 100 nm

Note 1 to entry: Properties that are not extrapolations from larger sizes are predominantly exhibited in this length range.

[SOURCE : ISO/TS 80004-1: 2016, definition 2.1]

Nanomaterial

Material with any external dimension in the *nanoscale* or having internal structure or surface structure in the nanoscale.

Note 1 to entry: This generic term is inclusive of *nano-object* and *nanostructured material*.

[SOURCE: ISO/TS 80004-1: 2016, definition 2.4]

Nanostructured material

Material having internal *nanostructure* or surface nanostructure.

Note 1 to entry: This definition does not exclude the possibility for a *nano-object* to have internal structure or surface structure. If external dimension(s) are in the *nanoscale*, the term nano-object is recommended.

[SOURCE: ISO/TS 80004-1: 2016, definition 2.7]

Engineered nanomaterial

Nanomaterial designed for specific purpose or function

[SOURCE: ISO/TS 80004-1: 2016, definition 2.8]

Manufactured nanomaterial

Nanomaterial intentionally produced to have selected properties or composition.

[SOURCE: ISO/TS 80004-1: 2016, definition 2.9]

Particle

Minute piece of matter with defined physical boundaries.

Note 1 to entry: A physical boundary can also be described as an interface.

Note 2 to entry: A particle can move as a unit.

Note 3 to entry: This general particle definition applies to *nano-objects*.

[SOURCE: ISO 26824:2013, 1.1]

3 Abbreviations:

CLS Centrifugal Liquid Sedimentation

DLS	Dynamic Light Scattering
ENMs	Engeneered nanomaterials
ICP-OES	Inductively coupled plasma optical emission spectrometry
NPs	Nanoparticles
PNEC	Predicted no-effect concentration**PSD** Particle Size Distribution
PVC	Polyvinyl chloride

4 Principle of the Method:

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:

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

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.

5 Description of the 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 has a smooth transition into the gradient (laminar flow). A procedure is selected to measure the specific type of (nano)particles. A certified calibration standard is used to determine the correct diameter-time relationship. Finally, the sample is injected and measured. The concentration of (nano)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.

5.1 Apparatus and equipment:

A. CLS 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 glass beakers.

H. Digital weight balance.

I. Thermometer.

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

K. Silica MNM dispersion, diameter: 100 nm.

L. Quality Control, PVC dispersion.

5.2 Health and safety precautions:

Prior to any use of this SOP a full risk assessment should be completed, considering all potential risks associated with chemicals equipment and use, in compliance with national regulation. Training of personnel should be completed before any person is working with the SOP.

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.

Instrument: The CLS system must not be used/cleaned with acetone or chlorinated solvents.

5.3 Applicability:

These protocol has been demonstrated for metallic, oxide and carbon-based nanomaterials.

5.4 Reagent preparation:

Sucrose solution

2 % and 8 % (m/m) sucrose solutions will be prepared. Two graduated glass beakers properly identified as 2 % sucrose and 8 % sucrose are required. Preparation:

1. 2 % sucrose: Place the 2 % sucrose beaker on the microbalance, making sure it is offset at zero with the beaker. Then add 0.2 g of sucrose to the beaker, followed by deionized water until a weight of 10 g is obtained.

2. 8 % sucrose: Place the 8 % sucrose beaker on the microbalance, making sure it is offset at zero with the beaker. Then add 0.8 g of sucrose to the beaker, followed by deionized water until a weight of 10 g is obtained.

Agitate each beaker gently until all the sucrose is dissolved. The beaker can alternatively mixed with an ultrasonication bath.

5.5 Procedure:

5.5.1 Starting the CLS equipment, injecting the sucrose gradient and dodecane

The equipment should be in “ON” position, 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 22000 rpm.

The sucrose gradient is injected with a series of sucrose mixtures in the following order:

Table 1. *Sucrose mixtures (8 and 2 %) and order of inyections to build the sucrose gradient inside the disk. * The first volume of 1.6 mL is introduced in the disk before turning on the instrument.*

Order	Sucrose 8 %	Sucrose 2 %	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

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, then press START to begin the disk rotation. Afterwards, when the disk reaches the set rotation speed (22000 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 8 % sucrose). Each time a total volume of 1.6 mL is injected.

Rigth after and using a new BD 3mL syringe inject 0.5 mL of dodecane. From that point, allow 20 minutes for the dodecane and gradient to stabilize.

5.5.2 Defining a sample procedure

A procedure is needed for each type of particle being measured. It 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. For silica particles the procedure parameters are described in Table 2. Note that the maximum and minimum diameters have to be adjusted to measure the particles within the selected range. The physical sample parameters (density, refraction index and absorption) are values taken from the literature (bulk material) or provided by the material supplier.

Table 2. *Parameters to be input for Silica measurement (example). * Calibration Standard Parameters should correspond to the calibration standard used at each lab.*

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 (□m)	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	No
Distribution Table	None
X-axis Scale	Log

In ‘New Name’ enter the name for the current procedure, ‘Silica MNMs’ for example. Click on ‘Save and Exit’.

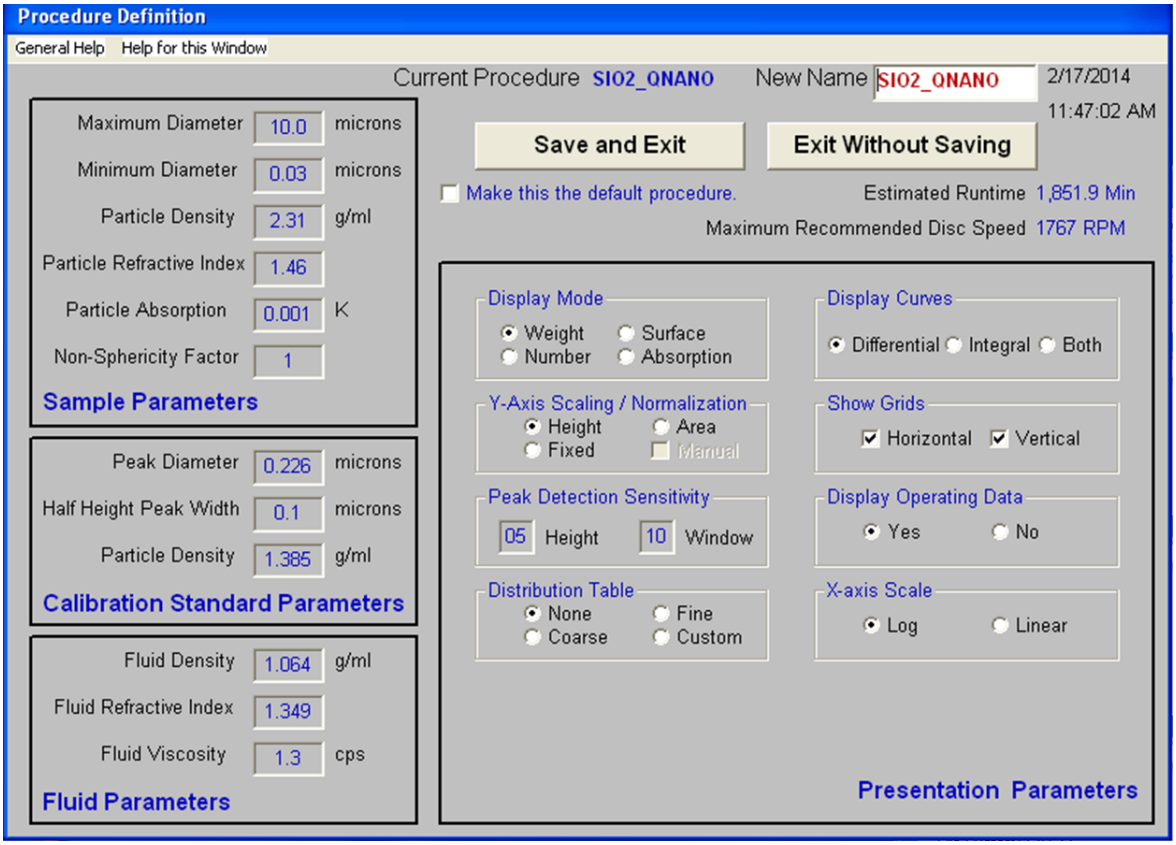

Figure 1. *Procedure definition window. It contains the parameters used for Silica measurements. Note that the parameters for the ‘Calibration Standard Parameemeters’ subsection depend on the specific calibration standard being used at each laboratory.*

5.5.3 Measurements

5.5.3.1 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’.

5.5.3.2 Measurements

1. Click on the button ‘Operate Analyzer’ from the main menu.

2. Type on the ‘Sample’ line: “name of your sample”. Click ENTER.

2.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.

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

2.b When the measurement is completed, introduce your sample 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.

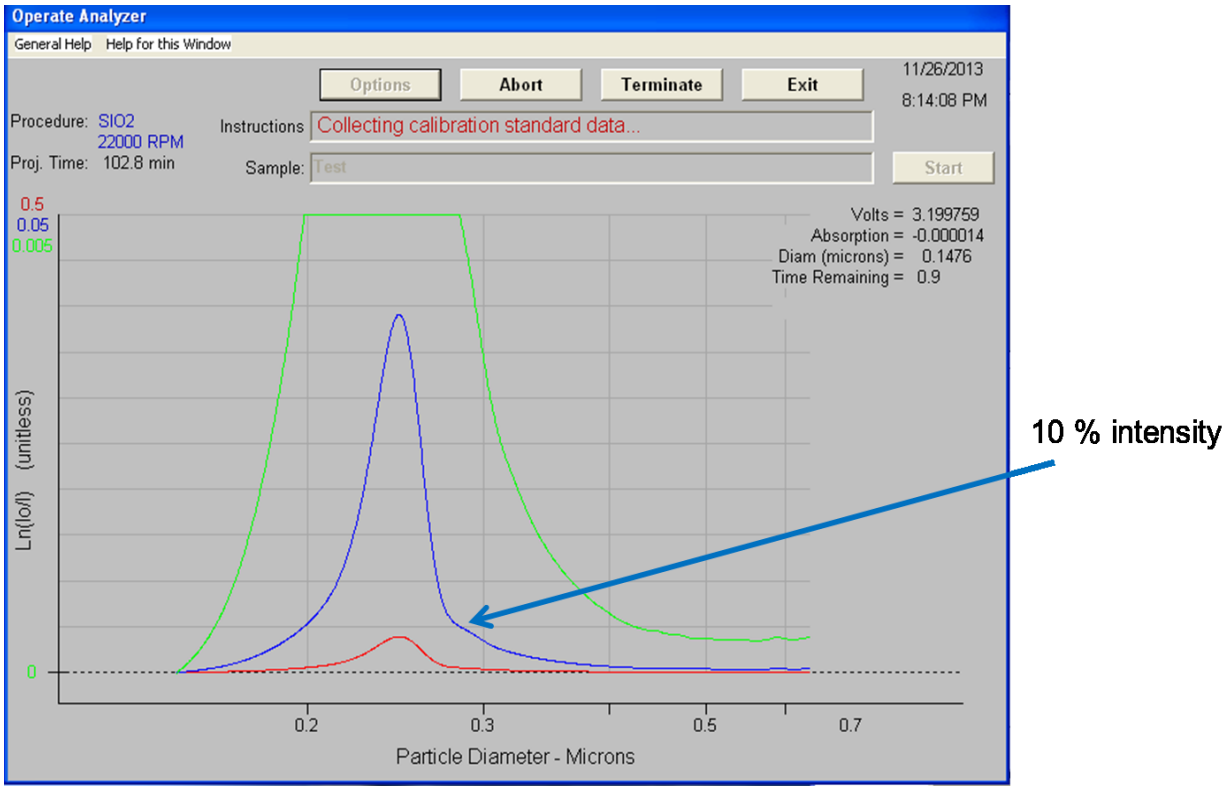

Figure 2. *Curve of the calibration standard. A good calibration is obtained when the blue curve is above the 10 % intensity level (first line from bottom-up, see arrow).*

5.5.4 Measurements in ecotox related medium

The CLS technique was used to determine the particle size distribution and the agglomeration state of ENMs in suspension, calculating hydrodynamic diameter. This parameter considers the environment of the particles and the solvation phenomenon. Many sources of uncertainties exist and can be related to sampling (weighing ENMs, volumetric operations such as pipetting, and dilutions), calibration (purity of standards and of reagents), instrument performance, input parameters (density, viscosity, refractive index of ENMs, of standard, of media…), different operators, matrix effects and sample effects. Uncertainties could be calculated by using a bottom-up approach (identification of all uncertainty sources, combination of individual measurement) or a top-down approach (quantification of repeatability and intermediate precision only).

Under our conditions, the top-down approach is more practical, however, it does not consider systematic errors. In order to evaluate reproducibility, an experiment was designed and was performed using this approach. A commercial solution from Benelux Scientific was used as a standard to avoid the errors related to the sampling. This solution contains PVC Nanoparticles with a certified size of 263 nm. This solution is homogenous and monodisperse; therefore, errors related to the matrix and samples effects may be avoided. Input parameters were found in the technical data sheet of the product. Measurements were performed 5 times for 5 consecutive days to minimise errors and to identify the uncertainties related to the instrument performance.

Particle size distribution are presented in different representations, in weight and/or in number of particles. Both representations appear in Figure **.** A bimodal distribution is observed in both cases (5 measurements per day during 5 days), but the signal is very narrow and is centered near 260 nm. Measurements are reproducible.

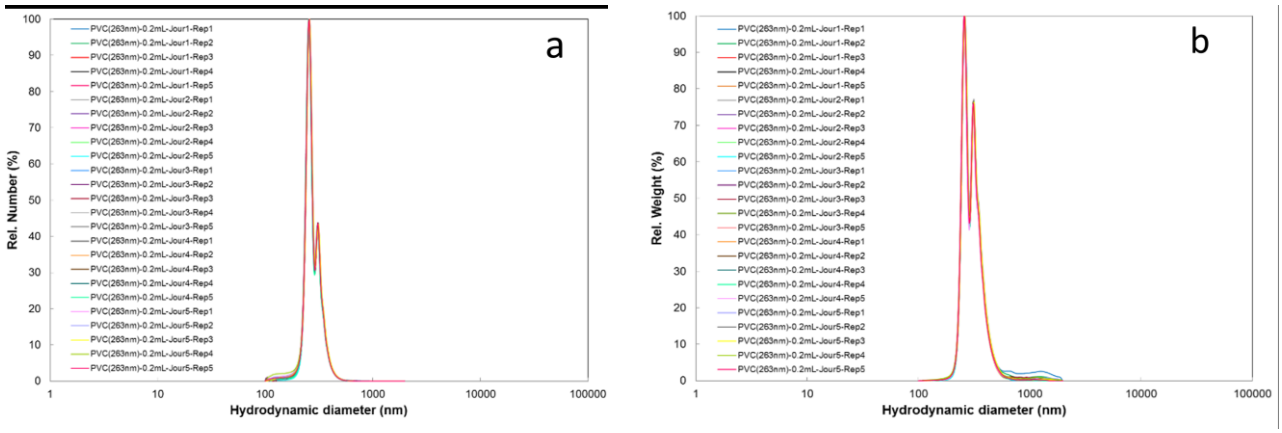

Figure 3 . *Particle size distribution (normalised) for PVC NPs (263 nm) provided by Benelux scientific a. in Number, b. in Weight related distributions.*

ANOVA treatment was used to calculate the uncertainty related to the instrument performance. It provides repeatability (S_{rep}) and intermediate precision (S_{ip}) values. A

coverage factor (k) of 2 was used in the calculations and the parameters n_{rep} and n_{days} are the number of replicates and the number of days of measurements respectively. Mean square (MS) are calculated accordingly. Equations are described below.

$$S_{rep} = \sqrt{Ms_{Within}}\ ,\ S_{ip} = \sqrt{\frac{Ms_{Between} - Ms_{Within}}{n_{rep}}}$$

$$U_{ANOVA} = k.\sqrt{\frac{S_{rep}^2}{n_{rep}} + \frac{S_{ip}^2}{n_{days}}}$$

$$U_{rel} = \frac{U}{Valeur} \times 100$$

Diameters of PVC NPs in both weight and number of particles are reported in Table 3 and in Table *4*4 respectively.

Table 3 . *Diameters of PVC NPs calculated by the CLS instrument from number related distributions.*

	Rep 1	**Rep 2**	**Rep 3**	**Rep 4**	**Rep 5**
Day 1	258.11	256.1	255.12	255.1	253.64
Day 2	257.59	257.1	256.66	255.97	255.25
Day 3	257.7	256.54	255.13	254.12	253.51
Day 4	257.4	256.47	255.31	255.31	253.99
Day 5	258.22	257.09	257.98	255.99	255.66

Table 4 : *Diameters of PVC NPs calculated by the CLS instrument from weight related distributions.*

	Rep 1	**Rep 2**	**Rep 3**	**Rep 4**	**Rep 5**
Day 1	261.08	258.96	258.23	257.85	256.61
Day 2	260.49	259.58	259.44	258.64	257.90
Day 3	260.54	259.3	257.8	256.73	256.05
Day 4	260.37	259.37	258.14	257.82	256.70
Day 5	261.34	260.12	260.66	258.57	258.19

ANOVA treatment was performed on the main intense signal only. Required data for ANOVA treatments are listed in Table 5. In number, the average value is 256.03 $\pm$ 2.82 nm and in weight is 258.78 $\pm$ 2.97 nm. The uncertainty related to the instrument is near 1.1 %, which is weak. From these results, we conclude that the CLS instrument is accurate and that the major source of error (if errors is > 1.1 %) comes mostly from the sampling stage.

Table 5 : *MS between and within group data are generated by ANOVA evaluation.* $\mathbf{S_{rep}}$ *and* $\mathbf{S_{ip}}$*are calculated by using MS values in uncertainties equations.* n_{rep} *and* n_{days} *are assumed to be 1 for 1 measurement. The factor coverage k is assumed to be 2.*

	Number	Weight
Average diameter (nm)	256.03	258.78
$Ms_{Between}$	2.291	2.248
Ms_{Within}	1.907	2.188
S_{rep}	1.381	1.479
S_{ip}	0.277	0.110
n_{rep}	1	1
n_{days}	1	1
K	2	2
U_{Mes} (nm)	2.82	2.97
U_{rel} (%)	1.10	1.15

5.5.4.1 Ecotox sample preparation

The NanoGENOTOX dispersion protocol and the SOP for calibration of Probe-sonicators for *in vitro and in vivo* testing were used to prepare the suspension. Experiments were performed in 2 different media: water and daphnia medium (Elendt M7). Briefly, a stock solution of 2.56 mg/mL in water was prepared and was sonicated for 15.40 min using a Branson Sonifer S-450D (Branson Ultrasonics, 300Watt, 10% Ampl, 13 mm horn). A suspension of 0.1 mg/mL in Elendt M7 medium (preparation medium is based on OECD TG-211 (2012), OECD TG-202 (2004) and Elendt (1990), with minor modifications in methodology for practical reasons) was

prepared from the stock solution. Measurements are done in triplicates for 3 consecutives days.

Sampling is done at 4 different times: 0h, 3h, 6h and 24h and at 3 different heights: Top (1.6 cm from the top reference), Middle (2.6 cm from the top reference) and Bottom (3.8 cm from the top reference). The vial dimensions are 2 cm wide by 4cm length. There should be a specific vial for each height. The high is marked outside the vial with a marker and the syringue should be introduced till that position only to take the sample. Samples are analysed inmediatly, for this the sampling is performed just when the instrument is ready to analyse the next sample. It has to be mentioned that an effort is needed to adequately programme the sampling in a way that samples are as fresh as possible (no waiting time before analysis). This is a factor that is sometimes neglected during the characterization of (nano)materials.

5.5.5 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), the measured particles sediment there and it may take a few cleaning cycles with a soft tissue and deionized water/ethanol until most of the remaining traces of particles are removed.The tissues, syringues and needles used should be discarded according to specific measures established in the institution for nanomaterials.

6 Data Analysis and Reporting of Data

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 where done, and click on the ‘OK’ button. This will make the software go to the window of the active procedure that contains 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.

6.1 Reporting the data

The Peaks and Half-Width of the each sample should be reported in an excel file with clearly identified 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 the determined peak and half-width should be reported.

Two types of graphs can be produced: a weight related distribution that highlights the contribution of heavy particles (agglomerates) and a number related distribution that highlights the contribution of more numerous particles (primary particles) present in the distribution. Both distributions are shown in Figure 4 with an example from ZnO NM-111 nanomaterial.

a. b.

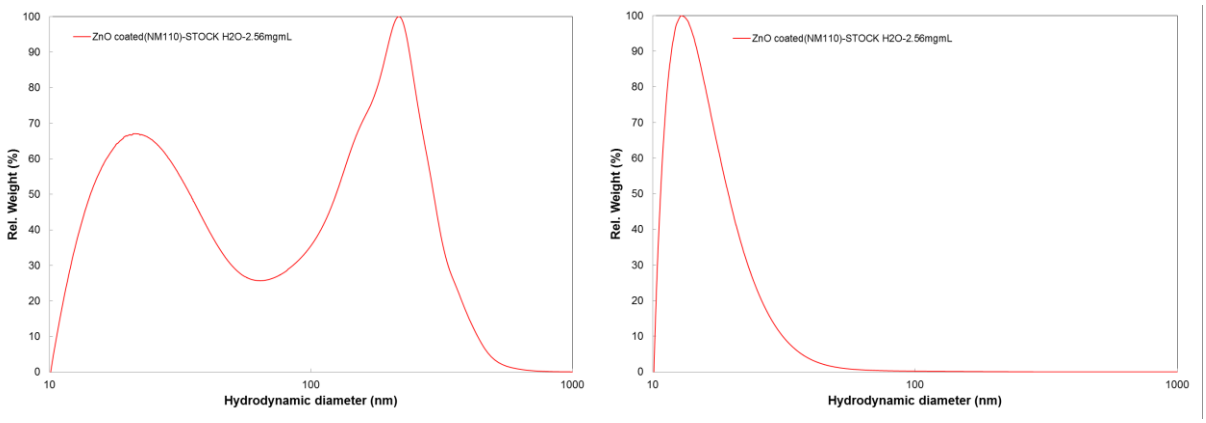

Figure 4. *Particle size distribution, a. Weight related distribution, b. Number related distribution of ZnO NM-111 suspended in H_2O (2.56 mg/mL).*

In general, weight related distributions are more insightfull for dose-related applications, given that weight is related to dose. Number related distributions are more adequated to describe applications where size of particles is of prime importance. As an example, the following Figure 5 illustrate the importance of sampling at different heights: the particle size distribution at the bottom is mainly composed of agglomerates while at the top there are no agglomerates.

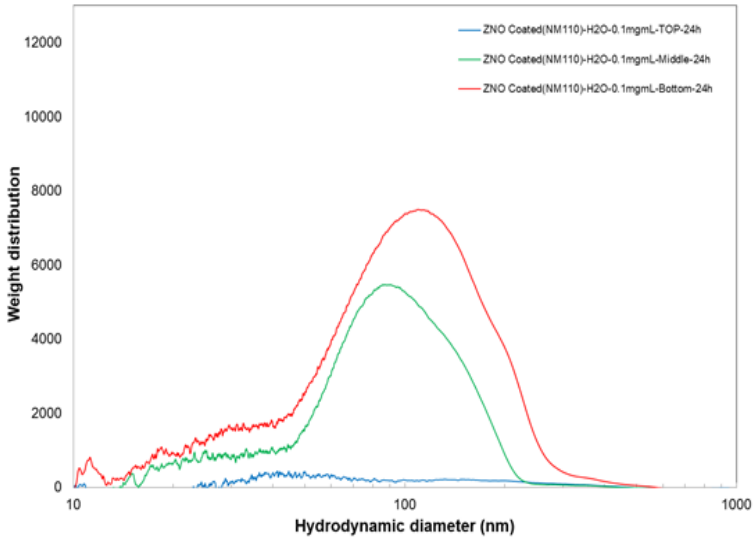

Figure 5. *Particle size distribution of ZnO NM-111 in H_2O (0.1 mg/mL) at different heights (Top, Middle and Bottom), at 24h.*

7 Publications:

8 References

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