

Report on the applicability of OECD TGs for determination of the environmental fate of ENMs

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Abstract

The main aim of Task 4.2 of RiskGONE project is to verify the applicability of OECD methodology for the determination of environmental fate of chemicals to engineered nanomaterials (ENMs) and suggest adaptations of the selected test guidelines (TGs), if needed. In this work, five OECD test guidelines were selected, to cover the main aspects of ENM environmental fate in different media: water, soil and fat. The selected TGs were: 1) TG111-Hydrolysis as a function of pH, 2) TG106-Adsorption-desorption using a batch equilibrium method, 3) TG312-Leaching in soil columns, 4) TG116-Fat solubility of solid and liquid substances, and 5) TG318-Dispersion stability of nanomaterials in simulated environmental media. The evaluation of their applicability for ENMs was carried out based on available OECD regulatory documents, expertise of partners involved in this task (CID, LIST, IMI and QSARL) and outcomes of other European initiatives, such as NanoFASE and NanoREG projects. The work performed in this task comprised two parts. The first one consisted of a detailed analysis of the procedures reported by the OECD TGs and the second regarded the experimental assessment of the proposed methodology by testing representative ENMs.

The main results and conclusions of the task are summarised within this deliverable (D4.8) which also proposes modifications on the existing methods that can be further considered for scientific publications and/or submitted as SFPS to regulatory bodies.



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1. Technical & Scientific progress

According to the updated Recommendation of the European Commission a nanomaterial is intended as a natural, incidental or manufactured material consisting of solid particles that are present, either on their own or as identifiable constituent particles in aggregates or agglomerates, and where 50 % or more of these particles in the number-based size distribution fulfil at least one of the following conditions:

(a) one or more external dimensions of the particle are in the size range 1 nm to 100 nm; (b) the particle has an elongated shape, such as a rod, fibre or tube, where two external dimensions are smaller than 1 nm and the other dimension is larger than 100 nm; (c) the particle has a plate-like shape, where one external dimension is smaller than 1 nm and the other dimensions are larger than 100 nm¹.

Due to their unique properties, the use of engineered nanomaterials (ENMs) in many technologies and products is increasing exponentially as well as their release into the environment. While for conventional chemicals several parameters for the prediction of environmental fate exist, the applicability of existing test guidelines (TGs) of the Organisation for Economic Co-operation and Development (OECD) for the determination of the environmental fate of ENMs should be verified. Since 2006, the OECD has coordinated a Testing Programme on the testing of manufactured nanomaterials via the Working Party on Manufactured Nanomaterials (WPMN). In 2009, the OECD published a “Preliminary Review of OECD Test Guidelines for their Applicability to Manufactured Nanomaterials”² in which 22 guidelines for the testing of chemicals were assessed and classified as: 1) applicable, 2) applicable under some circumstances or to some classes of nanomaterials, and 3) not applicable to manufactured nanomaterials or, if applicable, provide no useful information. Most TGs were considered as generally applicable but, up to date, only few TGs were specifically proposed for the analysis of the environmental fate of nanomaterials.

At this regard, the main objective of the *Task 4.2-Environmental fate* of RiskGONE project is to verify the appropriateness of selected OECD TGs for the testing of nanomaterials and the deliverable 4.8 reports the results of this task.

Initially, five test guidelines for the characterization of ENM’s behaviour and potential fate in the environment were proposed. The evaluation of their applicability to ENMs was performed based on available regulatory documents, expertise of partners involved in this task (CID, LIST, IMI and QSARL) and the outcomes of other European initiatives, such as NanoFASE and NanoREG projects, which were European projects specifically dedicated to the modelling of the environmental fate of ENMs. Table 1 summarizes the different properties considered within each TG and analysed by task 4.2 to verify the applicability of the selected OECD TGs to the environmental fate of ENMs.

Table 1. Proposed TGs for the evaluation of applicability of OECD methodology for ENMs.

OECD guideline	Property	Compartment	Applicable to ENMs
TG111	Hydrolysis as a function of pH	water	yes
TG106	Adsorption-desorption using a	soil	yes

	batch equilibrium method		
TG312	Leaching in soil columns	soil	yes
TG116	Fat solubility of solid and liquid substances	fat	yes
TG318	Dispersion stability of nanomaterials in simulated environmental media	water	yes

1.1. Description of the work

1.1.1 Theoretical evaluation of test guidelines: analysis and selection of existing OECD TGs

The work performed within RiskGONE Task 4.2 consisted in two phases. The first one was dedicated to a theoretical analysis of existing TGs debating the critical aspects of nanomaterial testing. The second part consisted of the experimental assessment of selected OECD protocols, by using reference nanomaterials.

OECD TG111: Hydrolysis as a function of pH

The first test guideline studied was the “OECD TG111: Hydrolysis as a function of pH”³. The method described by the TG is based on the incubation of the test substance in sterile aqueous buffer solutions of different pHs (4, 7 and 9), in the dark, under controlled laboratory conditions. The solutions are then analysed for the test substance and potential hydrolysis products at different time points. This means that this TG is applicable only to slightly volatile and/or non-volatile compounds with sufficient solubility in water. Therefore, this requirement makes the test guideline applicable only to those ENMs having a minimum water solubility, hindering for example, the testing of titanium dioxide NPs (TiO₂). The use of some water miscible solvents to increase the solubility of nanoparticles (such as ethanol, acetone, and acetonitrile) is allowed whether it can be shown that the solvent has no effect on the hydrolysis of the test substance.

Below are reported the testing conditions:

- If the water solubility of the test substance is lower than 2×10^{-2} M, a preliminary test can be performed. If less than 10% of hydrolysis is observed after 5 days of incubation at 50°C, the substance is considered hydrolytically stable.
- If the solubility of the substance is higher than 10%, the advanced test should be performed at the pH values at which the substance was found unstable.

In the advanced test, the stability of the samples is assessed not only as function of pH, but also by using different temperature conditions and at different end points to verify the order of the hydrolysis reaction.

Similarly to the considerations made by OECD², RiskGONE partners concluded that the TG111 is applicable only under specific circumstances and/or to some classes of ENMs. When ENMs dispersions are used, colloidal particles can remain in the medium if they do not hydrolyse completely. In this way, this test guideline can be updated and improved by better clarifying how to avoid the presence of colloidal particles on the results as well as including practical examples of its application to inorganic and organic nanoparticles. This guideline was selected to be furtherly assessed also from the experimental point of view.

OECD TG106: Adsorption-desorption using a batch equilibrium method

The second test guideline analysed was the TG106⁴. The aim of the method described in the TG is to estimate the adsorption/desorption behaviour of a substance in soils, by calculating/estimating/obtaining a sorption value which can be used to predict the partitioning under a variety of environmental conditions. For this purpose, known volumes of the test substance in CaCl₂ solution are added to soil samples, pre-equilibrated in CaCl₂ solution. The amount of the test substance adsorbed on the soil sample is calculated as the difference between the amount of test substance initially present in solution and the amount remaining at the end of the experiment, after centrifugation (indirect method). Centrifugation process should be temperature controlled and capable of removing particles larger than 0.2 µm from aqueous solution. Whether the difference in the solution concentration of the substance cannot be accurately determined, the application of a direct method is foreseen. However, it involves soil extraction with an appropriate solvent, which can make the analytical procedure more tedious. Examples of such cases are: 1) adsorption of the test substance on the surface of the test vessels, 2) instability of the test substance in the time scale of the experiment, 3) weak adsorption producing only small concentration changes in the solution and 4) strong adsorption yielding to low concentration which, therefore, cannot be accurately determined. According to the TG, this protocol can be applied to chemical substances for which an analytical method having a sufficient accuracy is available thus it is of difficult realization for substances with low water solubility ($S_w < 10^{-4} \text{ g l}^{-1}$). In addition, an important parameter that can influence the reliability of the results, especially when the indirect method is applied, is the stability of the test substance in the time scale of the test. Once again, OECD reported that this TG might be applicable only under some circumstances or only to some classes of manufactured nanomaterials. NanoFASE and NANoREG initiatives also studied the applicability of TG106 to ENMs. While there is no information about the conclusion of NanoFASE project, NANoREG concluded that this TG is partially or not applicable applicable to nanoparticles⁵.

RiskGONE project again agrees with the OECD position and proposes some adaptations in order to be able to use this test guideline for nanomaterial characterization. The very tiny dimensions of the ENMs and the presence of ions and other substances in soil samples may affect particle stability, inducing the formation of aggregates that can sediment upon centrifugation, even if they are not adsorbed on soil particles. If this occurs, the amount of adsorbed nanomaterial by soil could be erroneously overestimated. Considering this issue, the TG106 can be adapted by including a clarification on how to avoid possible overestimation of the amount of adsorbed nanomaterial.

OECD TG312: Leaching in soil columns

The impact of chemicals on soil is also covered by the TG312-Leaching in soil columns⁶. This guideline foresees that columns made of inert material are packed with soil. The surface of each soil column is treated with the test substance and/or with aged residues of the test substance. Then, artificial rain is applied to the soil columns and the leachate is collected. After the leaching process the soil is removed from the columns and is sectioned into an appropriate number of segments depending on the study requirements. The segments of soil should be extracted with an appropriate solvent. Each soil segment and the leachate are then analysed for the test substance and, if appropriate, for transformation products and/or other chemicals of interest. The test should be applied to chemicals that are supposed to be non-volatile in soil and water in the experimental conditions of the test. This requirement is normally not an issue for the most of ENMs. Nevertheless, the determination of ENMs in soil samples looks challenging, considering the scarce solubility of some inorganic ENMs and the complex chemical composition of soil, which includes the presence of organic and inorganic (nano)particles that can affect the results. Under the OECD WPMN initiatives related to “guideline revision”, NanoFASE project started working already in 2018 on the modification of the TG312. The preliminary testing of two reference nanomaterials (Ag and CeO₂ NPs) in different soils was included, aiming to strengthen the protocol. Moreover, in 2019 OECD published a work plan⁷ informing that a project led by Germany and Canada was working on a Guidance Document to support the implementation of the TG312 direct to Nanomaterial Safety Testing. The project has been concluded in 2021⁸, but so far, no updates have been found in the OECD library.

RiskGONE partners also concluded that the TG312 is potentially applicable to ENMs. However, the test guideline should be adapted including a clarification on how to avoid that the presence of colloidal particles in the test substance may have an impact on the leaching in soil columns. The inclusion of some practical examples of application of this TG to inorganic and organic nanoparticles would be also helpful.

OECD TG116: Fat solubility of solid and liquid substances

The next test guideline evaluated was the OECD TG116, “Fat solubility of solid and liquid substances”⁹. This test guideline dates from 1981 and aims to provide useful data for evaluating the storage of lipid soluble materials in biological tissues. The principle of the test is based on the solubility of the test substance in liquid fat (triglycerides). The fat solubility is defined as the mass fraction of a substance which forms a homogeneous phase with a liquid fat without giving rise to chemical reactions. The test substance is added in a standard fat, stirred, and centrifuged. One sample is taken from each saturated phase for further analysis. The sample is weighed, and the mass fraction is determined by using a substance-specific analytical method. The test can only be applied to pure substances which are stable at 50°C for at least 24h and not volatile under those conditions. According to OECD², this TG is considered applicable to manufactured nanomaterials.

After the analysis of the described method, we concluded that, on one hand, the protocol looks very simple and easy to perform but, on the other, an adequate method for the determination of ENMs in fat is required. This is not representing an issue in case of testing of some organic particles (i.e., lipid nanoparticles). However, this step may be challenging when commercial products containing inorganic functionalized nanoparticles (such as functionalized ZnO and TiO₂ nanoparticles used in sunblock lotions) are tested. Those ENMs cannot be considered as pure substances and additionally they have a

certain stability on fatty liquids given by the functionalization. Therefore, the application of the TG318 to understand their environmental fate might not be suitable.

In 2009, OECD recognized that there are other methods to verify the possibility of nanomaterials to accumulate in fatty tissues, but the properties of nanomaterials and their potential applications might require a proper guideline. More recently, the NanoFASE project worked on a potential adaptation of this TG but so far, no updates of this TG can be found in the OECD library.

OECD TG318: Dispersion stability of nanomaterials in simulated environmental media

The last analysed TG to study the environmental fate of nanomaterials was the TG318 “Dispersion stability of nanomaterials in simulated environmental media”¹⁰. This test guideline is quite recent (published only five years ago) and it represents the first OECD guidance document dedicated to analysing the fate of nanomaterials in aqueous media. The “dispersion stability” was identified as an important parameter affecting the environmental fate of nanomaterials. The objective of this test guideline is to offer an effective method to analyse the colloidal stability of a nanomaterial under relevant environmental conditions. For this purpose, the test involves: (i) the dispersion of the tested nanomaterial in water-based medium by using a calibrated sonication procedure, (ii) the incubation of the nanomaterial at different environmental conditions (different pHs, electrolyte concentrations and presence of natural organic matter – NOM), and (iii) the determination of the mass concentration of the nanomaterial in a set of test vials while the particles suffer agglomeration and settling in the different environments used. The conditions used in the test are mimicking the composition of phys-chem parameters found in 90-95% of natural freshwater bodies. The presence of NOM needs to simulate the interaction between the nanomaterial and the organic matter present in natural waters, supports pH stability through its inherent buffering capacity and promotes the dispersion stability. In turn, the use of an electrolyte such as $\text{Ca}(\text{NO}_3)_2$ aims to assess the impact of ionic strength on the colloidal stability of the particles. The electrical double layer (EDL) is the region near the particle surface where the electric potential of the surface is balanced by counter-ions in the solution. The higher is the electrolyte concentration and ion valence, the smaller is the distance from the particle surface at which the electric potential vanishes. Apart from many other forces, the electrostatic interaction is controlling the stability of the dispersion. For particles with the same polarity on their surface charge, low electrolyte concentration promotes stability, while high electrolyte concentration promotes their agglomeration and sedimentation. Finally, pH influences the surface charge of the particles by protonation or deprotonation of charged groups, affecting the magnitude and the polarity of the surface charge. The effect of pH is particularly important for electrostatically stabilized nanoparticles, once they are unstable at their isoelectric point (IEP), i.e., pH of Point of Zero Charge (PZC).

It has to be noted that this guideline is applicable to nanoparticles with a density higher than 1 kg/L and to dispersions with number of particles ranging from 0.5×10^{12} and 5×10^{12} particles/L within the analysed samples. The difference in terms of density between the particles and the medium as well as the particle size are significant factors that affect agglomeration of particles during the test. Nanomaterials with a density smaller than 1 kg/L will not settle down and will remain at the air-interface in the vessels. Moreover, if identical mass concentrations are used for particles of different size and density, results at fixed time points will vary just because of different number concentrations at the beginning of the test.

The mass concentration of dispersions must be determined by a precise and sensitive analytical method, such as Inductively Coupled Plasma Mass Spectrometry (ICP-MS) or Inductively Coupled Plasma Optical Emission Spectrometry (ICP-OES) methods, depending on the expected concentrations. In the first part of the test (screening test) (Figure 1), the test covers a range of electrolyte concentrations ($\text{Ca}(\text{NO}_3)_2$ 0, 1 and 10 mM), pHs (4, 7 and 9) and NOM. The stability of the particle is measured by the concentration of the nanomaterial on the supernatant in two time points: at the beginning of the test (0h) and at 6h of experiment, after application of a centrifugation step with particle size cut-off of 1 μm . According to the results obtained, particles are classified as follows:

- completely stable: $\geq 90\%$ of the nanomaterial remains in the dispersion at the end of the test;
- intermediate stability: the mass concentration of the nanomaterial in the dispersion is between 10 and 90%, depending on the condition applied (pH, ionic strength and presence of NOM); and
- not stable: $\leq 10\%$ of the nanomaterial remains in the dispersion at the end of the test.

The second part of the test is only recommended for particles with intermediate stability, for which the mass concentration is measured every hour during a period of 6 h, in presence or absence of NOM.

RiskGONE project considers this guideline crucial and encourage the use and application of this guideline. From a theoretical point of view, not further improvements seem to be necessary. To confirm this evaluation, the test guideline was selected to be assessed also from the experimental point of view.

1.1.2 Experimental evaluation of selected OECD test guidelines

OECD TG318: Dispersion stability of nanomaterials in simulated environmental media

Preparation of ENM dispersions

The first step of the experimental evaluation of the TG 318 was the selection of reference nanomaterials to be used. For unexperienced users of the test protocol, the TG recommends the use of three reference nanomaterials from JRC to validate the method: Ag NPs (NM-300K), as completely stable material; CNTs (NM-100) as not stable material; TiO_2 (NM-105) as material with intermediate stability). As starting point, it was decided to gain some experience with the test guideline by using a material with good colloidal stability, like silver nanoparticles. Since the supply of Ag NPs (NM-300K) from JRC was discontinued, it was decided to use commercial silver nanoparticles (PVP-coated silver nanopowder, Sigma-Aldrich, ref: 576832, particle size <100 nm). The protocol described in the TG 318 (not shown here) states that the preparation of 125 mL of a stock dispersion with a particle concentration not higher than 20X the concentration of particles in the analysed dispersion (0.5×10^{12} - 5.0×10^{12} part/L) is required. In addition, stock dispersions should be prepared by pre-wetting the nanoparticles during the previous 24h and by applying a sonication energy of 40 W through a 13 mm probe diameter (previously calibrated) during 10 min. By following these instructions, the concentration of silver nanoparticles within dispersion analysed was kept at 1.0×10^{12} part/L and the concentration of the stock dispersion at 10^{13} part/L (10X the final concentration of the particles in the

analysed dispersion). For the silver nanoparticles selected, it corresponded to a mass concentration of 0.048 mg/mL (6.86 mg silver nanopowder in 125 mL water).

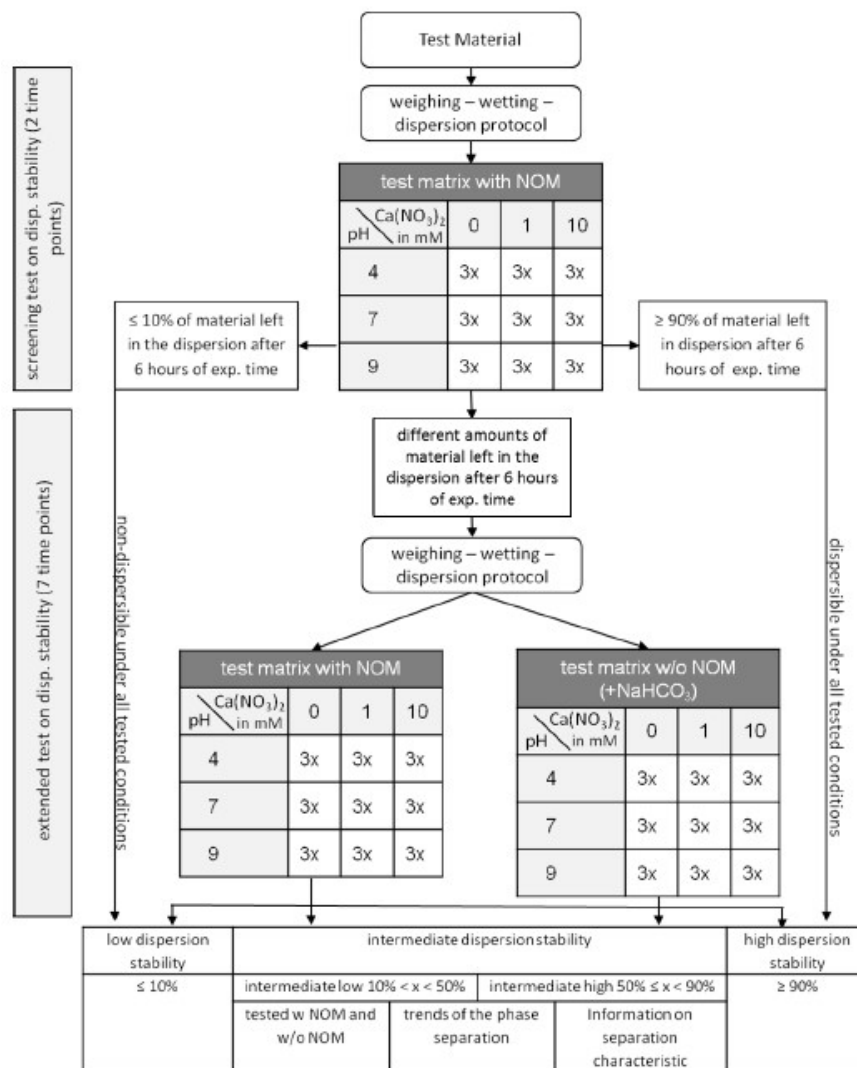
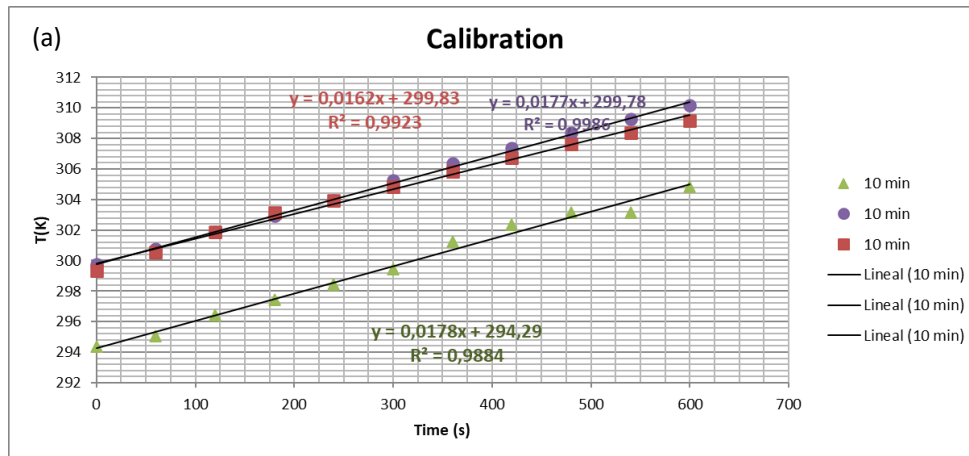


Figure 1. Decision-tree layout of the tiered testing for dispersion stability. For nanomaterials with intermediate dispersion stability, the extended test is required under all test conditions of the screening test.

The results of the sonicator calorimetric calibration are displayed in the Figure 2a. The delivered acoustic power was calculated by using the formula $P = dT/dt \cdot M \cdot C_p$, where dT/dt is the increase of the temperature of the medium during the experiment, C_p is the specific heat capacity of the medium (4.184 kJ/g*K for water), m is the mass of the medium (g). The dT/dt value was obtained by calculating the slope of the linear data fits shown in Figure 2a. The reason why the graphs are not on

top of each other is due to the different starting temperatures, but the slopes values are quite similar for the three calibrations performed. Thus, the average delivered power was estimated to be 36.0 ± 1.9 W. Nevertheless, after application of the delivered power recommended by the TG 318, it was not possible to get stable silver dispersions and sedimentation occurred immediately after the end of the sonication, as shown by Figure 2b. In addition, the measurements of the particle size distribution in the suspension were also not possible, because DLS analysis highlighted the presence of large particles, sedimentation phenomena or high polydispersity that made DLS technique (based on the movement of the particles by the Brownian motion) invalid for the characterization of hydrodynamic diameter. This result was quite surprising because the selected Ag NPs are provided with PVP as dispersant, a water-soluble polymer that should help particle stabilization in aqueous medium. To overcome this stability issues, the protocol was applied again on a new PVP-Ag NPs suspension prepared by decreasing the final volume (assuming to deliver higher sonication energy to the dispersion). Measurement of particle size by using DLS technique confirmed that the volume reduction resulted beneficial in increasing the stability of silver NP dispersions, due to the increase of the delivered sonication energy (DSE) per volume unit (the DSE applied was up to 3 times higher than the recommended by TG318). Figure 3 shows the hydrodynamic size distribution of silver nanoparticles in water. The average particle size obtained 190.0 ± 7.0 nm ($PDI = 0.431 \pm 0.028$). However, silver nanoparticles again sedimented quite fast, making impossible to use this dispersion as stock dispersion for the next steps of the protocol about ENM environmental fate study.



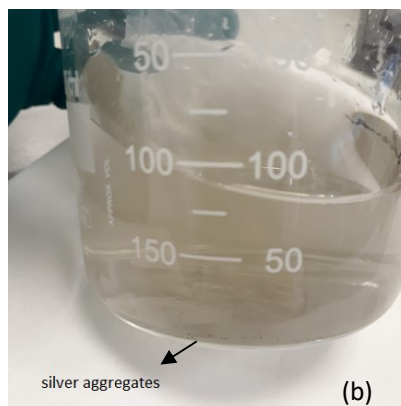


Figure 2. (a) Determination of the delivered acoustic energy by calorimetric measurements. (b) Silver NP dispersions (125 mL) after 10 min of sonication at 40 W.

	Size (d.nm):	% Intensity:	St Dev (d.nm):
Z-Average (d.nm): 198,1	Peak 1: 348,5	96,0	304,3
Pdl: 0,417	Peak 2: 4778	4,0	738,6
Intercept: 0,738	Peak 3: 0,000	0,0	0,000
Result quality : Good			

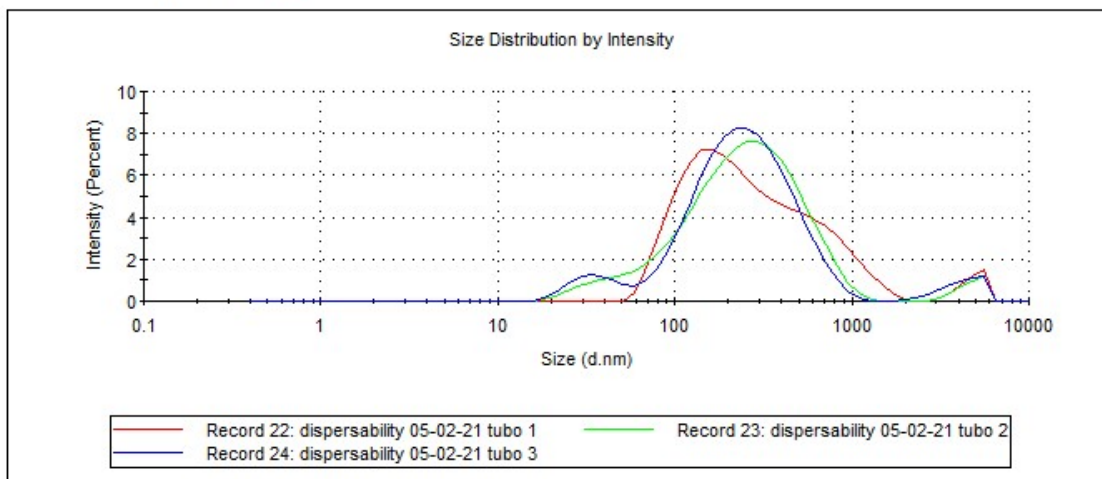


Figure 3. Hydrodynamic size distribution of silver nanoparticles in water obtained by dynamic light scattering technique.

To overcome the issues related to the poor colloidal stability of silver nanoparticles from Sigma-Aldrich, it was decided to use commercial silver nanoparticles already provided in dispersion form (colloidal citrate-coated silver nanoparticles, 0.1 gL⁻¹, particle size: 10 nm, Plasmachem). In this way, it was possible to skip step 1 to step 2 of the protocol, i.e., the preparation of ENM dispersions in different environmental conditions (screening test).

Preparation of ENM dispersions in different environmental conditions

Silver dispersions were prepared as follows. In a 50 mL plastic tube, known volumes (17.6, 88.0 or 176 μ L) of silver stock dispersion were added. Deionized water was then added up to 20 mL and 0.8 mL of NOM stock solution (prepared according to the TG 318 protocol) was also added to achieve a concentration of 10 mg/L DOC (dissolved organic carbon). Then, 0.1 M $\text{Ca}(\text{NO}_3)_2$ solution was added to achieve the desired concentration in the medium (0, 0.4 and 4 mL for 0, 1, and 10 mM, respectively). Afterwards, the volume was adjusted to 35 mL by addition of water and the pH was adjusted to 4, 7 or 9 by using small amounts of 0.1 M HNO_3 or 0.1 M NaOH solutions. The pH of the dispersion was checked again after 1 h of rest. Tubes were kept closed to avoid influence of CO_2 on the pH of the sample. Then, the volume of the dispersion was raised by water addition up to 40 mL and the tubes were sonicated for 30s in ultrasonic bath. Each test condition was analysed in triplicates.

Sampling and quantification of mass concentration in the supernatant using ICP-OES

The aliquots of supernatant from the test vials were taken from the top 0.5-1 cm volume of the dispersions. The recommended volume is 0.5 mL. At $t = 0$ h, 0.5 mL of sample was taken from the top of the tube and then put in a vial (15 mL). Then 9.5 mL of 0.235 %vol. HNO_3 was added to each falcon tube to digest particles for ICP-OES analysis. The tubes were vortexed during 30 s and remained in agitation at RT for 24h. Before analysis with ICP, samples were filtered using a 0.45 μ m filter. Just before starting the 6h experiment, samples were centrifugated during 4 min at 25°C and 439 rpm to promote the sedimentation of particle agglomerates larger than 1 μ m. Then, at time $t = 6$ h, 0.5 mL of sample was taken from the top of the tube, transferred to a vial and the particles were digested with HNO_3 solution during 24h. All the tubes were stored at the dark at 4°C prior ICP analysis. Determinations of silver concentrations were made using an ICP-OES instrument (Spectroblue FMX36, Spectro). Quantitative measurements were performed by means of a standard curve prepared by using silver concentrations between 0.02 and 0.2 mg/L.

Results

As mentioned earlier, TG 318 is applicable to particle dispersions containing a number of particles ranging from 0.5×10^{12} and 5.0×10^{12} particles/L in the analysed samples. In agreement with this recommendation, dispersions with number of particles equal to 1.0×10^{12} particles/L (0.044 mg/L) and 5.0×10^{12} particles/L (0.22 mg/L) were chosen to check the applicability of the test guideline. Moreover, the silver NP dispersions were exposed to different electrolyte concentrations ($\text{Ca}(\text{NO}_3)_2$ 0, 1 and 10 mM) in order to study the effect of this parameter on the colloidal stability of ENMs in the presence of NOM. According to the TG 318, presence of $\text{Ca}(\text{NO}_3)_2$ leads to agglomeration of particles in the extent proportional to the concentration of electrolyte, while NOM stabilizes the dispersions with electrolyte concentration.

Table 2 shows the silver concentrations measured for all experiments performed. For samples prepared by using particle concentrations of 1.0 and 5.0×10^{12} particle/L and the recommended ICP digestion procedure (0.5 mL dispersion + 9.5 mL HNO_3 solution), silver concentrations were quite small (≤ 0.01 mg/L) to be detected by ICP-OES technique, in which the limit of detection is 12 ppb (0.012 mg/L), according to the manufacturer. Due to this limitation, it was not possible to detect any

effect of the electrolyte concentration on the results obtained. For this reason, the next activities were focused on the optimization of the protocol to make possible the quantification of silver by ICP-OES technique.

To pursue this objective, the amount of the dispersion volume analysed by ICP-OES was increased from 0.5 to 1.5 mL and the initial particle concentration in the dispersions was increased up to 1.0×10^{13} part/L. For dispersions with lower particle concentration (5.0×10^{12} part/L), the results shown in Table 2 indicated that the silver concentrations were within the standard curve concentration range (0.02-0.2 mg/L) only when the volume of the dispersion taken was increased up to 1.5 mL. However, the increase of the initial concentration of particles up to 1.0×10^{13} part/L resulted in a detectable concentration only when the volume of silver dispersion taken was greater than or equal to 1.0 mL.

Table 2. Summary of Ag concentration results results obtained by analysing Ag NPs dispersions through ICP-OES at different time points.

Parameter studied	Number of particles (part/L) / mass concentration	Conditions	ICP-OES digestion procedure	Ag concentration	
				Expected concentration (mg/L)	Measured concentration (mg/L)
[Ca(NO ₃) ₂]	1,0 x10 ¹² / 0,044mg/L	Ag NPs + NOM pH 7 + Ca ²⁺ (0, 1 and 10 mM)	0.5 mL dispersion + 9.5 mL HNO ₃ sol.	0.002	All Ag concentrations at t=0 and t=6h smaller than 0.01 mg/L
Particle concentration/ [Ca(NO ₃) ₂]/ concentration of dispersion analysed by ICP-OES	5.0 x10 ¹² / 0.22 mg/L	Ag NPs + NOM pH 7 + Ca ²⁺ (0 and 10 mM)	0.5 mL dispersion + 9.5 mL HNO ₃ sol.	0.011	≤0.01 mg/L for all conditions and time points analysed (0 and 6h)
		Ag NPs pH 7 (no NOM and Ca ²⁺)	0.5 mL dispersion + 9.5 mL HNO ₃ sol.	0.011	
		Ag NPs pH 7 (no NOM and Ca ²⁺)	1.0 mL dispersion + 9.0 mL HNO ₃ sol.	0.022	At t=0h, 0.017 ± 0.001 At t=6h, 0.015 ± 0.002
		Ag NPs pH 7 (no NOM and Ca ²⁺)	1.5 mL dispersion + 8.5 mL HNO ₃ sol.	0.033	At t=0h, 0.025 ± 0.002 At t =6h, 0.028 ± 0.003
Particle concentration/ concentration of dispersion analysed by ICP-OES	1.0 x10 ¹³ / 0.44mg/L	Ag NPs pH 7 (no NOM and Ca ²⁺)	0.5 mL dispersion + 9.5 mL HNO ₃ sol.	0.022	At t=0h, 0.016 ± 0.001 At t=6h, 0.019 ± 0.001
		Ag NPs pH 7 (no NOM and Ca ²⁺)	1.0 mL dispersion + 9.0 mL HNO ₃ sol.	0.044	At t=0h, 0.034 ± 0.000 At t=6h, 0.036 ± 0.002

		Ag NPs pH 7 (no NOM and Ca ²⁺)	1.5 mL dispersion + 8.5 mL HNO ₃ sol.	0.066	At t=0h, 0.052 ±0.001 At t=6h, 0.062±0.008
Ag NPs: silver nanoparticles HNO ₃ sol.: in all samples the final concentration of nitric acid was 0.2% (% by volume).					

As 5.0×10^{12} part/L is the maximum particle concentration acceptable for the evaluation of the dispersion stability in environmental media (according to the TG318), the next experiments performed within RiskGONE WP4 task 4.2 were carried on by keeping this particle concentration and increasing the volume of the sample taken for acid digestion from 0.5 mL (as recommended by the TG 318) to 1.5 mL.

In addition, the TG318 also recommends the analysis of the colloidal stability of silver nanoparticles in all the simulated conditions (with NOM and different electrolyte concentrations) as well as the evaluation of potential release of silver ions in the medium before the end of the experiment at 6h. If the test nanomaterial dissolves in a considerable amount, it will turn in a false positive result since the released ions stay in the supernatant after the centrifugation step. Unfortunately, since October 2021 the ICP-OES equipment of CIDETEC (main partner responsible for experimental part of this work) has faced technical issues that made impossible the determination of silver by using this technique. Those technical issues have been solved recently and activities will be restarted in the following weeks. Results are expected by the end of August.

OECD TG111: Hydrolysis as a function of pH

Meanwhile the experimental evaluation of other OECD TG111 “Hydrolysis as a function of pH” has been carried out to assess the applicability of this guideline to ENMs. In the same manner of TG318, the TG111 aims to study the impact of chemical in waters, by studying the stability of test substance against hydrolysis at different pHs (4, 7, and 9), as well as the identity and rates of formation of hydrolysis products. As previously mentioned, this TG is only applicable to compounds with sufficient solubility in water. As previously mentioned, the test foresees a tiered approach depending on the water solubility. If the water solubility of the test substance is not higher than 2×10^{-2} M, a preliminary test can be performed. If less than 10% of the hydrolysis is observed after 5 days of incubation of the dispersions at 50°C, the substance is considered hydrolytically stable. If the solubility of the substance is higher than 10%, the advanced test should be performed at the pH values at which the substance was found unstable. Following this approach, the first step of this study was the selection of nanomaterials to be used as test substance. For this purpose, two ENMs already used in the framework of RiskGONE project have been chosen: ZnO (suspension form, Sigma-Aldrich, ERM00000063) and CuO (nanopowder, Plasmachem, ERM00000088). The information about the solubility of those ENMs at the different pHs indicated by the test is not completely known, but it is expected to be smaller than 10%. As in the experimental evaluation of TG318, issues with the sensitivity of ICP-OES technique used for the characterization step were observed, therefore it was decided to perform a “pre-validation” of the TG111 by investigating the effect of pH combined to the effect of the particle concentration in the dispersions. IMI kindly offered to contribute to the experimental work by performing the determination of the mass concentration by Atomic Absorption Spectrometry (AAS).

Preparation of ENMs dispersions at different pHs

ENM dispersions were prepared as follows. CuO NP dispersion was prepared following the sonication procedure like that one used in WP4-Round Robin 3 (Particles dispersion protocol for DLS and Z potential measurements of RiskGONE ENMs – RR3). Initially, a certain amount of CuO nanopowder was weighed in a glass vial and then sterile water was added to obtain a 1000 mg/L dispersion. The dispersion was sonicated at 100% amplitude (continuous mode) until a stable dispersion with the smallest possible size of aggregates was achieved (5 min). Afterwards, 0.5 mL of CuO stock dispersion was diluted in different volumes of buffer solution at different pHs (the preparation of buffer solutions is not detailed here but it can be found in the Annex I). In the case of ZnO suspensions, the dispersions were prepared by direct dilution of concentrated suspension in the buffer solutions. The samples were degassed using nitrogen during 5 min, the vials hermetically closed, and incubated at $50.0 \pm 0.5^\circ\text{C}$ in the dark for 5 days. Then, all the dispersions were centrifugated during 15 min at 3000 rpm. The supernatant of the samples was collected and stored in proper vials to be shipped to IMI (Croatia). The determination of the mass concentration of CuO and ZnO by AAS in IMI is currently in progress. As soon as the experimental work will be completed, the results will be reported.

Table 3 lists all the samples prepared during this part of the work.

Table 3. Dispersions prepared during the experimental activities direct to evaluation of OECD TG111.

ENM dispersion	Volume of stock dispersion (ml)	Buffer solution used	Volume of buffer solution (mL)
ZnO	0.5	pH 4	0.5
		pH 7 or	1.0
		pH 9	10
CuO	0.5	pH 4	0.5
		pH 7 or	1.0
		pH 9	10

2. Deviations from Description of Action – impact/how you cope with them

COVID-19 pandemic restrictions combined with technical problems encountered during the characterization of ENMs by ICP-OES equipment in CIDETEC (main partner responsible to perform the experimental activities) caused a serious delay on the activities of WP4-Task 4.2. Consequently, some experimental activities of this task are still in progress (they should have been finalized by M36). To minimize the impact of this deviation, a backup plan was agreed between CIDETEC and IMI, in which IMI offered its support for the chemical analysis of samples prepared by CIDETEC. Samples have been sent already to Croatia and they are currently under analysis by Atomic Absorption Spectrometry.

3. Conclusions

The evaluation of available OECD methods for the environmental fate of chemicals to be applied to ENMs has been performed in this work. Initially, a theoretical evaluation of critical aspects related to the applicability of the protocols to ENMs was done. Then, two test guidelines were chosen by RiskGONE partners for their experimental evaluation. RiskGONE consortium concluded that in principle all the selected TGs were applicable to nanomaterials, but some adaptation of the methods may be required. Based on the work carried out in this project, some modifications of the existing test guidelines have been identified and they will be furtherly evaluated by RiskGONE partners to see whether they can be translated in SPSF for the submission to OECD.

Below the main critical steps to be addressed for each TG:

- OECD TG111: revision of the TG to address the effect of the presence of colloidal particles in the test substance and inclusion of practical examples regarding the application of the test guideline to nanomaterials.
- OECD TG106: revision of the TG clarifying the impact of the test conditions on the colloidal stability of ENMs and suggesting how to avoid overestimation of adsorbed nanomaterial due to the formation of NPs aggregates that sediment upon centrifugation.
- OECD TG312: revision of the guideline including practical examples for the application of the guideline to inorganic and organic particles. Moreover, it is recommendable to show a method that makes possible to quantify properly the leaching of some nanomaterials, i. e., inorganic particles, from a material containing other colloidal particles, as soil.
- OECD TG116: adaptation of the protocol to be applied to not pure substances, e.g., multicomponent nanomaterials This modification will contribute to a more realistic guideline to evaluate the environmental fate of several commercial ENMs.
- OECD TG318: through the theoretical analysis of this TG any need for modification has been identified. However, the preliminary results obtained through the experimental evaluation of the TG indicate that i) the protocol for the dispersion preparation is not applicable for all the sizes of commercial silver particles, and ii) the particle concentration used for small size nanomaterials, such as 20 nm Ag NPs, should be high enough to get reliable results even when using sensitive techniques like ICP-OES. For these reasons, the analysis of a volume of dispersion bigger than the one foreseen by the original protocol is highly recommended. Therefore, it would be very helpful if this TG could include some explanation about the need of using bigger volumes when small size NPs are used to prepare the dispersions. In addition, although this TG mentions that the method is applicable to materials such as MWCNTs, results related to this material cannot be found in the test guideline. Therefore, the publication of results regarding the experimental evaluation of MWCNTs is also recommended.

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Annex I

Buffers used in the experimental evaluation of TG111 were prepared as follows.

Buffer pH 4: Using a 100 mL volumetric flask, 0.4 mL of 1M sodium hydroxide (NaOH) solution and 50 mL of 0.1 M potassium biphthalate solution were diluted by addition of water. After checking the pH by potentiometry, the final solution (pH 4.09) was sterilized by filtration using syringe filters (pore size 0,22 µm) and transferred to a very clean glass/plastic bottle and kept at RT.

Buffer pH 7: Using a 100 mL volumetric flask, 29.63 mL of 1M sodium hydroxide (NaOH) solution and 50 mL of 0.1 M monopotassium phosphate solution were diluted by addition of water. After checking the pH by potentiometry, the final solution (pH 7.07) was sterilized by filtration using syringe filters (pore size 0,22 µm) and transferred to a very clean glass/plastic bottle and kept at RT.

Buffer pH 9: Using a 100 mL volumetric flask, 21.3 mL of 1M sodium hydroxide (NaOH) solution and 50 mL of 0.1 M boric acid in 0.1 M potassium chloride solution were diluted by addition of water. After checking the pH by potentiometry, the final solution (pH 9.07) was sterilized by filtration using syringe filters (pore size 0,22 µm) and transferred to a very clean glass/plastic bottle and kept at RT.





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