A status report – July 2022

NanoHarmony

NANOMET

DEVELOPMENT OR REVISIONS OF OECD TEST GUIDELINE (TG) AND GUIDANCE DOCUMENTS (GD) APPLICABLE FOR NANOMATERIALS

Authors: Elisabeth Heunisch [1], Flemming Cassee [2], Eric Bleeker [2], Thomas Kuhlbusch [1], Mar Gonzalez [3]

[1] Federal Institute for Occupational Safety and Health (BAuA), Germany; [2] National Institute for Public Health and the Environment (RIVM), Netherlands; [3] Organisation for Economic Co-operation and Development (OECD)

1 Background

This document summarizes the current status of projects (aimed) to develop or adapt OECD Test Guideline (TGs) and Guidance Documents (GDs) for nanomaterials as well as the outlook per project[1]. The list was compiled in a common effort by EU projects NanoHarmony and NANOMET and is intended to be a living document that will be updated regularly. A large part of the document comprises activities in EU projects like NanoHarmony, Gov4Nano, RiskGONE and NANORIGO and national projects that contribute within the Malta Initiative to the development of TGs and GDs for nanomaterials. Apart from the projects supported by the OECD Working Group of National Co-ordinators of the TGs programme (WNT) or OECD Working Party on Manufactured Nanomaterials (WPMN), an additional number of standardization activities are presented in Chapter 4 that develop test methodologies for which the initiators foresee an OECD project status in the near future[2].

1.1 OECD Test Guidelines

Innovation is one of the key factors in securing the welfare of current and future generations. Legislation has to keep pace with innovative developments to ensure the trust of citizens in innovation, e.g. on safety of new developments. Appropriate and clear legislation is therefore a key factor for long-term investments. Where legislation is unclear, investors will be cautious.

International collaboration at OECD level is one element in addressing the global challenges associated with innovation. Such collaboration facilitates the sharing of knowledge, e.g. through the legal acceptance of data. The OECD Council Decision on Mutual Acceptance of Data (MAD) is a legally binding instrument in OECD member countries and non-OECD countries which have adhered to them. MAD is an important instrument in facing the global challenges of testing nanomaterials. In essence, MAD means that data collected in one country must be accepted (in a legal sense) in all countries, provided that (an) agreed OECD Test Guideline(s) has/have been used and the OECD Principles of Good Laboratory Practice have been applied during the collection of the data. MAD avoids the duplication of testing of substances, reduces the amount of animal testing and saves on resources[3].

Generally, Test Guidelines (TGs) and Guidance Documents (GDs) for the testing of 'traditional' chemical substances are also applicable to nanomaterials. Nevertheless, specific properties of nanomaterials require specific information needs. For a number of endpoints TGs/GDs may not sufficiently address such specific information requirements when dealing with nanomaterials. As a

[1] As of July 2022

[2] For further information and involvement in projects please contact nanoharmony@baua.bund.de and/ or the OECD Secretariat (mar.gonzalez@oecd.org). Also, please note that draft Test Guidelines and/ or Guidance Documents under revision are made available for public consultation at the following site: https://www.oecd.org/chemicalsafety/testing/chemicalstestingdraftoecdguidelinesforthetestingofchemicals-sections1-5.htm.

[3] See https://oe.cd/mad

result, some TGs/GDs may even be considered as not applicable for the testing of nanomaterials, while other TGs/GDs may only need relatively small adaptations.

1.2 The Malta Initiative

The Malta Initiative arose during the Maltese EU Council Presidency in 2017, when Germany initially approached the EU Directorate-General for Research and Innovation (DG RTD) to request political and financial support to develop and amend TGs and GDs to ensure that any nanospecific issues for fulfilling regulatory requirements are addressed. The Malta Initiative currently brings together a group of EU member states, the European Commission (notably the DG RTD, DG ENV, DG GROW and JRC[4]), the European Chemicals Agency (ECHA), industry and other institutions committed to this aim and welcomes additional international collaborators.[5]

In line also with existing procedures at the OECD, the Malta Initiative welcomes any country or organisation with expertise to become an active contributor, when they are interested in working on adapting existing OECD TGs or developing new OECD TGs and/or GDs.

The activities of the Malta Initiative are supported through national, international and EU resources by means of direct funding, in-kind contributions, or providing expertise.

Table 1 provides an overview of current OECD TG and GD projects for nanomaterials, their Test Guideline Programme (TGP) project number or TG/GD number for projects completed. Further details on the different projects are given in Chapters 2 and 3. Table 3 outlines standardization initiatives supported by the EU H2020 project RiskGONE that are intended to be submitted to the OECD in the near future. This list only shows examples for standardization activities towards OECD for nanomaterials and is not intended as an exhaustive list of all (EU) research project activities towards OECD TGs or GDs.

[4] Directorate-General for Environment (DG ENV); Directorate-General for Internal Market, Industry, Entrepreneurship and SMEs (DG GROW); Joint Research Centre (JRC), i.e. the Commission's science and knowledge service.

[5] Austria, Denmark, Finland, France, Germany, Greece, Italy, Luxembourg, Netherlands, Norway, Poland, Portugal, Romania, Slovenia, Spain, Sweden, Switzerland, United Kingdom, ECHA, European Commission and BIAC currently support the Malta Initiative.

Table 1: List of OECD projects on Test Guideline and Guidance Documents for nanomaterials, either ongoing or finished (2017 – June 22).

Project Title	Project ID or TG/ GD No.	Description under chapter	
Section 1: Physical Chemical Properties			
Test Guideline on Determination of the (Volume) Specific Surface Area of Manufactured Nanomaterials	TG 124	2.1	p. 9
Test Guideline on Particle Size and Size Distribution of Manufactured Nanomaterials	TG 125	2.2	p. 10
Determination of solubility and dissolution rate of nanomaterials in water and relevant synthetic biologically mediums	TGP* Project 1.5	3.1	p. 22
Identification and quantification of the surface chemistry and coatings on nano- and microscale materials	TGP Project 1.6	3.2	p. 23
New Test Guideline on Determination of the Dustiness of Manufactured Nanomaterials	TGP Project 1.8	3.3	p. 24
New Test Guideline on Determination of Surface Hydrophobicity of MNs	TGP Project 1.7	3.4	p. 26
Guidance Document on the determination of concentrations of nano-particles in biological samples for (eco)toxicity studies	TGP Project 1.10	3.5	p. 27
Guidance on Release Tests for Manufactured Nanomaterials	WPMN** Project	3.6	p. 29
(Update) Guidance Document on Sample Preparation and Dosimetry for the Safety Testing of Manufactured Nanomaterials (GSPD)	WPMN Project	3.15	p. 41
Section 2: Effects on Biotic Systems			
Technical recommendations for conducting assays with ENMs according to OECD Test Guidelines 201, 202 and 203	TGP Project 2.71	3.7	p. 30
Section 3: Environmental Fate and Behaviour			
Test Guideline No. 318: Dispersion stability in simulated environmental media	TG 318	2.3	p. 12
Guidance Document for the testing of dissolution and dispersion stability of nanomaterials and the use of the data for further environmental testing and assessment strategies	GD 318	2.4	p. 13
Test Guideline on dissolution rate of nanomaterials in aquatic environment	TGP Project 3.10	3.8	p. 32
Study Report on MNS Removal in Wastewater Treatment Plants: Activated Sludge Sorption Isotherm	Study Report No. 340	2.5	p. 14
Guidance Document on assessing the apparent accumulation potential for nanomaterials TG 305	TGP Project 3.12	3.9	p. 33
Guidance Document Behaviour in Soils Using TG 312 for NMs	GD 342	2.6	p. 15
Guidance Document and Test Guideline on Aquatic (Environmental) Transformation of Nanomaterials	TGP Project 3.16	3.10	p. 34
Assessment of the durability of NMs and their surface ligands in env. surroundings (biodurable/ biodegradable)	WPMN / No. 86	2.7	p. 16
Scoping review for a tiered approach for reliable bioaccu. assess. of MNs in environ. organisms minimising use of higher tier vertebrate tests	WPMN Project	3.11	p. 36

Table 2 (con´t): List of OECD projects on Test Guideline and Guidance Documents for nanomaterials, either ongoing or finished (2017 – June 22).

Project Title	Project ID or TG/ GD No.	Description under chapter	
Section 4: Health Effects			
Test Guideline Subacute Inhalation Toxicity: 28-Day Study	TG 412 (updated)	2.9	p. 18
Test Guideline Subchronic Inhalation Toxicity: 90-day Study	TG 413 (updated)	2.10	p. 19
Guidance Document on Inhalation Toxicity Studies	GD 39	2.11	p. 20
Study Report and preliminary guidance on the Adaptation of In Vitro Mammalian Cell Based Genotoxicity TGs for Testing of MNs	Study Report No. 359	2.8	p. 17
Applicability of the TG 442D *in vitro* skin sensitisation for nanomaterials	TGP Project 4.133	3.12	p. 37
Test Guideline on toxicokinetics to accommodate testing of nanoparticles	TGP Project 4.146	3.13	p. 38
Guidance *Document on* Integrated In Vitro Approach for Intestinal Fate of Orally Ingested Nanomaterials	TGP Project 4.158	3.14	p. 39

* **TGP** = Test Guideline Programme
** **WPMN** = Working Party of Manufactured Nanomaterials

Table 3: Examples of standardization activities aiming at OECD from the EU H2020 project RiskGONE (H2020-NMBP-13-2018 RIA).

Project Title	Description under chapter	
Particle counting	4.1	p. 43
Endotoxins	4.2	p. 44
Zeta potential	4.3	p. 45
New Comet assay to detect strand breaks and specific DNA lesions of Daphnia magna (or other species) exposed in vivo	4.4	p. 46
Daphnia magna reproduction test (with male induction)	4.5	p. 47
Technical recommendations for conducting the In Vitro Mammalian Cell Gene Mutation Test (OECD TG 476) with ENMs	4.6	p. 48
Colony forming efficiency (CFE)	4.7	p. 50
New in vitro guideline for comet assay to detect strand breaks and specific deoxyribonucleic acid (DNA) lesions	4.8	p. 51
Cell transformation assays	4.9	p. 52
Impedance-based label-free assessment of nanotoxicity	4.10	p. 53

2 OECD documents completed

2.1 Test Guideline No. 124 on Determination of the Volume Specific Surface Area of Manufactured Nanomaterials

Year published: 2022

Lead: Joint Research Centre (EU) **Category:** Physical Chemical Properties

Short Project Description:

The development of a TG for the determination of the Specific Surface Area and the Volume Specific Surface Area of Manufactured nanomaterials has been in the workplan of the OECD Test Guidelines Programme (WNT-TGP) since April 2018 led by the JRC. The determination of the Surface Area of Manufactured Nanomaterials is based on the Brunauer, Emmett and Teller (BET) and further theoretical and practical developments as reflected in several ISO standards or the work of e.g. the NanoDefine project. This technique is based on the physical adsorption of an inert gas on the surface of a solid material. The project also includes the use of gaseous pycnometry or other methods to address density and porosity via measurement of sample volume. An ad-hoc expert group to support the project was created and met in October 2018 (including among others: BAM, NPL, CSIC, INERIS, NPL, LNE, US-EPA). A draft Test Guideline and a SOP was developed in 2019. The experts also agreed on the organisation of an interlaboratory exercise in two phases and on a list of materials to be used in such exercise.

The draft TG was approved by the WNT on April 27th 2022 and endorsed by the Chemicals and Biotechnology Committee (CBC). The TG became publicly available on 30th June 2022.

The Test Guideline and the report on the interlaboratory comparison are available as:

- Test No. 124: Determination of the (Volume) Specific Surface Area of Manufactured Nanomaterials https://doi.org/10.1787/abb72f8f-en
- *Interlaboratory comparison on the determination of the Volume Specific Surface Area (VSSA) of Manufactured Nanomaterials*. Josefa Barrero, Chiara Senaldi, Rita La Spina, Juan Riego Sintes. EUR 30702 EN, Publications Office of the European Union, Luxembourg, 2021, ISBN 978-92-76-37685-9, https://doi.org/10.2760/41115, JRC124644.

More information: NANOMET - OECD

The scientific background for this activity was partially funded by the EU H2020 project Gov4Nano (H2020-NMBP-13-2018 RIA).

2.2 Test Guideline No. 125 on Particle Size and Size Distribution of Manufactured Nanomaterials

Year published: 2022

Lead: Federal Institute for Occupational Safety and Health (DE)

Category: Physical Chemical Properties

Short Project Description:

The TG for determining particle size distribution and fibre length and diameter distribution (OECD TG 110) had been identified by the OECD-WPMN as one of the TGs that is not adequate to investigate size and size distribution of nanomaterials. Rather than updating, a new TG for the determination of particle size and particle size distribution of nanomaterials (TG PSD) has been drafted. In 2017, the Federal Environment Agency (UBA) commissioned the Federal Institute of Occupational Safety and Health (BAuA; project coordinator) and the Federal Institute for Material Research and Testing (BAM) to develop a corresponding test guideline. In its April 2022 meeting the WNT approved the presented draft without any changes.

The size range for which the TG PSD is applicable was agreed to range from 1-1000 nm for particle and fibre diameters. This range surpasses the upper limit of size ranges currently used within most of the (regulatory) definitions of nanomaterials. This becomes necessary to determine, by means of one or more measurements, whether the substance under investigation is within or outside the size ranges of possible definitions. Additionally, the upper limit for fibre length was set at 20 µm. A fibre is defined as an object with a length to diameter ratio (aspect ratio) ≥ 3. Complementary a particle is defined as an object with an aspect ratio < 3.

The measurement range of the individual methods described in the TG PSD will in most cases not cover the entire specified size range. Therefore, it may be necessary to use more than one measurement method or measurement setup to cover the full size range if needed. The applicable methods are based on different measurement principles and may differ in their resulting values.

In preparation of the new OECD TG PSD, various measurement methods suitable for measuring particle size and size distribution of nanomaterials were considered for being included in the TG. In the selection of sufficiently widespread and reliably and reproducibly usable methods, a distinction was made between methods for particulate and fibrous nanomaterials, based on the structure of TG 110. For particulate nanomaterials, atomic force microscopy (AFM), centrifugal liquid sedimentation (CLS), dynamic light scattering (DLS), differential mobility analysis system (DMAS), (nano)particle tracking system (PTA/NTA), small angle X-ray scattering (SAXS), scanning electron microscopy (SEM) and transmission electron microscopy (TEM) were included in the draft TG PSD. The comparability and reproducibility of particles and fibres was determined and validated in a comprehensive interlaboratory comparison (ILC) in 2019. A further method, single particle mass spectrometry with inductively coupled plasma (sp-ICP-MS) could not be validated widely enough. It is included in Annex C of the TG, as it is a relevant and promising method in some cases. For this method several validations outside of this project are available for specific nanomaterials.

For fibrous nanomaterials, scanning electron microscopy (SEM) and transmission electron microscopy (TEM) are the only available methods to measure diameter and length and are included in the TG PSD. Both methods can determine length and diameter for each fibre separately hence enabling the determination of fibre size mixtures. Measurement instructions for both methods were validated in an ILC.

For the validation of the TG PSD the two conducted ILCs with altogether 32 participants on a total of 12 test materials were carried out successfully. The respective individual results of the participants were collected, evaluated and compared by BAM and BAuA. The complete results and all

conclusions are summarised in the validation report. The validation report has been published together with the TG.

Following the discussion with the joint WPMN/WNT Expert Group the draft TG and the Validation Report were send to WNT. In two commenting rounds the draft TG was further improved in terms of precision of method description and wording. There was no fundamental opposition on any parts of the draft TG at this time.

The draft TG was approved by the WNT on April 27th 2022 and subsequently endorsed by the Chemicals and Biotechnology Committee (CBC). The TG and the validation report are now publicly available.

The Test Guideline and the validation report are available as:

- Test No. 125: Nanomaterial Particle Size and Size Distribution of Nanomaterials https://doi.org/10.1787/af5f9bda-en
- Validation Report on Particle and Fibre Size Distribution Measurements of Nanomaterials. Supporting TG 125 on Particle Size and Particle Size Distribution of Nanomaterials. Series on Testing and Assessment. No. 352

More information: NANOMET - OECD

2.3 Test Guideline No. 318: Dispersion stability in simulated environmental media

		Year published:	2017
Lead:	German Environment Agency (DE)	**Category:**	Environmental Fate and Behaviour

Short Project Description:

The main purpose of this guideline is to assess the ability of a nanomaterial to attain a colloidal dispersion and to conserve this dispersion under environmentally relevant conditions. The test procedure involves a dispersion of the nanomaterial with the aid of a calibrated sonication procedure and the determination of the mass concentration of the nanomaterial in a set of test vials while the particles undergo homoagglomeration and settling in environments of different hydrochemistry.

The purpose of the validation study supporting Test Guideline 318 was to learn about the applicability and accuracy of proposed experimental routines, determine whether the described procedures are interpreted and implemented correctly, identify the factors that can affect results variability and perform the analysis of statistical variability. It reports the design of the round robin test and describes the procedures (participating laboratories; study design; chemicals tested; validation of the results; results; and conclusions.)

The Test Guideline and the validation report are available as:

- Test No. 318: Dispersion Stability of Nanomaterials in Simulated Environmental Media https://doi.org/10.1787/2074577x
- Report of the validation study supporting the development of the draft (new) TG 318 on dispersion behaviour of nanomaterials in different environmental media. Series on Testing and Assessment No. 276 ENV/JM/MONO(2017)28

More information: NANOMET - OECD

2.4 Guidance Document 318 for the testing of dissolution and dispersion stability of nanomaterials and the use of the data for further environmental testing and assessment strategies

		Year published:	2020
Lead:	German Environment Agency (DE)	**Category:**	Environmental Fate and Behaviour

Short Project Description:

The activity was led by Germany as WNT project 3.9. The objective of this activity was to support the development of a new OECD GD on the testing and interpretation of data regarding dissolution rate and dispersion stability (homo- and hetero-agglomeration) of nanomaterials in the environment.

The GD features an important building block for the testing of solubility, dissolution rate, and dispersion stability of nanomaterials. It supports interpretation and presentation of data coming from OECD TG 318 on dispersion stability of nanomaterials in environmental media. Furthermore, it informs how to determine dispersion stability based on heteroagglomeration of nanomaterials with natural occurring matter which is not addressed in the TG 318. It gives interim guidance on determination and interpretation of solubility and dissolution rate of nanomaterials in environmental media while the intended TG on this endpoint is still pending (currently developed within WNT project 3.10). It also presents a testing strategy to inform further environmental testing based on data on these endpoints.

At WNT-32 in April 2020, the GD was successfully adopted and published in summer 2020 as Guidance Document No. 318 The GD is accompanied with an MS Excel tool to facilitate its use.

The Guidance Document and the MS Excel tool are available as:

- Guidance Document for the Testing of Dissolution and Dispersion Stability of Nanomaterials, and the Use of the Data for Further Environmental Testing and Assessment. Series on Testing and Assessment No. 318. ENV/JM/MONO(2020)9 (Excel)

More information: NANOMET - OECD

The project was funded by the German Federal Ministry for the Environment, Nature Conservation, Nuclear Safety and Consumer Protection.

2.5 Study Report on MNs Removal in Wastewater Treatment Plants: Activated Sludge Sorption Isotherm

Year published: 2021

Lead: Environmental Protection Agency (US)

Category: Environmental Fate and Behaviour

Short Project Description:

The document includes an annex with a detailed description of the procedure for measuring the net removal extent to which a MN distributes between activated sludge and water in wastewater treatment systems. The goal of this testing is to provide sufficient information to predict the removal of a test MN in a wastewater treatment through association with sludge. The distribution of conventional contaminants between supernatant and biomass states is often described by a sorption isotherm. These isotherms can be used to develop mass balances expressions for WWTP unit processes to estimate the amount of the chemical that will be removed during wastewater treatment. The Activated Sludge Sorption Isotherm, in Fate, Transport, and Transformation Test Guidelines (OPPTS 835.1110) method uses freeze-dried biomass and has been validated for neutral and ionized organic chemicals and dissolved metals. However, the OPPTS 835.1110 method has recently been demonstrated ineffective for predicting the removal of MNs during wastewater treatment. The freeze-drying significantly alters the physical size and structure of the biofloc which reduces interaction with the MNs. MN interactions with biofloc under most WWTP conditions reach a steady-state condition related to aggregation and/or dissolution, rather than thermodynamic equilibrium that exist for some organic chemicals (e.g., neutral organic molecules). Over time the processes that bring MNs into association with larger suspended solids (heteroaggregation) and those involved in the release from heteroaggregates (break-up) may reach a steady state. This steady state describes the maximum amount of MNs that may be removed by settling alone. This removal will vary as a function of mixing conditions and the chemistry of the suspended solids and nature of the MNs under testing.

Evaluations of the net removal of MNs observed at bench scale conditions provide useful information on what might be expected at full scale. The test procedures presented here are formulated to be used as a screening level assessment for potential releases from wastewater treatment plants and the resulting concentrations in receiving water bodies.

The document is available as:

- Study Report on a test for removal in wastewater treatment plants of gold manufactured nanomaterial (mn): activated sludge sorption isotherm. Series on Testing and Assessment No. 340. ENV/CBC/MONO(2021)15 (Annex: Excel File)

More information: NANOMET - OECD

2.6 Guidance Document 342 on Behaviour in Soils using TG 312 for NMs

Year published: 2021

Lead: Environment and Climate Change Canada (CAN) / German Environment Agency UBA (DE)

Category: Environmental Fate and Behaviour

Short Project Description:

This activity was led by Canada and Germany. The SPSF to this activity was accepted by OECD WNT in April 2017.

The objective of this activity was to improve the applicability of OECD TG 312 “leaching in soil columns” for the testing of mobility of nanomaterials in soils. Environmental behaviour and fate of nanomaterials is distinctively driven by kinetic processes (agglomeration, sedimentation), but there was no method available to appropriately determine fate of nanomaterials in soils.

While the existing OECD TG 312 was considered generally applicable to test nanomaterials, additional guidance with specifications for the testing of engineered nanomaterials was needed in order to reliably report on the mobility and fate of NMs in soils. Furthermore, proper guidance to predict NMs behaviour should rely on advanced/available modelling of environmental behaviour of NMs and the use of physical-chemical predictors (as alternatives to conventional coefficients) for soil transport predictions which are not yet available.

Thus, the use of OECD TG 312 for determining the fate of nanomaterials in soil becomes particularly important, and a guidance for using the TG was needed. Elements which need to be considered in such a guidance inter alia include the preparation of stock and test suspension, the application of the test substance to the test system, choice of appropriate test duration and flow rate, reliable and robust (and cost-effective) analytics as well as appropriate data interpretation.

As there was no explicit funding to guide this activity, development of the GD was mainly executed by an international core group of experts on a voluntary basis. The draft GD was commented by the OECD Joint Expert Group on Environmental Fate of Nanomaterials three times in 2018 and 2019. A core element of GD development was an interlaboratory comparison (ILC) test which ran in 2019 with 2 nanomaterials in 2 soils to which 7 labs from 4 countries contributed data. Based on the observations of the ILC, the draft GD was revised and submitted to WNT for the first commenting round in December 2020. In general, strong support for the proposed document was received and the comments on clarifications and editorial changes were considered in a final version of the draft document. As these comments were rather minor, the draft GD was submitted to WNT and successfully adopted at its 33th meeting in April 2021.

The Guidance Document and its accompanying ILC report are available as:

- Report of the inter-laboratory comparison testing for the GD to support implementation of Test Guideline No. 312 for Nanomaterial safety testing. Series on Testing and Assessment No. 341. ENV/CBC/MONO(2021)16
- Guidance Document on testing Nanomaterials using OECD TG No. 312 “Leaching in soil columns". Series on Testing and Assessment No. 342. ENV/CBC/MONO(2021)17

More information: NANOMET - OECD

2.7 Assessment of the durability of NMs and their surface ligands

Lead: National Institute for Occupational Health (SA)

Year published: 2018

Category: Environmental Fate and Behaviour

Short Project Description:

The aim of this project was to emphasize the importance as well as describe methodologies to assess the biodurability of nanomaterials (NMs). Biodurability of NMs is an important property that needs to be investigated as it may determine their potential to cause harm to humans and the environment. Biodurability of NMs may be determined through their ability to release ions or undergo enzymatic or chemical disintegration.

In vitro and in vivo tests were therefore identified and also the biological media that were used to determine dissolution rate and measure biodegradation. These data helped calculate the biodurability of NMs. The role of physicochemical properties and the surface ligands and coating in affecting the rate of dissolution and degradation was also discussed. Finally, the importance of the validation of the identified in vitro and in vivo techniques was emphasized to help increase their potential to predict the biodurability of NMs.

The document is available as:

- Report in the OECD Series on the Safety of Manufactured Nanomaterials No 86. ENV/JM/MONO(2018)11

More information: NANOMET - OECD

2.8 Study Report and preliminary guidance on the Adaptation of *In Vitro* Mammalian Cell Based Genotoxicity TGs for Testing of MNs

Lead: Joint Research Centre (EU) + UK/DE[6]

Year published: 2022

Category: Health Effects

Short Project Description:

The OECD test guideline 487 (July, 2016) is the current guideline under the Mutual Acceptance of Data (MAD) agreement that instructs how an *in vitro* micronucleus assay should be performed. The procedures described in the guideline are, however, directed at testing chemicals but not specifically engineered nanomaterials (ENMs). In the test guideline this deficiency is noted, yet a recommendation of a suitable adaptation of the protocol for the testing of nanomaterials is not given. Thus, to fulfil the current regulatory requirement for testing of ENMs for possible mutagenicity, one should follow the current procedure of the guideline, which is not the optimal procedure from a scientific point of view. Parameters such as the treatment interval or selection of the test concentration are a few examples of aspects, which need specific consideration when testing ENMs; a pulse treatment of cultures with ENMs do not provide sufficient time for the internalization process of a particular material and ENMs can per se only be tested at precipitating levels. Since there is no official document on how to adapt the OECD 487 protocol for ENM testing, there was a certain level of urgency in providing a guidance on the necessary adaptations, which would allow a more relevant assessment of the mutagenic potential of ENMs and avoid repetition of inadequately performed studies. This preliminary guidance document therefore aims at addressing this gap in the guidance available.

1. The Study Report shares the lessons learnt from work carried out so far under the project and guides the users of the Test Guideline 487 when testing ENM. The whole study project was divided into 3 phases: 1 - Clarification of main technical issues; 2 - Interlaboratory comparison and 3 - Guidance development.
2. This document will be refined in future with the testing of additional ENMs.

The document is available as:

- Study Report and Preliminary Guidance on the Adaptation of the *In Vitro* micronucleus assay (OECD TG 487) for Testing of Manufactured Nanomaterials. Series on Testing and Assessment No. 359. ENV/CBC/MONO(2022)15

More information: NANOMET - OECD

[6] JRC (EU) performed preliminary experimental work / UK & DE wrote the study report

2.9 Test Guideline No. 412: Subacute Inhalation Toxicity: 28-Day Study

Lead: Netherlands and United States

Year published: 2018
Category: Health Effects

Short Project Description:

This revised Test Guideline 412 (TG 412) has been designed to fully characterize test article toxicity by the inhalation route following repeated exposure for a limited period of time (28 days), and to provide data for quantitative inhalation risk assessments. It was updated in 2017 to enable the testing and characterisation of effects of nanomaterials tested. Groups of at least 5 male and 5 female rodents are exposed 6 hours per day for 28 days to a) the test chemical at three or more concentration levels, b) filtered air (negative control), and/or c) the vehicle (vehicle control). Animals are generally exposed 5 days per week but exposure for 7 days per week is also allowed. Males and females are always tested, but they may be exposed at different concentration levels if it is known that one sex is more susceptible to a given test article. This guideline allows the study director the flexibility to include satellite (reversibility) groups, bronchoalveolar lavage (BAL), lung burden (LB) for particles, neurologic tests, and additional clinical pathology and histopathological evaluations in order to better characterize the toxicity of a test chemical.

The Test Guideline is are available as:

- Test No. 412: Subacute Inhalation Toxicity: 28-Day Study
  https://doi.org/10.1787/9789264070783-en

2.10 Test Guideline No. 413: Subchronic Inhalation Toxicity: 90-day Study

Lead: Netherlands and United States

Year published: 2018
Category: Health Effects

Short Project Description:

This revised Test Guideline 413 (TG 413) has been designed to fully characterize test article toxicity by the inhalation route following repeated exposure for a period of 90 days, and to provide data for quantitative inhalation risk assessments. It was updated in 2017 to enable the testing and characterisation of effects of nanomaterials tested. Groups of at least 10 male and 10 female rodents are exposed 6 hours per day for 90 days to a) the test chemical at three or more concentration levels, b) filtered air (negative control), and/or c) the vehicle (vehicle control). Animals are generally exposed 5 days per week but exposure for 7 days per week is also allowed. Males and females are always tested, but they may be exposed at different concentration levels if it is known that one sex is more susceptible to a given test chemical. The results of the study include measurement and daily and detailed observations (haematology and clinical chemistry), as well as ophthalmology, gross pathology, organ weights, and histopathology. This Test Guideline allows the flexibility to include satellite (reversibility) groups, interim sacrifices, bronchoalveolar lavage (BAL), lung burden (LB) for particles, neurologic tests, and additional clinical pathology and histopathological evaluations in order to better characterize the toxicity of a test chemical.

The Test Guideline is are available as:

- Test No. 413: Subchronic Inhalation Toxicity: 90-day Study
  https://doi.org/10.1787/9789264070806-en

2.11 Guidance Document No. 39 on Inhalation Toxicity Studies

		Year published:	2018
Lead:	Netherlands and United States	**Category:**	Health Effects

Short Project Description:

The Guidance Document 39 on Acute Inhalation Toxicity Testing was published in 2009. Following the revision of Test Guidelines 412 and 413 to accommodate nanomaterials. This new edition of Guidance Document 39 reflects changes made in these two Test Guidelines and provides guidance relevant to the conduct of these TGs for the safety testing of nanomaterials. Although it is recognised that the current structure does not explicitly differentiate guidance applicable to acute studies from guidance applicable to long term studies, this revised edition will assist regulators in implementing the TGs 412 and 413 for nanomaterials safety testing. The reader is referred to the text of respective Test Guidelines where explicit requirements are typically provided

The Guidance Document is are available as:

- Guidance Document on Inhalation Toxicity Studies. Series on Testing and Assessment, No. 39. ENV/JM/MONO(2009)28/REV1

3 OECD projects under development

3.1 Determination of solubility and dissolution rate of nanomaterials in water and relevant synthetic biologically mediums

Project ID: TGP Project 1.5 **Expected year to finish:** 2023
Lead: National Research Centre for the Working Environment (DK) **Category:** Physical Chemical Properties

Short Project Description:

The GD is anticipated to contain information and guidance on test systems, test conditions, test medium compositions, quantification methods and calculations and measurands for determination of solubility and dissolution rates of (nano)material substances in water and biologically relevant synthetic test media. It is anticipated that the biologically relevant test media will include simulant fluids for the representing lunglining, phagolysome, and gastro-intestinal tract. Test methods in the GD will include batch reactor and continuous flow cell methods with real-time monitoring of experimental conditions as well as coupled measurements of dissolution when possible.

The methods will be recommended based on result from intra- and interlaboratory comparison testing conducted within the OECD-project.

The expected outcome is a consensus report and a new GD providing guidance and procedures for determining the solubility limit and dissolution rate of (nano)material substances and provide a solution for testing in addition to OECD TG105. The guidance document will furthermore give recommendations and examples on how to apply the data for modelling purposes.

The scientific background for this activity was partially funded by the EU H2020 projects Gov4Nano (H2020-NMBP-13-2018 RIA) and NanoHarmony (H2020-NMBP-34-2019 CSA).

3.2 Identification and quantification of the surface chemistry and coatings on nano- and microscale materials

Project ID: TGP Project 1.6 **Expected year to finish:** 2024
Lead: National Research Centre for the Working Environment (DK) **Category:** Physical Chemical Properties

Short Project Description:

It is anticipated that the GD will guide the user in a tiered approach to identify, analyse, and decide on the presence of surface chemical modifications and give strategies for their specific analysis and quantification. The GD will contain context, definitions and guidance on the strategy for stepwise identification and quantification of surface-chemical modifications (surface chemistry, coating and functionalization) as well as recommended structure for data reporting.

The GDs may include references to already existing documents from standardization organizations. anticipated to include methods for i.a.:

1) Measurement of mass loss during thermal treatment such as the furnace method for water loss and loss-on-ignition measurements or Thermogravimetric Analysis (TGA) with or without in-line measurement of evaporated substances and decomposition products.
2) Bulk inorganic and organic chemical analysis using non-destructive (e.g., X-ray Fluorescence, XRF) and destructive methods (combustion chemical analysis or Inductively Coupled Plasma-Mass Spectrometry; ICP-MS)
3) In situ analytical techniques such as Transmission Electron Microscopy (TEM) and X-ray Photon Spectroscopy (XPS)
4) Organic chemical extraction and analysis for identification of molecular structures such as Gas Chromatography–Mass Spectrometry (GC-MS), High Performance Liquid Chromatography Mass Spectrometry (LC-MS) and Direct Infusion Electrospray Ionization Mass Spectrometry (ESI-MS).
5) Recommendation of applicable benchmark materials for the different methods
6) The most suitable technologies will be selected and appropriate test measurements conducted for their mutual approval. Testing will include intralaboratory and interlaboratory comparison studies to establish a foundation for information of reproducibility, precision and accuracy of the measurements, as well as comparability between methods.

The scientific background for this activity was partially funded by the EU H2020 projects Gov4Nano (H2020-NMBP-13-2018 RIA) and NanoHarmony (H2020-NMBP-34-2019 CSA).

3.3 New TG on Determination of the Dustiness of Manufactured Nanomaterials

Project ID: TGP Project 1.8 **Expected year to finish:** 2024 (TG); 2025 (GDs)

Lead: National Institute for Industrial Environment and Risks (FR) **Category:** Physical Chemical Properties

Short Project Description:

This overall project aims at determining the dustiness of manufactured nanomaterials (MNs) and its subsequent use in the frameworks of worker exposure and explosive atmosphere (ATEX) safety. The proposal is designed in two successive steps by intending to develop a Test Guideline (TG) describing methods of measuring dustiness of nanomaterials and an associated Guidance Document (GD), specifying how to apply the data generated from the TG in worker exposure modelling and ATEX safety.

The TG is also intented to cover fibrous nanomaterials, with a specific focus on sampling methods during dustiness testing and morphological/counting analysis via electronmicroscopy techniques.

This set of two envisioned documents will further aim at:

- Developing a tentative criteria for nanomaterial dustiness ranking and worker exposure assessment usable in regulatory contexts (e.g., respirable mass or number concentrations for particles or fibers released).
- Harmonizing current descriptions of standards and accepted methods for performing dustiness testing of powder materials and their existing test results.
- Assessing the applicability and limitations of the proposed dustiness testing methods to support the current and foreseeable future regulatory needs and data requirements in the field of i) exposure categorization and quantitative exposure modelling, and ii) ATEX safety (Hazard zoning at work places).

Recent progress and outlook:

There are two independent dustiness interlaboratory comparisons (ILCs) conducted – one for granular nanopowders and one for fibrous/high aspect ratio nanomaterials (HARN).

The ILC measurements on testing the dustiness of granular nanomaterials have been completed. Six dustiness test methods have been tested by different international partners. A total of 13 partners have contributed so far in the ILC. All performed tests used 6 different materials, ranging from low dustiness to high dustiness and covering different chemical compositions as well as properties.

The ILC on testing the dustiness of HARN materials was started, with the collaboration of 15 international partners. Three dustiness test methods will be tested and compared. Scanning electron microscopy will be used for analysing the samples and counting as well as classifying the aerosol composition. The choice of fibrous nanomaterials (HARN), to be tested in the ILC, has been proposed and preliminary testing of dustiness of these fibrous materials was performed.

For exposure modelling related aspects, handling energy factors that link the energy applied during the dustiness test to the energy applied during workplace powder handling scenarios have been experimentally determined for small rotating drum and continuous drop dustiness methods. The experimentally determined handling energy factors have been used together with the dustiness index to characterise the emission source of specific workplace powder handling scenarios, and

used as input for modelling to predict nanoparticle concentrations.

For ATEX related aspects, measurement of explosivity and total dustiness of representative granular combustible materials has been completed.

The status of the work was discussed with the international expert group and presented at the Meeting of the Joint WNT-WPMN Expert Group on Physical-Chemical Properties of Nanomaterials (January 2022).

The dustiness results of the ILC on granular nanomaterials as well as the status of the ILC on HARN materials will be shared with OECD experts. .

Submission of a drafts TG on the determination of dustiness of manufactured nanomaterials and draft GDs on the use of dustiness data for exposure modelling and ATEX assessments are planned for June 2023, which will include analysis and reporting on the dustiness ILC results and related aspects to exposure modelling and ATEX.

However, because of their differences in scope, two separate GDs could be produced rather than one global GD as previously anticipated: one GD on the use of dustiness test results for exposure modelling and a second one on the use of dustiness test results for ATEX safety.

The scientific background for this activity was partially funded by the EU H2020 projects Gov4Nano (H2020-NMBP-13-2018 RIA) and NanoHarmony (H2020-NMBP-34-2019 CSA).

3.4 Test Guideline on Determination of Surface Hydrophobicity of MNs

Project ID: TGP Project 1.7 **Expected year to finish:** 2023
Lead: Joint Research Centre (EU) **Category:** Physical Chemical Properties

This Test Guideline (TG) describes a method to determine the surface hydrophobicity of manufactured nanomaterials (powdery or suspension) as dispersed in an aqueous solution by measuring their binding rate to different engineered surfaces (collectors) and mathematically deriving their polar component of the surface free energy using the XDLVO (eXtended Derjaguin Landau Van Overbeek) theory.

Short Project Description:

In 2019 an Expert Group was established and meetings held including experimental demonstration of testing. A first draft test protocol was established based on the scientific literature, followed by identification of testing and data generation needs in order to: i) optimize the test protocol; ii) organise the interlaboratory comparison (ring trial); iii) if relevant and feasible, carry out a full performance validation; and iv) if relevant and feasible, further investigate the relationship between hydrophobicity (measured via the proposed method) and cellular uptake or bioaccumulation in organisms.

In 2020: interested laboratories were identified and comments received on the project and protocols and the optimization was initiated. It was temporarily interrupted due to Covid-19 in spring, after which the interlaboratory comparison (ring trial) was initiated in autumn with 10 participant laboratories that confirmed their interest (+JRC). A set of five materials was sent together with the SOP and a test kit. The Covid-19 pandemic caused important delays and changes in participation: 4 of the confirmed laboratories declined their participation and 3 did not manage to perform the test within the deadline (fixed at end of 2020).

Early in 2022 a first draft TG submitted to the Expert Group for review/comments by February. A revised version will be prepared taking into account comments made and then circulated together with the draft ILC results during the 3rd quarter of 2022.

The TG is expected to be submitted for approval in 2023.

3.5 Guidance Document on the determination of concentrations of nanoparticles in biological samples for (eco)toxicity studies

Project ID: TGP Project 1.10 **Expected year to finish:** 2023
Lead: UK Health Security Agency (UK) **Category:** Physical Chemical Properties

Short Project Description:

A number of current and proposed OECD Test Guidelines require the nanomaterial content within biological samples to be quantified, in particular the content/concentration of nanoparticles (NPs) present and information on their size distribution, but at present there is limited guidance on the appropriate approaches to use under different circumstances (e.g. different biological matrices, nanomaterial types and sizes and expected concentrations). In particular, authoritative guidance on sample preparation prior to the use of specific techniques (e.g. spICP-MS), and the limitations of each technique in terms of, for example, limits of detection both in terms of concentrations and particle size is lacking. Guidance is therefore required in this area to ensure appropriate techniques and protocols are used to produce meaningful results.

The agreed amendments to REACH Annexes include a greater focus on inhalation exposures and enhanced requirements regarding toxicokinetics for NMs. Such assessments can require NP contents of organs/tissues to be generated. The WPMN approved project on revised/new Toxicokinetics TG for NMs will not define how this data can be generated so this project will address this need. From an ecotoxicity perspective, bioaccumulation assessments also require information on NP contents in biological matrices,. Given the matrix similarities it is relevant to include both within a single document.

The expected outcome of the OECD project is a Guidance Document on the determination of concentrations of nanoparticles in biological samples. This is expected to contain

(a) descriptions of the available analytical techniques (imaging approaches, spICP-MS etc.) with reference to relevant OECD Guidance Documents, Test Guidelines and other relevant international standards (e.g. ISO), including details on their constraints, including limits of detection (both material and particle size specific) and the uncertainties on the results;

(b) detailed guidance on appropriate sample preparation protocols to be used to prepare biological samples for analysis using the chosen techniques, this will be related to the biological matrix, the characteristics of the nanomaterial (form, size) and the expected concentration level; and

(c) a series of decision flow charts designed to allow the user to easily identify the most appropriate approach for the specific circumstances.

Recent progress and outlook:

- OECD Meeting on the joint WPMN WNT Ad Hoc Expert Group 22 Sept 2021
- Presented project at WNT Joint meeting NM Phys-Chem Jan 2022
- NanoHarmony Review of literature on spICP-MS has been completed and published Laycock A, Clark NJ, Clough R, Smith R, Handy RD. Determination of metallic nanoparticles in biological samples by single particle ICP-MS: a systematic review from sample collection to analysis. Environ Sci Nano. 2022 Feb;9(2) 420-453. doi:10.1039/d1en00680k. PMID: 35309016; PMCID: PMC8852815. NH Workshop held on analytical techniques for carbon-based materials

- Planning of additional experimental studies progressed, such as an experimental study of CRMs for spICP-MS started OECD
- Start drafting Structure/Contents of GD
- Further Meeting of Ad Hoc Expert Group
- NanoHarmony: Continue with Experimental studies in support of project (storage, treatment, new NMs/matrices).
- Draft deliverable – 'Scientific document to support OECD activities on the development of GD on the determination of concentrations of ENMs in biological materials'

The scientific background for this activity was partially funded by the EU H2020 project NanoHarmony (H2020-NMBP-34-2019 CSA).

3.6 Guidance on Release Tests for Manufactured Nanomaterials

Project ID: WPMN Project **Expected year to finish:** 2024
Lead: Federal Institute for Occupational Safety and Health (DE) **Category:** Physical Chemical Properties

Short Project Description:

The objective of this project is to develop a new guidance on release tests for manufactured nanomaterials (MNs) and their possible applications. The foreseen outcome is a guidance for producers, processors and (commercial) users of MNs and MN-containing products as well as institutions, which assess the safety of consumer products. All types of MNs will be considered and different physical states (i.e. in powder form, dispersed in a liquid, embedded in a composite, attached to a tissue) will be taken into account. A decision framework is envisioned to aid in choosing appropriate release test methods for a given process. The guidance will conceptually link release processes to available release tests. Possible applications of release test data for (regulatory) assessments such as exposure assessment, lifecycle assessment and safer and sustainable by design approaches will be outlined.

Recent progress and outlook:

The concept of this project was presented by Germany at the 21st WPMN meeting in June 2021 and welcomed by the delegations. The Steering Group on Exposure Management and Mitigation further discussed and developed the proposal and it was circulated to the WPMN for comments, which were received in February 2022. The kick-off meeting was held on 28th of March 2022. The following partners build the project team: BAuA (Germany), UBA (Germany), BfR (Germany), NRCWE (Denmark) and ECHA. The project team will meet regularly and continue the establishment of the guidance. The project status was presented during the 22nd WPMN meeting in June 2022.

3.7 Technical recommendations for conducting assays with ENMs according to OECD Test Guidelines 201, 202 and 203

Project ID: TGP Project 2.71 **Expected year to finish:** 2024
Lead: National Institute of Agricultural and Food Research and Technology (ES) / National Institute for Industrial Environment and Risks (FR) **Category:** Effects on Biotic Systems

Short Project Description:

Results obtained for MNs when applying the current OECD test guidelines or ISO standards, originally developed for soluble (or for highly soluble) chemicals, have shown very large variability. This has raised questions about the applicability of these tests for MNs. In the particular case of ecotoxicity, the critical issue is that standard testing with MNs involves exposures of test organisms to dispersions or to colloidal systems, rather than dissolved chemicals. Exposure is further complicated by the dynamic nature of MN in aqueous media, resulting in aggregation/agglomeration, sedimentation, and physical interaction with the organisms during the test. Consequently, these uncertainties in exposure raise questions on the fundamental principle of the current test guidelines or standard protocols, which aim at ensuring comparability of the results performed with the same protocol.

The REACH regulation establishes in Annexes VII to X the physico-chemical, toxicological, and ecotoxicological information requirements for assessing the hazard of substances considering tonnage thresholds for substances placed on the market. In the ecotoxicological information requirements, these annexes mention in relation to aquatic toxicity the necessity of using data about short-term toxicity in invertebrates (preferred species Daphnia sp.); about growth inhibition in alga; and about short-term toxicity in fish. The toxicity of a substance on alga is determined by means of OECD TG 201, the short-term toxicity in Daphnia through TG 202 and, finally, the short-term toxicity in fish can be assessed by means of TG 203. In Europe, the corresponding test methods for these three TGs are included in the Council Regulation No 440/2008 on test methods and its subsequent amendments.

Taking into account the special case of MNs, their particular physico-chemical properties and in general low solubility, the REACH regulation has been amended so that it could cover this type of substances (Regulation (EU) 2018/1881). This amendment indicates that, for nanoforms, the ecotoxicological studies (i.e., growth inhibition of aquatic plants, short term toxicity on invertebrates and fish) may not be waived on the basis of high insolubility in water alone.

At the OECD, the recent GD 317 on Aquatic and Sediment Toxicological Testing of Nanomaterials, in addition to the characterization methods and sampling frequency of MNs in test suspensions, provide some general information on the influence of protocol setup, including e.g. test chamber material, dimensions, volume, water renewal frequency, and use of interpretative controls. However, these recommendations are usually general and there is a need to clarify and bring pragmatic and validated protocols that ensure the relevance of the results obtained.

Considering all of the above it is essential for OECD TG 201, 202 and 203 to develop appropriate methodologies that ensure the applicability of these TGs to MNs.

The main aim of this work is to bring technical recommendations on how to apply to MNs the commonly used aquatic short-term ecotoxicity tests (TGs 201, 202, 203), which are required in REACH and other regulations, and for the classification and labelling of chemicals according to the GHS. As these three tests deal with the study of acute toxicity of substances in the three

representative taxa considered in regulation for the aquatic compartment, they will be considered in parallel.

The expected outcomes are proposals of protocol adaptations and/or specific technical recommendations on how to apply TG 201, TG 202 and TG 203 to MNs. These protocols will appear as annexes of the GD 317 on Aquatic and Sediment Toxicological Testing of Nanomaterials.

Recent progress and outlook:

In the last 12 months experimental work was done to optimize the protocols for conducting the three assays (TG201, 202 and 203). Eight NMs of different nature and solubilities have been tested: bentonite (NM600), CNT (NM400 and NM401), SiO2 (NM200), TiO2 (NM101 and NM104) and ZnO (NM111 and NM110). In addition, the SPSF has been drafted, sent to the WPMN and WNT and the comments addressed. In April 2022 the SPSF was approved and the project included in the work plan of the Test Guideline Programme.

A second virtual workshop with Ad Hoc Expert Group was organized in the framework of the H2020 project NanoHarmony in connection with the OECD WPMN. In the next months, the transferability of the optimized test protocols on the selected ENMs will be assessed to conclude on the suitability of the proposed improvements. In addition, the technical annexes to GD 317 on Aquatic and Sediment Toxicological Testing of Nanomaterials will be drafted. A third workshop with the expert group will be organized before the submission of annexes to the Joint WPMN/WNT Expert Group on Ecotoxicity and Environmental Fate Testing of MNs.

The scientific background for this activity was partially funded by the EU H2020 project NanoHarmony (H2020-NMBP-34-2019 CSA).

3.8 Test Guideline on dissolution rate of nanomaterials in aquatic environment

Project ID: TGP Project 3.10 **Expected year to finish:** 2025
Lead: German Environment Agency (DE) **Category:** Environmental Fate and Behaviour

Short Project Description:

Here, a standard protocol for solubility and dissolution rate in environmental media via batch and dynamic method is anticipated. With this project, the WNT project 3.10 has been continued. The method will be harmonised with the proposed TG for determination of solubility and dissolution rate of nanomaterials in water and relevant synthetic biologically media (TGP project 1.5) and the proposed GD on environmental abiotic transformation of nanomaterials (TGP project 3.16) as far as appropriate. The project is funded by the German Federal Ministry for the Enviroment, Nature Conservation, Nuclear Safety and Consumer Protection. The project is realised by the University of Vienna (AT) and the Fraunhofer IME (DE).

The relevance and potential methodological strategy to test dissolution rate in environmental media based on batch test and dynamic system was already included into the GD 318 on dissolution and dispersion stability of nanomaterials. However, a harmonised and standardised approach is crucially needed.

Recent progress and outlook:

With regard to the development of the static batch test system to determine solubility and dissolution rate, thermodynamic modelling of solubility and formation of low-soluble species as a function of pH (using PhreeqC[7] and available species from databases – Zn, Cu, Ag) was performed. In addition, a 96-well ultrafiltration system was applied with polyethersulfone (PES) membranes using specific molecular weight cut-offs.

With regard to the development of the dynamic test system to determine dissolution rate, flow-through experiments were conducted with different Ag NMs (NM 300k and Nanocomposix 80 nm) in the standard setup originating from the EU H2020 Gracious project, using different loadings, including also experiments using a medium analog to the one used in TG 318 and at different pHs.

The work on the batch test method is planned to be finalised until end of 2022, while work on the dynamic method will continue until summer 2023. This will be followed by a first draft of the test protocol for the interlaboratory comparison (validation) which is intended to take place in autumn 2023.

[7] PHREEQC is a computer program designed to perform a wide variety of aqueous geochemical calculations (https://www.usgs.gov/software/phreeqc-version-3).

3.9 Guidance Document on assessing the apparent accumulation potential for nanomaterials TG 305

Project ID: TGP Project 3.12 **Expected year to finish:** 2023
Lead: National Institute of Agricultural and Food Research and Technology (ES) **Category:** Environmental Fate and Behaviour

Short Project Description:

Spain and UK are working at the OECD level on the development of a Guidance Document to apply the OECD 305 Test Guideline on bioaccumulation in fish after dietary exposure.

The overall objective is to develop protocols that would allow the applicability of OECD 305 to MNs. The specific objectives are 1) to establish a good methodology to include MNs in fish feed so that they are not released to the water previously to its ingestion by fish; 2) to identify the most appropriate analytical techniques to determine the presence and concentration of the different types of MNs (metal, organic and C-based) and their transformed forms in solid matrices (fish feed and fish tissues); 3) to verify the applicability to MNs of the equations used in the current TG 305 for the calculation of a biomagnification factor (BMF) and the appropriateness of the approaches proposed for chemicals to derive a bioconcentration factor (BCF) from a BMF; 4) to identify for which MNs the water exposure could also be used.

Recent progress and outlook:

An appropriate method has been developed for preparation of spiked diet for dietary bioaccumulation testing using NM-aqueous suspension (Gov4Nano D2.24). A revision on the analytical techniques to determine nanomaterials and its transformation products in fish tissues has been reported in Gov4Nano D2.25. Aqueous and dietary bioaccumulation experiments were performed with two NMs. Spherical and rod-shaped CuO NMs were selected to investigate the influence of shape and exposure route on bioaccumulation potential as well as the presence of ions or nanoparticles in tissues. To address the effects of surface coating and exposure route on bioaccumulation COOH and PEG coated CdTe QDs were used. Gov4Nano D2.26 was recently finished with information on the bioaccumulation studies. An interlaboratory comparison study for feed spiking will be performed. A draft guidance document is under preparation and it will be presented to the ad hoc expert group for further discussion and development. The expected time for completing this project is 2023.

The scientific background for this activity was partially funded by the EU H2020 project Gov4Nano (H2020-NMBP-13-2018 RIA).

3.10 Guidance Document and Test Guideline on Aquatic (Environmental) Transformation of Nanomaterials

Project ID: TGP Project 3.16 **Expected year to finish:** 2024
Lead: University of Vienna (AT) and Germany **Category:** Environmental Fate and Behaviour

Short Project Description:

With regard to chemical substances the solid-phase transformation of nanomaterials can be considered a degradation process since the pristine properties are lost in the process.

The aims are to develop a Guidance Document for the determination of abiotic transformation processes of nanomaterials and their coatings under relevant environmental (including WWTP) conditions. It complements the TG on dissolution of nanomaterials by determining the effect of the extrinsic properties of environmental aquatic media on the solid material (not the dissolved phase).

Among the various possible reactions of NM with freshwater chemical species a few relevant transformation reactions and pathways will be identified and possible experimental approaches, relevant media compositions and analytical strategies will be developed and a proof of principle will be performed.

In a later stage another objective is to support or replace in-vitro experiments for transformation with in-silico solutions using and adapting existing geo- and hydrochemical models (e.g. PhreeqC8, WHAM9) to predict the transformation in a given water chemistry. These models are developed on bulk materials and consider thermodynamic equilibrium, which may not be adequate for nanomaterials. Adaptions, sensitivity analysis and validations will be required and will result in reduction of testing effort and/or inform the testing to meet optimized conditions for improved performance (reduced cost per sample). The endpoints which will be addressed by the GD are transformation and degradation of the nanomaterial.

Recent progress and outlook:

The conceptual paper on dissolution/transformation experiments in flow-through devices (continuous flow) was accepted for publication in the scientific journal “Nanomaterials” (https://doi.org/10.3390/nano12030519)

Experiments on the realistic NM transformation in natural sediments, analysed with a reference method (XAS) were completed and a manuscript submitted to Environmental Science & Technology.

Experiments with the same NM exposed in natural sediment by the NM-on-TEM-grid methodology have been completed and the manuscript is drafted.

Major outcome: the TEM grid method is suitable to qualitatively observe realistic transformation processes. It has been assigned the method of choice for these processes.

Completing the last deliverable in Gov4Nano Task 2.7 is foreseen to be finalised by October 2022. The deliverable and information generated will be used to develop the OECD Guidance Document.

[8] PHREEQC is a computer program designed to perform a wide variety of aqueous geochemical calculations (https://www.usgs.gov/software/phreeqc-version-3).

[9] The Woods Hole Assessment Model (WHAM) is a general state-space age-structured stock assessment framework designed to include environmental effects on population processes (https://timjmiller.github.io/wham/)

A new research project is funded by the German Federal Ministry for the Environment, Nature Conservation, Nuclear Safety and Consumer Protection (see TGP project 3.10 in section 3.8). The aim of this new project is to develop a technical guideline in parallel to the guidance document on transformation. The work on both documents will be harmonized and aligned.

The scientific background for this activity was partially funded by the EU H2020 project Gov4Nano (H2020-NMBP-13-2018 RIA).

3.11 Scoping review for a tiered approach for reliable bioaccumulation assessment of MNs in environmental organisms minimising use of higher tier vertebrate tests

Project ID: WPMN Project **Expected year to finish:** 2023
Lead: University of Plymouth (UK) **Category:** Environmental Fate and Behaviour

Short Project Description:

Currently, bioaccumulation potential is assessed using TG305, which is an in vivo fish study, with a dietary exposure method proposed for nanomaterials (compare TGP Project 3.12). The traditional trigger for testing has been the octanol-water partition coefficient (log KOW), but this is problematic for nanomaterials, and in the absence of log KOW data, the current default is to conduct TG305. This situation needs to be resolved to reduce the burden of testing work, the use of animals, and to save money. Handy et al 2018 (https://doi.org/10.1039/C7EN01139C), proposed a framework for a tiered approach to bioaccumulation testing that include four tiers: tier 1 - new chemical trigger relevant to nanomaterials; tier 2 – invertebrate data and read-across approaches; tier 3 in vitro fish testing; tier 4 in vivo fish testing (TG 305). This idea was presented to the OECD Working Party on Manufactured Nanomaterials and will act as a thought starter to reconsider how best to evolve a testing strategy that is efficient for nanomaterials and also minimises the use of vertebrate animals. The ultimate outcome is intended to be a Guidance Document detailing a tiered testing strategy that has been agreed by consensus building, but the project will first deliver a scoping review. In future, linking a set of test guidelines and guidance documents to support the lower tiers is foreseen.

The scientific background for this activity was partially funded by the EU H2020 project NanoHarmony (H2020-NMBP-34-2019 CSA).

3.12 Applicability of the Test Guideline 442D *in vitro* skin sensitisation for nanomaterials

Project ID: TGP Project 4.133 **Expected year to finish:** 2023
Lead: Federal Office of Public Health (CH) **Category:** Health Effects

Short Project Description:

This project deals with the ARE-Nrf2 luciferase method, which supports the discrimination between skin sensitisers and non-sensitisers in accordance with UN GHS. In particular the KeratinoSensTM test method (TG442D) has been evaluated for its use with manufactured nanomaterials. If required by this evaluation, recommendations for adaptations to the test are envisaged. The project started with a literature review to explore existing studies on toxicological and immunomodulatory effects of nanomaterials on keratinocytes including applied test media. In a second step the applicability of TG 442D (KeratinoSensTM test method) for nanomaterials has been investigated using a wide set of nanomaterials. The results were compared to existing in vivo data (from the literature review and the OECD testing programme). Finally, all results were evaluated and recommendations regarding adaptations of TG442D were made. Expert workshops were carried out to establish collaborations and to facilitate mutual exchange of information regarding skin sensitisation and nanomaterials.

Recent progress and outlook:

The results from testing selected nanomaterials within KeratinoSensTM were compared to in vivo data from literature. Unfortunately, only a basic (yes/no) in vitro to in vivo correlation could be made, mainly due to a lack of available in vivo data.

The expert workshop held in December 2021 lead to recommendations for adaptations of OECD TG 442D (KeratinoSensTM test method). The recommendations included viability assessment of the cells, dispersion of nanomaterials, and use of DMSO during exposure of nanomaterials to the cell system, testing of leachates of nanomaterials, exposure time and endotoxin measurements of nanomaterials. Now the Study Report is being written, results from Gov4Nano will also be included in this report. A first draft was recently submitted to the OECD WNT experts for comments.

The scientific background for this activity was partially funded by the EU H2020 project Gov4Nano (H2020-NMBP-13-2018 RIA).

3.13 Test Guideline on toxicokinetics to accommodate testing of nanoparticles

Project ID: TGP Project 4.146 **Expected year to finish:** 2025
Lead: National Institute for Public Health (NL) / UK Health Security Agency (UK) **Category:** Health Effects

Short Project Description:

This project will develop the scientific basis for the development of a new TG for in vivo toxicokinetic studies applicable to nanoparticles. The aim is to determine the minimum requirements of the study design for in vivo toxicokinetic studies, including the dosing regimen (administration mode, level and/or range, frequency and duration), the duration of the post exposure period, the time points for determining organ or tissue burdens (during and post exposure), and the key organs, tissues and/or excreta to be analysed. These minimum requirements should be sufficient to determine the absorption, distribution, potential accumulation and clearance of the nanoparticles. What is sufficient depends on the anticipated presence of nanoparticles in the different target tissues and the ability to detect the nanoparticles or determinants thereof (e.g. the radiolabel, fluorescent functional group or chemical components of the nanoparticles) in tissues. The anticipated presence and ability to detect the nanoparticles in the target organs is likely to be governed by the uptake and translocation of the particles to target organs, the dissolution kinetics of the particles in relevant physiological media, and the limit of detection of the particles or determinants thereof in the target organs.

The number of published toxicokinetic studies with nanoparticles is limited, especially with respect to studies in which the distribution of nanoparticles beyond the organ of entry is measured on multiple time points during and after the exposure period. Based on the ISO Technical Report and an inventory within the EU NanoSafety Cluster and the OECD Ad Hoc Expert group on toxicokinetics, the following available and planned toxicokinetic studies have been identified to provide useful data to determine the minimum requirements of the study design of oral and inhalation toxicokinetic studies for moderately (SiO_2) and slowly (CeO_2 and TiO_2) dissolving nanoparticles. These will be used as a starting point for development towards the guidance on toxicokinetic studies.

Recent progress and outlook:

Several experimental studies have been performed to generate data on non-soluble and poorly soluble nanomaterials including silica oxide, cerium oxide and titanium dioxide as well as a polymer. These data will be used in computational modelling, for which focus will be on studies that conducted to determine local concentrations in a toxicity study. It will be investigated whether general a priori information on the solubility of ENMs in physiological (fluid) matrices may help define optimal design for an experimental kinetics study. The rationale for this being that solubility of an ENM will be related to elimination of the ENM in the body which, in turn, will determine the time frame in which significant distribution will take place. The objective of this study is to relate the solubility of an ENM to the minimum post-exposure duration of an in vivo study. Furthermore, we aim to gain insights into the minimum required number in vivo sampling points and the timing of those sampling points. The outcomes of this study will include a quantitative linear model relating solubility to the post-exposure duration, and a qualitative analysis of the necessary time points of in vivo sampling.

The scientific background for this activity was partially funded by the EU H2020 project NanoHarmony (H2020-NMBP-34-2019 CSA).

3.14 Guidance Document on Integrated *In Vitro* Approach for Intestinal Fate of Orally Ingested Nanomaterials

Project ID: TGP Project 4.158 **Expected year to finish:** 2023
Lead: Istituto Superiore di Sanità (IT) **Category:** Health Effects

Short Project Description:

The project is aimed to establish in vitro experimental procedures able to evaluate NMs fate after oral exposure and it was initially included in June 2019 in the WPMN programme of work. Its expected outcome is the production of a consensus document finalized to the elaboration of a new Guidance Document (GD) defining conceptual framework and procedures to determine NMs behaviour in a simulated in vitro intestinal environment. It is expected to report: i) best conditions for in vitro NMs simulated digestion; ii) best conditions for in vitro NMs translocation/internalization.

An interlaboratory comparison (ILC) for each endpoint is planned to verify reliability and reproducibility of selected protocols. Considerations on relevant aspects linked to the intestinal fate of NMs will also be included in the GD, providing science-based tools for NMs hazard identification and grouping. Scientific rational of the project is also matter of study in NanoHarmony project.

The work in the NanoHarmony project consists of two phases: 1) analysis of scientific background on intestinal digestion/absorption processes of NMs performed by survey of literature data. 2) set up of the most reliable experimental procedures for both simulated digestion with synthetic digestive fluids (in collaboration with TGP project 1.5) and NMs translocation/internalization in an advanced in vitro model of the intestinal barrier. These procedures will be verified by appropriate ILCs using selected NMs.

Recent progress and outlook:

(1) Scientific background

State-of-the-art analysis of the consolidated in vitro models for simulated digestion and for the intestinal barrier, including analytical techniques for NMs detection, and in vitro/in vivo correlation, was performed on several NMs relevant for oral absorption (e.g. SiO2, TiO2, ZnO, silver). Information was collected according to criteria and templates defined and agreed with OECD and other international experts.

The analysis was presented in the dedicated session “Data gap identification related to the intestinal fate of ingested nanomaterials” at the first NanoHarmony international expert workshop (3-5 November 2020). The session was aimed to identify data gaps related to the two main steps of the experimental model, i.e. ENMs fate in simulated gastro-intestinal fluids and internalization/translocation of digested ENMs through the intestinal barrier. About 30 experts from academia, governmental institutes and EU bodies (e.g. JRC and EFSA), international organizations (notably OECD) as well as commercial companies actively joined the session.

Results of the state-of-the-art analysis and data gap evaluation were reported in the NanoHarmony deliverable D1.16.

Preparation of a scientific paper summarizing results of the state-of-the-art-analysis is scheduled. Other relevant ingested NMs e.g. copper and iron will be included as well. This manuscript will provide a relevant scientific basis for GD elaboration.

(2) Setup of the experimental procedure for interlaboratory comparison

A preliminary protocol for interlaboratory comparison (ILC) on simulated in vitro digestion was set up. So far, two laboratories (EcamRicert and IIT) have confirmed their participation to the ILC. In January 2022 experimental activity has started in the IIT laboratory. For simulated digestion three NMs (TiO_2 – NM-104, SiO_2 - NM-203 and ZnO – NM-110) were selected. A delay in these experiments is expected due to the lack of supply of some essential materials for protocol execution caused by the Covid-19 pandemic.

Strengths and weaknesses and relevant parameters of the above-mentioned protocol, as well as of the protocol for the tri-culture intestinal barrier model were discussed in the specific session on “Intestinal fate determination” during the 2nd NanoHarmony expert meeting (November 2021).

ILC experiments for simulated digestion will be completed and digested TiO_2, SiO_2 and ZnO NMs samples will be collected for the subsequent uptake/translocation studies through the tri-culture intestinal barrier model. This second ILC is scheduled for autumn 2022. To date, four laboratories will be involved, ISS, List, Solvay, and University of Barcelona.

The scientific background for this activity was partially funded by the EU H2020 project NanoHarmony (H2020-NMBP-34-2019 CSA).

3.15 (Update) Guidance Document on Sample Preparation and Dosimetry for the Safety Testing of Manufactured Nanomaterials (GSPD)

Project ID: WPMN Project **Expected year to finish:** 2023/2024
Lead: Joint Research Centre (EU) **Category:** Physical Chemical Properties, Environmental Fate and Behaviour, Health Effects

Short Project Description:

The Working Party on Manufactured Nanomaterials (WPMN) discussed in March 2021, the need for updating the OECD Guidance on Sample Preparation and Dosimetry for the Safety Testing of Manufactured Nanomaterials, published in 2012. The proposal was agreed by the WPMN in June 2021. To this effect, an Ad hoc group was established under the leadership of the EU Joint Research Centre (JRC), to do a thorough review of the document, and identify sections that needed to be updated, deleted or added. Experts for several OECD delegations are supporting the JRC in this endeavour. The project kick-off meeting was held on 1 December 2021, followed by four meetings held between January and June 2022.

Recent progress and outlook:

Lead authors per section were identified and prioritisation on the sections and sub-sections to update was set. Highest priority is to update section V “Specific Considerations” (V-A. Physical Chemical Properties, V-B. Ecotoxicity Studies, V-C. Degradation, Transformation, and Accumulation, Studies, V-D. Health Effects). Followed by the update of sections III “Considerations on Appropriate Dose-Metrics” and IV “Common Issues Regarding Sample Preparation and Dosimetry”. Sections I “General Introduction”, and II “Terminology” will be updated at later stage. A first literature search was carried out and a list of updated references for Section V is available, however, it would be reassessed and possibly refined once the first full draft of this section is available.

Lead authors of Section V agreed to review their specific sub-sections to identify areas that need to be: a) deleted, b) updated to reflect new scientific knowledge c) extended. An initial revision of sub-section V-A “Specific considerations- Physical Chemical Properties” was carried out by the lead author (EU-JRC). Based on the feedback provided by Ad hoc group members on the review of Subsection V-A “Physical-Chemical Properties”, it was agreed to review the list of physical-chemical endpoints currently included to consider whether to keep them. Also, to consider the potential inclusion of new endpoints. Further input on the approach to the update provided by Ad hoc group members is: to expand section III with a sub-section on dissolution studies; to include health effects related to exposure and dosimetry experiments with medical implant materials, durability, and long-term studies in section V-D; and to inform readers that the GSPD complements the guidance on sample preparation and dosimetry also provided in specific OECD Test Guidelines.

The plan is to circulate a proposal for a draft final version of the updated GSPD to the WPMN for information and written feedback for the WPMN23 meeting (26-30 June 2023).

4 Activities underway relevant to the OECD

The document under this section are not at OECD level, but are relevant for potential future developments on Test Guidelines and Guidance Documents. The list presented here is not exhaustive.

4.1 Particle counting

Status: Under discussion
Lead: Luxembourg Institute of Science and Technology (LU)
Category: Physical Chemical Properties

Short Project Description:

Among the objectives of RiskGONE project there is the development of guidance documents (GDs) for the experimental techniques used to characterize the physicochemical properties of selected engineered nanomaterials (ENMs).

ISO/DTR 24672 is seen as the reference Guidance Document for the measurement of the particle number concentration, but it is still under development.

To support advancements on this topic, part of the RiskGONE-WP4 work has been dedicated to the validation of the Particle Tracking Analysis (PTA) as fast and easily accessible technique for the determination of particle number concentration in water-based media. In addition, several commercial instruments are available on the market thus this technique could be considered suitable either for academia or industry purposes.

Recent progress and outlook:

A Standard Operating Procedure (SOP) about the determination of the number concentration of ENMs in water-based media by PTA has been drawn by LIST. The SOP has been consolidated and the reproducibility of the results has been evaluated through an ILC exercise in collaboration with other partners of the project.

In particular, the protocol has been adapted to the application of PTA (by NanoSight) to the selected ENMs. Optimal operating conditions to overcome weaknesses and limitations of the method have been identified.

Although no calibration is specifically required to run PTA measurements, the performance of the instrument should be verified by using a standard quality control helping to ensure intra and interlaboratory data comparability. Reference materials are available for other kind of instruments, such as DLS; unfortunately, no standards for particle concentration calibration using PTA are available yet. The next working period will be thus dedicated to the evaluation of potential benchmarks to verify the performance of PTA-based systems like NanoSight.

4.2 Endotoxins

Status: Under discussion
Lead: Luxembourg Institute of Science and Technology (LU)
Category: Health Effects

Short Project Description:

Among the objectives of RiskGONE project there's the development of guidance documents (GDs) for the experimental techniques used to characterize the physicochemical properties of selected engineered nanomaterials (ENMs). The GDs will be then part of a framework for the governance of ENMs and will be later available to the regulatory agencies dealing with this topic.

Several ENMs have been reported to trigger inflammatory responses in different in vitro and in vivo models. Lately, awareness has risen on the possibility of ENMs contamination by bacteria and bacteria components, i.e. endotoxins. Endotoxins can bind to the surface of ENMs leading to misinterpretation of results, or altered results, when it comes to in vitro and in vivo investigations such as the release of inflammatory cytokines.

To address this issue part of RiskGONE work was focused on the establishment of a harmonised protocol for the determination of ENMs endotoxin content, starting from already available documents including the European Standard EN ISO 29701, specific projects deliverables and relevant literature. At this aim, the chromogenic version of the Limulus Amebocyte Lysate (LAL assay) was selected as method to be reviewed and adapted to ENMs specific features and tested through interlaboratory comparison (ILC) exercises for its validity and reproducibility.

Recent progress and outlook:

The series of ILCs was completed involving three different institutes (NILU, LIST and CSIC) in different stages of the exercise. A Standard Operating Procedure (SOP) was then drawn by LIST and NILU, reviewing and adapting the kit manufacturer's instructions for the chromogenic version of the Limulus Amebocyte Lysate (LAL assay) and the EN ISO 29701 and trying to better understand and address the interference issue.

A range of compounds were tested at different concentrations selected within the range of the exposure conditions used for the human hazard assessment in vitro studies (1 - 100 µg/ml). This approach enabled the validation of the results obtained in nanotoxicology testing. The relevance of applying ENM depyrogenation (as preliminary step before testing) and the introduction of mock and spiked controls have been also evaluated. An adapted Data Collection Template was drafted and specific acceptance criteria taking into account potential interference issues have been defined.

In the next period the work will be focused on a further validation of the results obtained on those ENMs considered as relevant for RiskGONE activities. NILU and LIST will work together with IDEA to consolidate the Data Collection Template for the next uploading on the eNanoMapper platform.

4.3 Zeta potential

Status: Under discussion
Lead: Luxembourg Institute of Science and Technology (LU)
Category: Physical Chemical Properties

Short Project Description:

Among the objectives of RiskGONE project there is the development of guidance documents (GDs) for the experimental techniques used to characterize the physicochemical properties of selected engineered nanomaterials (ENMs). The behaviour of ENMs in water-based media is a hot topic to understand the different phenomena occurring at the interface when particles are dispersed in liquids prior hazard assessment using in vitro or in vivo systems.

The zeta potential is an important parameter associated with the surface functionality and/or with the stability of dispersed particles. The main focus of zeta potential analysis is obtaining information on the surface charge of a material. Electrostatic repulsion between particles depends on the value of zeta potential. The higher the zeta potential, the stronger the repulsion, the more stable the system become.

Recent progress and outlook:

A consolidated pre-validated standard operating procedure for zeta potential determination by dynamic light scattering (DLS) of selected ENMs has been prepared. Interlaboratory comparison (ILC) exercises have been performed among task partners to evaluate the validity and the reproducibility of the proposed guidance document. Limitations and operating conditions have been evaluated after ENM dispersion in simple medium (water) as well as complex medium (cell culture medium). The procedure was sufficiently accurate to allow the different partners to classify the various ENMs within the same range of zeta potential identifying the suspension stability (i.e. flocculation or coagulation, incipient instability, moderate stability, good stability, excellent stability). In next period part of the work will be dedicated to zeta potential determination of ENM specifically relevant for RiskGONE activities. This means performing the measurements using biological and environmental relevant media and applying realistic concentrations.

4.4 New Comet assay to detect strand breaks and specific DNA lesions of Daphnia magna (or other species) exposed in vivo

Status: Under discussion
Lead: University of Birmingham (UK) **Category:** Effects on Biotic Systems

Short Project Description:

The Comet Assay, or Single Cell Gel Electrophoresis assay (SCGE), is a simple, quick and inexpensive technique able to detect DNA damage in the form of strand breaks generated by the action of genotoxic agents. Virtually, any eukaryotic cell can be used for the test, without any prior knowledge of the genome structure. It has been a widely and successfully applied test for over 30 years on fungi, plants, invertebrate, fish and mammalians, humans included (Pellegri et al. 2020, https://doi.org/10.1016/j.scitotenv.2019.135780). In 2016 the Organisation for Economic Co-operation and Development (OECD)adopted the Comet Assay in a test guideline for mammalian in vivo biomonitoring (OECD TG 489), as well as accepted by the Food and Drug Administration (FDA) and World Health Organization (WHO). Its use in environmental biomonitoring for daphnia has recently been demonstrated (Pellegri et al. 2020, https://doi.org/10.1016/j.scitotenv.2019.135780).

The comet assay is a well-established method for assessing genotoxicity of nanomaterials, although there are some concerns regarding interference of nanomaterials with the shape of the Comet tails.

Whole organism exposures will be standardised to perform Comet assay in parallel with the OECD TG 202 (acute Daphnia immobilisation) and OECD TG 211 (Daphnia reproduction) assays to extend the data available and support deeper understanding of adverse outcomes and Mode of Action.

Harmonisation and optimisation of the SOPs is needed for extraction of the tissue following exposure.

4.5 Daphnia magna reproduction test (with male induction)

Status: Under discussion
Lead: University of Birmingham (UK) **Category:** Effects on Biotic Systems

Short Project Description:

OECD Test No. 211: Daphnia magna Reproduction Test assesses the effect of chemicals on the reproductive output of Daphnia magna Straus. To this end, young female Daphnia are exposed to the test substance added to water at a range of concentrations (at least five). For semi-static tests, at least 10 animals at each test concentration and for flow-through tests, 40 animals divided into four groups of 10 animals at each test concentration are used. The test duration is 21 days. The total number of living offspring produced per parent animal which does not die accidentally or inadvertently during the test and the number of living offspring produced per surviving parent animal at the end of the test are reported. The study report also includes: the daily counting of the offspring, the daily recording of the parent mortality, the weekly measurement of oxygen concentration, temperature, hardness and pH values and the determination of the concentrations of test substance. Optionally other effects can be reported, including the sex ratio of the offspring. The reproductive output of the animals exposed to the test substance is analysed, by comparing it with that of the control in order to determine the lowest observed effect concentration (LOEC) and hence the no observed effect concentration (NOEC), and by estimating the concentration that causes an x % reduction in reproductive output by means of a regression analysis.

UoB has been applying this test to a wide range of nanomaterials including silver and TiO2 with a range of variants, as well as assessing the impact of water type (without/with Natural Organic Matter) and ageing of the particles for different periods of time (Ellis et al, Small, 2020, 2000301; Ellis et al, Environ Sci: Nano, 2020, 7, 1136-1149). Our SOP will be further specified, extended to the wider set of RiskGone NMs, and tested via a set of interlaboratory comparisons in RiskGONE.

4.6 Technical recommendations for conducting the *In Vitro* Mammalian Cell Gene Mutation Test (OECD TG 476) with ENMs

Status: Under discussion
Lead: Swansea University (UK) **Category:** Health Effects

Short Project Description:

Evaluation of in vitro genotoxicity induced by a test agent relies on a battery of assays capable of measuring different forms of DNA damage. For chemicals, the recommendation is to use the Bacterial Reverse Mutation (Ames) Test to detect mutagenicity (OECD TG 471) and the In Vitro Micronucleus Assay (OECD TG 487) for gross chromosomal damage, including aneugenicity and clastogenicity. As it is now well accepted that the Ames test is not suitable for nanomaterials, the recommendation is to substitute this with the In Vitro Mammalian Cell Gene Mutation Test (OECD TG 476). Data generated for MNs using OECD TG 476 have shown some inconsistencies due to variation in the standard operating procedures (SOPs) applied. Thus, this project will establish a harmonized SOP for MNs with the In Vitro Mammalian Cell Gene Mutation Test using the HPRT gene, based on the testing in an adherent (V79) and a suspension (TK6) cell line.

Once the harmonized SOP has been established, its reproducibility and transferability will be evaluated through three rounds of interlaboratory comparison testing. At each stage of testing, the SOP will be refined as necessary to generate a robust protocol that can be readily applied by independent laboratories to evaluate the mutagenicity of MNs.

The expected outcomes of this project are the proposal of protocol adaptations and/or specific technical recommendations on how to apply OECD TG 476 to MNs.

Recent progress and outlook:

In the past 12 months the progress made on the HPRT forward mutation assay has encompassed two interlaboratory comparison (ILC) trials conducted in two independent laboratories, i.e. Swansea University (SU) and The Norwegian Institute for Air Research (NILU). Each group prepared their own SOPs which specified the use of one of two cells lines: with minor adaptations from the current OECD test guidance for use with engineered nanomaterials (ENMs). SU prepared an SOP specifying the use of suspension, human lymphoblast (TK6) cells and NILU prepared the SOP specifying the adherent, Chinese hamster lung fibroblast (V79) cells. The data from the first ILC experiments revealed a good level of concordance between both independent laboratories for both cell lines. The level of concordance however was closer in the TK6 cells as opposed to the V79 cell line. This cytotoxicity and mutagenicity data was generated using zinc oxide (ZnO) and titanium dioxide (TiO2). Throughout both SOPs a positive control of methyl methane sulfonate (MMS) was used which consistently provided a strong mutagenic response. The degree of concordance was statistically evaluated by QSAR Lab Ltd for precision and accuracy in the data. Following ILC1, ILC2 introduced three new ENMs; copper oxide (CuO), tungsten carbide-cobalt (WC-Co) and multi-walled carbon nanotubes (MWCNTs). There were also minor modifications made to each SOP to better harmonise the data generated, this included example colony formation images, cell titration calculations, background, and positive mutation frequencies for healthy cells. There was also an opportunity to generate training videos for each SOP which were also incorporated into the SOPs to provide a clear audio-visual cue for intricate phases of the assay, such as the plating and scoring. The data generated in ILC2 is currently being finalised and being prepared for statistical analysis. The finalised data pertaining to the V79 exposures however appears to show a good degree of concordance. With respect to the experimental work, SU has

completed the TK6 and V79 experiments and have uploaded the relevant data into the templates. NILU will soon be finalising the TK6 data following unforeseeable setbacks. All data for V79 cells has been finalised for ILC2.

The outlook for the next 12 months involves completing the manuscript which has been under draft following the completion of ILC1. This paper will focus upon the 5 tested ENMs, their cytotoxicity and mutagenicity (associated with the HPRT gene) in the context of an interlaboratory study. The paper will also provide guidance on the necessary adaptations required to incorporate ENMs into mutagenicity testing, this will be addressed by discussing interference, required (additional) centrifugation stages, and the dispersion / sonication technique employed. Currently, we are refining the presentation of the data (displayed as graphs) in the Results section to better harmonise between the two SOPs which offer slightly alternate ways to present the cytotoxicity and mutagenicity. The manuscript is largely prepared with the statistical analysis of ILC1 also being included. The remaining ILC2 data is currently being finalised at NILU; this will then be followed by the interlaboratory statistical analysis before inclusion into the manuscript.

4.7 Colony forming efficiency (CFE)

Status: under discussion
Lead: Norwegian Institute for Air Research (NO) **Category:** Health Effects

Short Project Description:

Validated and sensitive, interference-free screening methods for cytotoxicity of nanomaterials (NMs) are needed. The CFE assay is a label-free method for assessment of basal cytotoxicity. The method is specifically suitable for assessment of NMs toxicity in vitro being non-colorimetric and non-fluorescent, and avoiding interference with the readout of the test method, which is commonly seen with optical detection methods, metabolic assays and enzymatic assays. The ultimate index of cytotoxicity is loss of cell viability. The CFE assay follows this, and cytotoxic effects are measured by reduction in the number of colonies formed. Additionally, cytostatic effects can be indicated by reduced colony size. Exposure is on individual cells growing in small inoculum attached to a surface, in contrast to other cytotoxicity assays based on confluent cells. The CFE assay is shown to be a sensitive test assay for potential toxic effects of NMs in human, mammalian and fish cells. An advantage is also the more sub-chronic exposure scenario.

The CFE assay has been optimized and standardized for NMs testing by the JRC's Nanobiosciences Unit and validated in the interlaboratory comparison study of the Colony Forming Efficiency assay for assessing cytotoxicity of nanomaterials (https://doi.org/10.2788/406937). NILU has further optimized and validated the assay by increasing the throughput by applying the 6- or 12-well format, to better comply with a screening approach for testing of NMs.

The aim of this project is to harmonize and validate the SOP developed for chemical substances for NM and for 12-well format, to test reproducibility and transferability and evaluate it through three rounds of interlaboratory comparison testing. At each stage of testing, the SOP will be refined as necessary to generate a robust protocol that can be readily applied by independent laboratories to evaluate the cytotoxicity of NMs. The expected outcome of this project is the proposal of protocol standardization with specific technical recommendations and application for a new OECD TG on CFE.

4.8 New *in vitro* guideline for comet assay to detect strand breaks and specific deoxyribonucleic acid (DNA) lesions

Status: Under discussion
Lead: Norwegian Institute for Air Research (NO) **Category:** Health Effects

Short Project Description:

There is an urgent need for developing robust in vitro genotoxicity testing methods to cope with the ever-increasing range and number of NMs. The alkaline comet assay (single cell gel electrophoresis) in its basic form detects DNA strand breaks (SBs) and alkali-labile sites. A simple modification of the comet assay, incorporating the bacterial DNA repair enzyme formamidopyrimidine DNA glycosylase (Fpg), allows sensitive detection of oxidised DNA bases, as the enzyme converts oxidised purines to breaks.

The enzyme-linked comet assay has been applied in numerous studies of NMs and it is the most used method to assess genotoxicity of NMs. Previous projects such as NanoTEST, NanoGENOTOX, NANoREG, and NanoREG2 tested large group of NMs with the aim of developing a standard SOP for the use of this assay. The comet assay is a fast and reliable method, readily adapted to a high throughput mode and for 3D models. There is in vivo comet assay OECD TG 489. A new in vitro comet assay TG, employed in initial screening, can enormously speed up the testing of NMs, greatly reducing the costs of testing, not to mention saving animals.

The aim of this project is to harmonize the SOP, to test reproducibility and transferability and evaluate it through three rounds of interlaboratory comparison testing based on the testing in an adherent (A549) and a suspension (TK6) cell line.

At each stage of testing, the SOP will be refined as necessary to generate a robust protocol that can be readily applied by independent laboratories to evaluate the genotoxicity of NMs.

The expected outcome of this project is the proposal of protocol standardization with specific technical recommendations and application for new OECD TG on in vitro comet assay.

4.9 Cell transformation assays

Status: Under discussion
Lead: Luxembourg Institute of Science and Technology (LU)
Category: Physical Chemical Properties

Status: Project in preparation
Lead: Norwegian Institute for Air Research
Category: Health Effects

Short Project Description:

The cell transformation assay (CTA) is an approach that makes use of the phenotypic morphological transformation of cells as a marker of carcinogenicity. Cells transformed in vitro have been shown to induce tumours when injected into immunosuppressed animals. Cells used in CTA are either primary cells derived from rodent embryos, such as the Syrian hamster embryo (SHE), or stable cell lines such as mouse BALBc 3T3, Bhas42 and C3H/10T cells. The CTA with Bhas 42 cells is an in vitro carcinogenicity test measuring morphological transformation of cells as foci derived from a single cell. It can detect both genotoxic and non-genotoxic carcinogens (OECD Guidance Document No. 231). The Bhas 42 CTA can distinguish between tumour initiating and tumour promoting activity. As an initiation assay to test tumour-initiating activity, cells at low density are treated with a test chemical for three days. As a promotion assay to test for tumour promoting activity, near confluent cells are treated with a test chemical for 10 days. The CTAs can be useful to identify non-genotoxic carcinogens and thus could be included as an integral part of a battery of in vitro tests in order to predict carcinogenic potential of chemicals and also NMs. The performance of the Bhas42 CTA has been conducted on a large set of chemical substances, and OECD guidance documents are developed (OECD Guidance Document No. 231).

The aim of this project is to harmonize SOP developed for chemical substances for NM (OECD Guidance Document No. 231), to test reproducibility and transferability and evaluate it through interlaboratory comparison testing. The expected outcome of this project is the proposal of protocol standardization with specific technical recommendations and application for new a OECD TG on Bhas 42 CTA.

4.10 Impedance-based label-free assessment of nanotoxicity

Status: Under discussion
Lead: University of Bergen (NO) **Category:** Health Effects

Short Project Description:

The aim is to develop label-free methods that are less prone to interferences caused by nanomaterials. Impedance-based methods are label-free and suited for high-throughput testing. Impedance-based real-time monitoring can detect relevant concentrations and timepoints to be used for more in-depth mechanistic studies. Such methods are very useful for initial nanotoxicity screening. Moreover, they are more labour- and time- efficient and since they do not require chemical reagents, are more environmentally friendly.

Recent progress and outlook:

SOPs have been developed for nanotoxicity testing based on label-free impedance-based live monitoring of adherent cells using an xCELLigence system (Agilent) and for single cells using impedance flow cytometry (AmphaZ30, Amphasys AG). The following SOPs were established by the UiB team in the FP7 project "NANoREG" and the NFR NANO2021 project (NorNANoREG) for nanotoxicity testing using human cells:

NANoREG D5.07 SOP 02 Real-time label-free impedance-based nanotoxicity assessment

NANoREG D5.07 SOP 04 Label-free nanotoxicity assessment by impedance-based flow cytometry

These SOPs were further developed within the NFR project NanoBioReal and the H2020 project RiskGone. Within the RiskGone project, the use of real-time impedance monitoring was extended from testing of nanomaterials' effects on human cell lines to red trout gut cells and zebra fish embryo cells in collaboration with the University of Birmingham.

In the RiskGONE project current cytotoxicity and genotoxicity TG methods together with other methods with high throughput potential are compared. The impedance-based nanotoxicity testing is included in this exercise.

To mimic the real-life type of exposure to NMs, the UiB group has also developed a microfluidic platform that uses custom-made microfluidic chips with embedded electrodes for live monitoring of cells, tissues and organson-a-chip by impedance sensing and live microscopy, under controlled dynamic exposure to nanomaterials. The chips are constructed by the University of Bergen.

The group has developed cyclic voltammetry as a label-free electrochemical methods to assess the oxidative stress of NMs in collaboration with HVL.

SOPs and training videos are currently prepared for real-time impedance-based monitoring and impedance flow cytometry and will be uploaded on the RiskGONE website (https://riskgone.wp.nilu.no).

The SOPs can be ready to be forwarded for TG assessment by the end of the RiskGONE project (2023).

Cyclic voltammetry for oxidative stress assessment needs further optimisation. We envision that by the end of the RiskGONE and NanoBioReal projects (2023), a SOP will be produced.

The microfluidic system and the 3D models (microvasculature and lung) are more complex and need further optimisation. We envision that two SOPs will be produced by the end of the RiskGONE and NanoBioReal projects.