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ECOTOXICOLOGY AND ENVIRONMENTAL FATE OF MANUFACTURED NANOMATERIALS: TEST GUIDELINES

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This publication was developed in the IOMC context. The contents do not necessarily reflect the views or stated policies of individual IOMC Participating Organizations.

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FOREWARD

As part of its Programme on the Safety of Manufactured Nanomaterials, and in particular work on the testing and assessment of manufactured nanomaterials, OECD initiated a series of expert meetings to improve the applicability of the OECD Test Guidelines to nanomaterials. With this in mind, the Working Party on Manufactured Nanomaterials agreed to address the ecotoxicology and environmental fate of manufactured nanomaterials.

The OECD Expert Meeting on Ecotoxicology and Environmental Fate took place on 29th-31st January 2013 in Berlin, Federal Press Office. The event was hosted by the German delegation and funded by the German Federal Ministry of the Environment, Nature Conservation and Nuclear Safety (BMU) as well as the United States Environment Protection Agency (US EPA).

This document presents a detailed report of the discussion and recommendations derived from the discussion. An accompanying document includes the presentations given at the workshop [ENV/JM/MONO(2014)1/ADD]. A short meeting report was also published in Science of the Total Environment[1].

This document is published under the responsibility of the Joint Meeting of the Chemicals Committee and Working Party on Chemicals, pesticides and Biotechnology of the OECD.

[1] http://www.sciencedirect.com/science/article/pii/S0048969713013387

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Abbreviations and definitions

ADME:	Absorption, distribution, metabolism, and excretion
BAF:	Bio accumulation factor
BCF:	Bio concentration factor
BET:	Brunauer, Stephen; Emmett, Paul Hugh; Teller, Edward. Determination of the specific surface area of a solid material by gas molecule adsorption
BMF:	Bio magnification factor
CCC:	Critical coagulation concentration
DLS:	Dynamic light scattering
EELS	Electron energy loss spectroscopy
EDX:	Energy-dispersive X-ray spectroscopy
EM	Electron microscopy
ENM:	Engineered nanomaterials
ENP:	Engineered nanoparticle
ESEM:	Environmental scanning electron microscopy
FFF:	Field flow fractionation
GD:	Guidance document
GL:	Guideline
GSPD:	Guidance Document on Sample Preparation and Dosimetry
HNMR:	Hydrogen nuclear magnetic resonance
ICP-MS:	Inductively coupled plasma mass spectrometry
IS:	Ionic strength
ISO:	International Organization for Standardization
NOM:	Natural organic matter
MAD:	Mutual acceptance of data
PAH:	Polycyclic aromatic hydrocarbon
PEC:	Predicted environmental concentration
PTA:	Particle tracking analysis
OECD:	Organisation of Economic Co-operation and Development
OM:	Organic matter
RA:	Risk assessment
REACH:	Registration, Evaluation, Authorisation and Restriction of Chemicals for legislation
ROS:	Reactive oxygen species
SEM:	Scanning electron microscopy
SPM:	Scanning probe microscopy
TG:	Test guideline
WPMN:	OECD Working Party on Manufactured Nanomaterials
XPS:	X-ray photoelectron spectroscopy

In this document the general term ***aggregation*** was used to encompass the more specific term agglomeration in referring to interactions between nanomaterials (homoaggregation) and between nanoparticles and other natural colloids.

EXECUTIVE SUMMARY

The OECD Expert Meeting on Ecotoxicology and Environmental Fate took place on 29th-31st January 2013 in Berlin, Federal Press Office. The event was hosted by the German delegation and funded by the German Federal Ministry of the Environment, Nature Conservation and Nuclear Safety (BMU) as well as the United States Environment Protection Agency (US EPA).[2]

The aim of the meeting was to (i) discuss the applicability of existing OECD Test Guidelines (TGs) related to the fate and ecotoxicology of nanomaterials, and (ii) identify whether there is a need to amend current TGs or to develop new ones.

The workshop was organised in two sections. The first section dealt with ecotoxicology whilst the second section addressed fate and behaviour. A summary of the main points discussed as well as the main conclusions reached is provided below. A detailed description can be found in Session Four: Conclusions and Recommendations.

Applicability of the OECD TG

- The OECD Test Guidelines (TG) 201; 211; 222; 225; 305; 315; and 317 are applicable to ENMs (engineered nanomaterials). TG 105 is not appropriate for nanomaterials, a recommendation was made to develop a TG that addresses the dissolution behaviour of ENM. In addition TG 106 for measuring the adsorption and desorption behaviour of a substance in soils cannot be applied for the testing of nanomaterials, since no valid differentiation between adsorbed and not adsorbed ENM is possible.
- Specific recommendations regarding the application of each TG to ENM and details about the data analysis, acceptable loss of the ENM during the test, endpoints, test procedure, selection of the test media, target organism, can be found in the different sections of this document.

Characterisation

- Dissolution, dispersability, agglomeration, degradation and transformation were identified as the first needed information.
- A decision tree / tiered approach should be established as prior tests before further ecotoxicology or fate tests are conducted.
  - First step – dissolution and dispersability → new TG needed
  - Second step – agglomeration state and dispersion stability → new TG needed
  - Third step – bio-degradation → a new TG must be developed.
  - Fourth step – abiotic degradation → definition of important parameters.

[2] The summary of the discussion was prepared by Dana Kühnel (Helmholtz-Centre for Environmental Research - UFZ, Leipzig, Germany) and Carmen Nickel (IUTA e.V., Air Quality & Sustainable Nanotechnology, Duisburg, Germany).

- The importance of testing transformed and aged ENMs instead - or as an addition to - pristine materials was highlighted. As such, environmental tests should also be conducted with aged ENMs. A Guidance Document (GD) for pre-treatment scenarios of ENMs should also be developed in order to harmonize the processes related to aging and transformation.
- A decision tree / tiered approach should be conducted before soil / sediment testing is developed, including the dissolution and dispersability step.

Detection

- Guidance is needed for detection techniques in soil and sediment.
- The quantification of ENM accumulating in organisms as well as the state of the ENMs in soils/sediments are needed for regulatory purposes.

The application of the ENM to the test system was discussed in all sessions.

- For ecotoxicity testing, no clear statement of the best spiking (wet or dry) procedure was made.
- Dietary spiking should be favoured in OECD TG 305.
- Wet spiking should be favoured in OECD TG312 and TG315.
- Wet or dry spiking should be favoured in OECD TG 317.

Stock suspension preparation for aquatic ecotoxicity testing

- One suggestion was to include decision trees into the OECD Guidance on Sample Preparation and Dosimetry (GSPD), with 3 tiers covering the following issues:

  - Tier 1: Stock / stem suspension preparation
  - Tier 2: Preparation of exposure solution
  - Tier 3: Conducting the test

- The same stock suspensions should be used for both, aquatic ecotoxicity and environmental fate tests.

Further comments

- To promote the comparability of results of ecotoxicity and fate tests, the same test conditions should be used (e.g. the application of the ENM to the test media, physicochemical conditions and parameters of the suspension and test media, etc.).
  At the very least, the same stem suspensions should be used.
- Given its importance for the fate and transport of ENMs in the environment, the type of NOM which is used for the testing or which is already available in the system must be specified.
- K_{ow}, BCF or BMF cannot be directly used for the characterisation of ENM behaviour in the environment. In addition, other "nanorelevant" endpoints must be identified, such as uptake rate, internalisation rate, attachment efficiency. Further studies should be carried out to verify which endpoint are the most appropriate.

The table below provides an overview of the applicability of the TGs discussed during the expert meeting in different environmental compartments. TG 105 and TG 106 were considered not applicable for NM. For all other guidelines, NM-specific amendments or guidance was demanded.

Table 1: Overview of the Test Guidelines (TGs) and Guidance Documents (GDs) discussed during the expert meeting in the two breakout sessions

	Breakout group on Ecotoxicology	Breakout group on Fate & Behaviour	Joint remarks for ecotox. and fate & behaviour testing
Aquatic tests - ***Session 2***	**TG 201** (Freshwater Alga and Cyanobacteria, Growth Inhibition Test) **TG 202** (Daphnia sp. Acute Immobilisation Test) **TG 211** (Daphnia magna Reproduction Test) **TG 225** (Sediment-Water Lumbriculus Toxicity Test Using Spiked Sediment)	**TG 105** (Water solubility) **TG 305** (Bioconcentration: Flow-through Fish Test) (additionally discussed: **GD 29** and biodegradation tests in general)	• Preparation of stock suspensions should follow the same protocol • Careful NM characterisation
Soil & sediment tests - ***Session 3***	**TG 222** (Earthworm Reproduction Test (Eisenia fetida/Eisenia andrei)) **TG 225** (Sediment-Water Lumbriculus Toxicity Test Using Spiked Sediment)	**TG 106** (Adsorption) **TG 312** (Leaching in Soil Columns) **TG 315** (Bioaccumulation in Sediment-dwelling Benthic Oligochates) **TG 317** (Bioaccumulation in Terrestrial Oligochaetes)	• Spiking procedure should follow the same protocol • Careful NM characterisation

Rough summary of needs for further research in the near future

Ecotoxicology

- A robust physical-chemical characterisation for biological assays.
- An improvement of understanding on homo- and hetero-agglomeration processes.
- An improvement of understanding of interferences of NM with assays.

Aquatic Toxicity:

- Chronic toxicity tests (growth, reproduction, energetics, new materials) under environmentally realistic concentrations, ADME / ecotoxicokinetics.
- Effects of pristine vs. aged ENMs.
- The interaction of ENMs with sense organs of animals should be studied more in detail.

Soil & sediment:

- A robust measurement method for ENM quantification in environmental media.
- Assessment of the bioavailable fraction.
- Spiking technique.
- Aggregation, transformation, dissolution.
- Applicability of TG to other organisms.

Measurement Techniques

- Need for more sensitive measurement techniques for the identification and quantification of ENMs under environmentally realistic concentrations or in different compartments and/or tissues or cells was identified.

Fate and behaviour

- Analysis of dissolution, dispersability and transformation processes in environmental media.
- Tests should be conducted with aged/transformed ENMs as well as pristine ENMs.
- More information concerning degradation and transformation processes.
- The use of different soil types or reference soils for the environmental fate.
- The exposure pathway of ENM to the natural compartments was identified as an important factor affecting the fate and behaviour of ENMs.
- Chronic studies.

INTRODUCTION

The OECD Expert Meeting on Ecotoxicology and Environmental Fate took place on 29th-31st January 2013 in Berlin, Federal Press Office. The event was hosted by the German delegation and funded by the German Federal Ministry of the Environment, Nature Conservation and Nuclear Safety (BMU) and the United States Environment Protection Agency (US EPA). The agenda can be found in Annex I.

The rapporteurs of the meeting were Dana Kühnel[3] (UFZ, Germany) and Carmen Nickel[4] (IUTA, Germany). Seventy-seven participants from the OECD member countries as well as stakeholder representatives from industry, academia and NGOs took part in the meeting (see the participants list in Annex II – list. The meeting was opened by the welcome address of Jochen Flasbarth[5], president of the German Federal Environmental Agency (UBA).

The main objective of this meeting was to assess the applicability of existing Test Guidelines (TGs) to nanomaterials, with a view to:

1. Identify the needs for (i) updating the (OECD) TGs related to environmental fate and ecotoxicology, and (ii) developing new (nanospecific) TGs. The latter aim includes identifying further steps and specifying which kinds of changes should be made.

2. Identify specific needs for developing/updating existing guidance documents (GDs) (such as the "Guidance on Sample Preparation and Dosimetry"[6]), including identifying the need for additional sections for fate and ecotoxicology testing of nanomaterials.

3. Develop separate specific or adapt existing GDs for environmental fate and ecotoxicology testing of nanomaterials.

The topics were discussed in two breakout sessions. The first breakout session dealt with ecotoxicology issues; the second with fate and behaviour topics. Each breakout session was split between the water and soil/sediments compartments. Selected TGs (see Table 1) and related nano-specific issues were discussed.

The conclusions from each breakout group were discussed and summarised by all participants in the plenary session.

[3] Helmholtz-Centre for Environmental Research - UFZ, Leipzig, Germany

[4] IUTA e.V., Air Quality & Sustainable Nanotechnology, Duisburg, Germany.

[5] The welcome address can be found in Annex III – Welcome address by Jochen Flasbarth

[6] OECD, 2012. Guidance on Sample Preparation and Dosimetry for the Safety Testing of Manufactured Nanomaterials [ENV/JM/MONO(2012)40].

SESSION ONE - GENERAL ASPECTS OF ENVIRONMENTAL TOXICITY AND FATE OF MANUFACTURED NANOMATERIALS [7]

Chair: *Susanne Walter-Rohde (UBA, GER)*
Rapporteurs: *Dana Kühnel (UFZ, GER) and Carmen Nickel (IUTA, GER)*

OECD Guidance Document on Sample Preparation and Dosimetry: Overview, and Areas for Environmental Protocol Development

Phil Sayre (US-EPA)

Dr. Sayre provided an overview of the sections of the OECD Guidance on Sample Preparation and Dosimetry[8] (hereafter GSPD) related to environmental toxicity and fate of nanomaterials. He stressed that the GSPD is a living document (currently considerably useful to regulatory authorities), but where updates should be included whenever our knowledge on manufactured nanomaterials (hereafter MN) increases.

Developing an understanding of the physical-chemical properties of MN is of major important as this knowledge is crucial for ecotoxicity, degradation, as well as transformation studies. The physical-chemical properties of MNs are not steady, but may change with sample preparation, choice of media, dispersant use, presence of environmental ligands, and other factors. Hence, differences in sample preparation may affect toxicity. To give material-specific recommendations for dispersion methods in biological media, a category approach has been suggested (based on ENM properties). As an initial step, the idea of standardized sonication procedures for making stem/stock solutions for environmental testing was suggested. This would provide an understanding of the exact composition of stem solutions in terms of size distribution and energy input to sonication, and would enable replication by others. In this way, a comparable solution can be used for all ecotoxicity and environmental fate tests for a single nanomaterial and due to this the fate and effect information can be better related as suggested in the GSPD. Publications that provide standardised preparation methods for metal oxide powders, based on titanium dioxide, are already available (as cited in slides[9]). These methods also provide a general calorimetric approach to measuring energy delivered to all nanomaterials during sonication, and therefore allow for the standardisation of energy input with a view to lessen inadvertent damage to nanomaterials and increase comparability of results. However, many unknowns were addressed by Mr. Sayre: e.g. limited knowledge on tests which indicate retention of nanomaterials in wastewater treatment plants, methods for reactive oxygen species estimation, and methods for estimation of octanol water partitioning coefficient (log kow) equivalent information for nanomaterials. In addition to the protocol for nanomaterial retention in sewage sludge, other protocols were also considered as essential, such as those that would predict movement of nanomaterials in subsurface and lotic sediments. Protocol improvements to existing OECD guidelines were also identified (e.g. guidelines focusing on the dissolution kinetics of soluble nanomaterials such as silver).

Discussion and remarks

- Dissolution kinetics for nanomaterials need to consider effects of environmental ligands. Dissolution kinetics form foundation work in both ecotoxicity and fate for soluble nanomaterials.

[7] All presentations can be found in their entirety in Annex IV.

[8] Guidance on Sample Preparation and Dosimetry for the Safety Testing of Manufactured Nanomaterials (2012) [ENV/JM/MONO(2012)40]

[9] Taurozzi, Hackley & Wiesner. 2011. Nanotoxicology /// Taurozzi, Hackley & Wiesner. 2012. Nanotoxicology.

NIST Special Publication 1200-3, in conjunction with CEINT, http://www.nist.gov/mml/np-measurement-protocols.cfm

- There is a need for using standard approaches for preparing ingstem/stock solutions. These approaches could be developed by using well-known materials such as TiO_2 and may additionally accompanied by selected ecotoxicity tests which may be streamlined using a category approach.
- As outlined in the GSPD, there is a need for developing guidance on dosimetrics for ecotoxicity.

The OECD Test Guideline Programme (Ecotoxicity and Fate): Appropriateness of Test Guidelines / Needs for Testing Manufactured Nanomaterials
Jukka Ahtiainen (TUKES, FIN)

Dr. Jukka Ahtiainen presented insights into the handling of guidance apart from scientific issues. For ENMs in the environment, the applicability of a TG (nano-relevance, dosing & dosimetry) as well as the relevance of endpoints should be taken into consideration. This leads to the question of whether new endpoints for the testing of ENMs are needed. According to Mr. Ahtiainen, ecotoxicology, degradation and bioaccumulation are rather well covered by existing TGs. However, specific guidance (i.e. GDs) is needed on sample preparation and dosimetry when using existing TGs. New internationally harmonized and validated methods are needed for physical-chemical properties and ENM detection. Care should be taken that the GDs developed are not out-of-date by providing updates or new TGs. Because mutual acceptance of data (MAD) applies to TGs but not to GDs, Dr. Ahtiainen discussed whether ENM specific guidance should be given in the annex of a TG. However, it may be possible that if the nano-specific guidance is given in an OECD GD, it could be seen as refinement of the TG, and MAD would apply to the data developed. This would also be a more feasible solution. He also stated that guidance should not run over regulation.

Discussion and remarks

- OECD guidelines are used as references in literature as a kind of quality asset, but often ENM specific changes are not addressed in these GDs.
- For the refinement of guidelines, this kind of information is needed.
- GDs may be helpful in using and refining TGs.
- GDs cannot be included in TGs of a more practical use for regulatory use.
- Implementation into REACH, as ECHA regulation is based on REACH .

Characterisation of ENM in Environmental Compartments: Knowledge, Challenges, and Perspectives
James Ranville (Colorado School of Mines, US)

Dr. James Ranville gave a presentation focusing on the current knowledge of ENM characterisation and presented the endpoints which can be measured (e.g. size, surface charge, shape). He also indicated that it is not clear which of these parameters are critical for ecotoxicity and environmental fate. Currently, the identification of critical parameters is a challenge due to the lack of knowledge and sensitive measurement techniques for environmental compartments. Beside the ENM parameter, it is also unclear as to which environmental compartments should be examined. One should firstly define which parameter or compartments are important for the exposure and risk assessment. It was also mentioned that pristine ENPs are the most studied but are likely to be the least relevant to risk characterisation, due to aging processes in the environment and the application of coated ENMs.

He also highlighted the challenges of ENM characterisation in environmental media due to the presence of interfering particles and presented promising techniques (i.e. Field Flow Fractionation (FFF) or single particle ICP-MS). The advantage of single particle ICP-MS is that this method is not fundamentally new compared to FFF, where many tools should be investigated.

Discussion and remarks

- Particle number concentrations as well as surface area are important metrics for the characterisation of ENMs.
- FFF, PTA, spICP-MS are promising tools for the detection of ENMs in environmental media. The advantage of PTA and spICP-MS are that they are not fundamentally new techniques compared to FFF. For FFF many tools should be investigated and validated whereas for PTA and spICP-MS existing tools can be used.
- There was also a discussion focused on how to detect the surface area of nanoparticles or aggregates in the environment. In this respect, the BET method, used for the characterisation of the dry powder of ENM, can be applied, although its values should not be transferable to the conditions in the environment. Another possibility for surface area calculation is based on measured particles' size distributions. This method can exhibits several limitations because it is dependent on several assumptions (e.g. spherical particles). The detection of the particle size distribution in the environment is also difficult and important. A third method available is HNMR, which can measure the particle or agglomerate surface area of ENMs in suspension. There are several disadvantages associated to this method: (i) it requires pre-validation, and (ii) it requires a stable suspension with a high concentration (depending on the ENM, around 1 %) which can be very difficult to prepare.
- Currently, no sensitive measurement capable of directly detecting the surface area of the ENM in environmental media is available.
- The available surface area should be measured instead of the total surface area.
- The transformation of ENM in natural media can change environmental behaviour.
- Depending on which technique is used, the measurement can alter the ENM and thus the environmental behaviour. This is important if additional measurements are to be conducted with the same sample.

Exposure Routes of ENMs in the Environment
Martin Scheringer (ETH Zürich, CH)

Dr Scheringer introduced the multimedia mass balance models and presented an example of a multimedia mass balance model with exposure routes of TiO_2 ENM to the Rhine River. For each reservoir modelled, all inputs and outputs should be specified.

Typically, mass balance approaches are valid for ENMs, but the following detailed information should be provided:

- Flux processes;
- Chemical properties of the ENM:
  - rate constants for dissolution, transformation,
  - rate constants for homo- and heteroaggregation,
  - attachment efficiencies for homo- and heteroaggregation,
  - identification of the environmentally relevant chemical form of ENPs;
- Environmental conditions:
  - pH,
  - Temperature,
  - Particle background,
    Concentrations of divalent ions, NOM and type of SPM.

In many cases, this information is not available for ENPs, and should be measured in further projects.

The fact that the identification of essential information is important for the definition of acceptable simplifications of the model was also mentioned. Furthermore, for the sake of harmonisation, a consistent format for the reporting of data should be used.

Mr. Scheringer highlighted heteroaggregation as a key process and the attachment efficiency α_{het} as a key parameter that should be measured as one nanospecific endpoint. He highlighted the need for techniques which can identify these factors.

Discussion and remarks

- For the modelling, it is important to estimate if comparable metrics are available for water, soil and air compartments (e.g. which type of particle diameter is measured - hydrodynamic, electric mobility, aerodynamic). Converting different data can be problematic if the validity of the data used is not known.
- Bioaccumulation is not included in the presented models; this should be one of the next steps.
- The differentiation between ENM uptake and attachment is currently problematic, but has been identified as an important parameter. There is also a need for sensitive measurements.
- Heteroaggregation with other particles in river water can lead to higher agglomeration and sedimentation but also to higher transport and lower sedimentation; this can also be included in the model as a fraction which is still dispersed.

Ecotoxicology of ENM – Knowledge, Challenges, and Perspectives
Richard Handy (University of Plymouth, UK)

Richard Handy gave a summary of the knowledge and challenges, as well as the progress made, in modifying regulatory tests for the ENM testing. ENM toxicity has been reported in mg/l levels (acute lethal concentration). However, there are still weak datasets (e.g. for chronic toxicity). Growth was found to be unaffected by ENM, but effects on reproduction have been reported. The mechanisms of action of ENM are not yet fully understood - in particular, more knowledge regarding uptake, ADME, target organs, and sub-lethal effects needs to be generated. Professor Handy suggested making greater use of existing data (e.g. on metal toxicity for comparison). According to the TG OECD 210, enough knowledge has been generated to modify guidelines. Some ENMs showing toxicity are not part of the sponsorship programme. In general, there has been an improvement in the understanding of particle behaviour in test media as well as progress in methodology. Nonetheless, more efforts are needed in linking exposure to uptake.

Environmental Fate of ENMs – Knowledge, Challenges, and Perspectives
Graeme Batley (CSIRO, AUS)

In his talk, Graeme Batley summarised how the high variability in ENM materials (e.g. carbon based, metals, and modified surfaces) may lead to a high variability regarding their environmental fate. Various homo- and heteroaggregation processes occur, depending on ENM properties and environmental conditions/compartments (e.g. coating, ionic strength, pH, interaction with colloids/NOM). Synthetic or natural substances lead to a modification of surface properties, mostly resulting in ENM stabilisation. The dependence of degradation / transformation / sedimentation processes on ENM properties is not fully understood and can be affected by additional substances (humic substances, surfactants) in the environment. Regarding ENM fate in wastewater systems, the removal efficiency is high for some materials, but it is also known that surfactants can influence the removal efficiency of ENM. This has to be taken into account for wastewater systems. However, the assessment of environmental exposure is currently solely based on models, and hence real data is lacking. Therefore, realistic and relevant environmental studies are needed. Dr. Batley concludes that with current usage, minimal environmental risks are expected. This, however, may change if usage increases in the future.

General Conclusions and Remarks

Potential interactions of ENM with sense organs were discussed. It was observed that these interactions could lead to **channel/sensor damage and blockage**. The discussions focused in particular on whether these effects may explain some of the observations made (e.g. interference of ENMs with *Daphnia* reproduction). The blockage of the olfactory bulb of fish may interfere with smelling, although direct effects on the brain were considered unlikely. Further issues examined were the presence of ENMs in animal gut, which may exert several effects, such as the reduction of food intake and energy input, changes in gut mobility, or effects on nerves or smooth muscle fibres. However, the interaction of ENMs with animal sense organs is poorly studied,. In particular, ENM interference with calcium signalling by ROS generations (which may influence behaviour or smelling response) should be studied further.

Another discussion point was interactions of ENMs with constituents of natural water.

For CeO_2-NP, a fast agglomeration and fast settling in natural water was observed, illustrating the variability of ENM behaviour under environmental conditions. Participants also discussed **heteroaggregation** as being the main aggregation process under environmental conditions, whereas homoaggregation was deemed to be a more important process in the laboratory. However, water characteristics are too different to predict heteroaggregation. Hence, participants could not identify a way to deal with that fact. Furthermore, heterogeneous mixtures of natural colloids were identified as an additional variable in the agglomeration process. Indeed, the type of the natural colloids may influence agglomeration. It was pointed out that heteroaggregation can change the **transport behaviour** of ENMs in the environment and should therefore be addressed. In general, there are many complications in this field and future research is needed.

One focus of the discussion was the consideration and inclusion of natural variability and environmental conditions in the ecotoxicity test guidelines. The following questions were raised:

- Is an extrapolation from a few OECD field tests to a wide variety of environmental conditions possible?

- Do we need **more realistic TGs** and if we do, is this possible?

It was noted that natural variability and its effect on organisms is difficult to transfer to TGs. Additionally, the organisms themselves bring variability into tests (over time e.g. algae exudates) - a fact not considered in TGs for chemicals. Rather, the user needs to better understand the limitations of the TGs. Hence, only nano-specific issues could be considered in TGs. For example, heteroaggregation should be addressed in the tests, as it is the main process under real environmental conditions and can change the transport behaviour in the environment. Another issue addressed by the participants was that ENM surface area is an important metric for ENM and limitations in determining it. Only a few instruments are available and the measurement methods should be validated. Furthermore, the total surface area can lead to wrong interpretation of data due to heteroaggregation and a reduced available surface area. The alteration of ENMs during measurement should be taken into account.

The participants explored the relationship between observed effects and environmental concentrations. Currently, no knowledge on **actual environmental concentrations** exists. The main constraint is finding a measurement technique sensitive enough to detect ENM at **low concentrations** in natural media. Furthermore, some participants pointed out that effect concentrations and predicted environmental concentration (PECs) are probably orders of magnitude apart, and hence it is difficult to come to any conclusions on toxicity. It was proposed to check for more sensitive endpoints. In any case, however, the link to PECs is still difficult to establish. Tests should also be improved with regard to the mechanical

effects observed only at ENM concentrations considered to be so high that (according to the TGs) they become irrelevant. At PEC, however, no mechanical effects may be evident. It was noted that modelling information on particle behaviour could be considered to a greater extent in toxicity tests than currently done. Bioaccumulation as well as ENM attachment to organisms should be modelled, allowing differentiation between uptake and attachment.

Further improvements of TGs were discussed. In general, participants identified missing information on **particle behaviour over time** in ecotoxicity experiments (e.g. DLS overtime). This would lead to a proper process of understanding and assessment. Additionally, several issues related to the transferability of procedures established for chemicals to ENMs were discussed, e.g. according to TGs for chemicals, a 20% loss compared to the **nominal concentration** is considered acceptable, but it remains unclear whether this is an acceptable procedure for ENMs. Likewise the usage of solvents is practiced for chemicals; hence it was discussed whether dispersants such as NOM or polysaccharides are acceptable to prevent ENM sedimentation processes during tests. Another option raised was to perform tests under differing conditions (e.g. pristine / aged / modified ENMs) and compare results. It was mentioned that other **abiotic factors** such as UV light or water chemistry influence ENM and should be considered, as should the aging of released materials. However, it was noted that product testing is not covered by OECD TG and only original material is tested.

After Session One, the group was split into two breakout sessions, one dealing with the OECD TG for ecotoxicological testing, the other dealing with OECD TG for fate and behaviour testing.

SESSION TWO - ENVIRONMENTAL TOXICITY AND FATE OF MANUFACTURED NANOMATERIALS- COMPARTMENT WATER[2]

Ecotoxicology (Compartment Water)

Chair: *Steve Diamond (US EPA)*
Rapporteur: *Dana Kühnel (UFZ, GER)*

Background: Different approaches currently exist on how to disperse and apply nanomaterials in media for aquatic ecotoxicity studies. A review of the literature reveals that a wide range of dispersing techniques – and various combinations of these - are used, including the application of solvents, dispersion or stabilising agents, bath or probe sonication, stirring, bead milling, etc. Furthermore, the methods vary with respect to applied concentrations, time, etc. (Klaine *et al*. 2008, Handy *et al*. 2008, Hund-Rinke *et al*. 2010, Handy *et al*. 2011[10]). It is suggested that the properties of the dispersions depend on the dispersing method. Also, it is suggested by some authors that nanomaterials can be significantly altered as a result of the preparation method (Crane *et al*. 2008[11], Handy *et al*. 2011). As known also from other test substances, the interaction of solvents may result in toxic by-products or alter properties and toxicity of the nanomaterial. The group should develop recommendations on a reliable sample preparation procedure.

Dispersion Protocols for Aquatic Ecotoxicology
Nanna Hartmann (JRC, EC)

Nanna Hartmann gave an overview on current dispersion practices performed for aquatic *in vivo* and *in vitro* tests. This is part of her work within the MARINA project (Task 3.5). Available dispersion protocols from projects (NANOGENOTOX, PROSPECT, ENPRA, NANOMMUNE) and institutes for standardised methods (NIST/CEINT) were reviewed. The preparation of stock dispersions is crucial when testing for the hazard potential and fate of ENMs as their properties may be modified depending on the dispersion method applied. This, in turn, may influence the (eco)toxic effects of the ENM and hamper

[10] Klaine, S.J.; Avarez, P.J.J.; Batley, G.E.; Fernandes, T.F.; Handy, R.D.; Lyon, D.Y.; Mahendra, S.; McLaughlin, M.J.; Lead, J.R. (2008): Nanomaterials in the environment: behavior, fate, bioavailability, and effects. Environ. Toxicol. Chem, 27, 1825-1851.

Handy, R.D., von der Kammer, F., Lead, J.R.; Hassellov, M.; Owen, R.; Crane, M. (2008): The ecotoxicology and chemistry of manufactured Nanoparticles. Ecotoxicology, 17, 287-314.

Hund-Rinke, K.; Schlich, K.; Wenzel, A. (2010): TiO2 nanoparticles – Relationship between dispersion preparation method and ecotoxicity in the algal growth test. Umweltwiss Schadst Forsch, 22, 517-528.

Handy, R.D.; Cornelis, G.; Fernandes, T.; Tsyusko, O.; Decho, A.; Sabo-Attwood, T.; Metcalfe, C.; Steevens, J.A.; Klaine, S.J.; Koelmans, A.A.; Horne, N. (2011): Ecotoxicity test methods for engineered Nanomaterials: Practical experience and recommendations from the bench. Environ. Toxicol. Chem.

[11] Crane, M.; Handy, R.D.; Garrod, J.; Owen, R. (2008): Ecotoxicity test methods and environmental hazard assessment for engineered Nanoparticles. Ecotoxicology. 17 (5), 421-437.

comparability between tests. The development and refinement of dispersion protocols for ENMs can assist to minimise artefacts caused by undesirable modifications of the nanomaterials and provide a more realistic picture of the nanoparticle toxicity. Dr. Hartmann stated that a good dispersion method should be practically and scientifically sound. At the same time, harmonisation is needed to reduce variability between tests and ensure comparability between studies. However, the choice of appropriate dispersion method depends both on the test to be performed and the type of ENM to be dispersed. Several key parameters for the dispersion of ENMs were identified: (i) ultrasonication procedures (e.g. sonicator type, energy output, amplitude, and delivered energy), (ii) water quality/composition, (iii) pre-wetting steps, (iv) stabilising / dispersing agents, and (v) stock concentrations. A high degree of variation in some parameters was identified in the various reviewed protocols. Sonication was identified as a very important parameter. For example it may enhance the dissolution of metal ENM. To date, no studies that systematically examine the influence of different sonication methods, settings and time on the outcome of ecotoxicological tests were identified. Another important issue was the pre-wetting with ethanol, which could be applied only to those that are hydrophobic or (for harmonisation) to all nanomaterials. An OECD TG should define what degree of variation may be allowed, and which variables should be harmonised.

Discussion and Remarks

- Pre-wetting with ethanol was critically discussed, including the evaporation of ethanol during sonication.
- There was a discussion around the quantification of energy delivered to the sample during sonication. This is for example described in details in the NIST/ CEINT protocol (1200-2, developed only for TiO_2 P25) [12] where the effective power delivered to a liquid sample (dispersion) is calculated based on measurements of the temperature increase in the sonicated liquid over time for a given sonicator device output power setting. Control of energy input during sonication was suggested.
- Comparison between different sonication instruments is difficult with respect to delivered energy. Available methods to calculate the introduced energy were discussed.
- Guidance is needed on how to quantify energy for individual systems, perhaps building on calorimetric approaches.
- For some ENMs (e.g. for hydrophilic materials), sonication may not be necessary (but could be applied for harmonisation purposes).
- An ENM category approach was proposed also for dispersion protocols, where the dispersion method would be tailored for each category.
- Harmonised approaches are under development (e.g. in the MARINA project), but may be rather complex.
- Decisions are needed on which parameters to harmonise. A TG should give ranges for these parameters and accept a certain degree of variability.

[12] Taurozzi, Hackley, Weisner. 2012. NIST Special Publication 1200-2. Preparation of Nanoparticle Dispersions from Powdered Material Using Ultrasonic Disruption

General conclusions and recommendations

The appropriateness and practicability of a TG on dispersions were discussed. It was stated that several points should be considered: energy input, chemical mediation, or natural mitigating factors. The dispersion methods lead to a **change in ENM properties** and hence demand a thorough characterisation and concentration determination to be able to (qualitatively and quantitatively) quantify exposure. Furthermore, the type of used **dosimetry** was discussed, including the identification of which are the most appropriate measures and bulk concentration, surface area, aggregate / agglomerate number, size distribution etc. Here, no consensus among participants was reached. To cover the broad and diverse group of ENMs and corresponding demands on dispersion, a material-by-material guidance in one separate GD, rather than in each individual TG, was suggested. Giving a material-by-material guidance in each single TG was considered to expand the extent and scope of a TG. Another recommendation was to formulate respective dispersion protocols for classes or groups of ENMs. However, addressing this point, it was noted that the ENMs we are working with today may not be relevant for the future. In addition, the uniqueness of ENMs, e.g. releasing ions or undergoing agglomeration should be properly addressed. Finally, it was discussed that the selection of a dispersion protocol also depends on the test system used.

The requirements for ENM stock suspensions were discussed by the participants, e.g. whether the degree of **monodispersity** should be as high as possible or whether a range in size distribution is feasible. It was suggested to take the properties of ENMs in an actual application or product as orientation, as the biological effects or the mode of action may be ENM size dependent. Furthermore, there was a discussion focused on whether the 80% target concentration in the test system (as suggested in TGs for chemicals) is also an appropriate level for ENMs. The participants stated that more data on that issue are needed, e.g. with regard to **settling and sedimentation processes** leading to a loss of ENM from test media. Two approaches were suggested to address this point: renewal of media and/or dosimetric adjustments to account for settling and sedimentation.

Participants nonetheless agreed that the nominal concentrations given rarely reflect the actual concentration in the test solutions. It was reported that in some cases **irreversible ENM aggregation by sonication** occurs, and such issues could be addressed by standardized approaches to stem suspension preparation. The dilution of stock dispersion in the media used in a test leads to an additional change in particle behaviour due to the differences in medium composition. This adds more variability to the system as **different matrices are used as test media**. With regard to this issue the participants stated that the stock suspension shall ensure the **reproducible preparation** of subsequent test dilutions. Hence the emphasis may not be on making a stable stock solution but rather on ensuring good test dilutions (regarding stability and reliable concentrations). It was discussed how the big variations in stock suspension parameters between different labs can be tackled. It was suggested to change only one parameter at a time when comparing protocols.

When stock suspensions are transferred to test media, parameters such as higher ionic strength and media composition are likely to influence processes like sedimentation and aggregation / agglomeration. However, all relevant parameters for these processes could not be identified by the participants. The extent of the **influence of aggregation / agglomeration on available surface area** also remained unclear. Regarding the biological assays, uptake kinetics are influenced by specific parameters (e.g. by sedimentation) - hence it was suggested to adopt the test duration for ENM. An alternative suggestion has been to use renewal protocols (where the test solution is replaced at defined point in times by a new one), but this would be dependent on sedimentation time (acute, chronic).

To be able to give advice on dispersion for the large variety of different ENMs, the creation of decision trees was suggested. The aim is to provide guidance for the preparation of stock dispersions

considering **different types for the same material and coatings** (e.g. different sizes, shapes or porosities), where the information about the material is available.

Regarding the use of stabilisers, the participants differentiated between human toxicity testing, where **proteins** are used to stabilize ENMs in the test dilutions, and ecotoxicity testing. For the latter, the inclusion of **NOM** into test dilutions (not stock) was discussed, but no consensus was reached. Batch-to-batch variations should be considered for both ENMs and stabilizers. It was suggested to harmonise the procedure for fate testing and ecotoxicology testing by applying the same protocols. In addition, the characterisation of ENM suspensions throughout the dispersion protocol development was strongly recommended. This would allow adapting the dispersion protocol to the requirements (e.g. in case of monodisperse materials, no further sonication is necessary), hence chemical-physical characterisation is essential before deciding on a protocol.

The participants discussed the consequences that nano-specific issues in dispersion preparation may have in the regulatory context. Is the information on dispersion procedures and characteristics available and/or **relevant for regulators**? It was stated that, in general in regulatory testing, not all these variables are considered, and the testing is just performed according to the guidelines. Again, the regulatory practice for chemicals is not working for ENMs. The reason for this is that the need for ecotoxicity tests is specified based on **modelling approaches**, but these are not yet feasible for ENMs. Furthermore, it is thought that the concept of **equilibrium**, which most TGs for chemicals rely on when focusing on kinetics, does not apply to ENMs. Knowledge on particle kinetics over the whole test duration would be necessary, but doubt was expressed as to whether it would be possible to include this in a TG. Hence, the participants agreed on gathering more knowledge on that issue and then deciding on whether to prepare a TG for dispersion. As a compromise, it was suggested to agree on a dispersion protocol which ensures stability over 24 h or another defined period. Guidance on the relevant parameters to measure as well as on the frequency of measurements is needed. It was suggested to insert pre-tests for ENMs (compared to chemicals) which aim to capture the most important parameters to look at and to decide on the test system. Finally, the question was brought up of how meaningful dose-response curves for ENMs are, given these uncertainties.

No consensus on ONE sample preparation was reached. Hence, as a specific proposal for sample preparation, the participants agreed on the generation of decision trees (applying to both stock suspensions and test dilutions) and an update of the GSPD. The **decision trees** were suggested to follow e.g. the draft in the paper from the NanoImpactNet environmental workshop[13] and shall be included into the GSPD. In order to form that decision tree, a grouping/categorization approach was favoured, meaning that ENM parameters lead to a final decision on the dispersion protocol. It was suggested to select materials with a great wealth of knowledge (e.g. Ag and TiO_2) as a starting point. The decision tree is also intended to **show gaps** and should be flexible enough to allow the inclusion of new materials once they appear. The participants discussed the issue of **coatings** and suggested to consider them in the trees at least for each **category** of material, because the physical chemical properties of the coating will also affect dispersion. Furthermore, the selection of other starting points was considered (e.g. mechanistic or chemical based endpoints).

Furthermore, **guidance on ENM characterisation** was suggested (e.g. guidance on determination of surface area, monodispersity, agglomeration etc). To start with the practical work in drafting decision trees, an **expert workshop** should be organised. It was suggested to start with a collection of knowledge available from various sources (e.g. EU projects). The idea of organising the workshop in parallel to the International Conference on the Environmental Effects of Nanoparticles and Nanomaterials in Aix-en-Provence (France) in July was expressed. Richard Handy took responsibility for that.

[13] Stone V. et al. (2010), Sci Tot Env. 408, 1745–1754. doi:10.1016/ j.scitotenv.2009.10.035

Considering the wealth of knowledge on chemicals, to get an idea of how much natural variability can be incorporated into a TG, would also be an important step in this process.

Water Tests: OECD Test Guidelines 201; 202; 211; and 225
Teresa Fernandes (Heriot-Watt University, UK)

Algae and *Daphnia* tests were the focus of Teresa Fernandes' talk. Changes in the fate of ENMs in the presence of NOM (e.g. humic acid) have been observed, leading to interactions and changes in bioavailability, and hence toxicity of ENMs. For algae, the endpoint biomass or growth was considered. Different test media are recommended in the guidelines for algae. For both chemicals and ENMs, this may result in different outcomes in terms of toxicity. The exposure of algae is strongly influenced by performing the tests either under static or shaking conditions. The supply of CO_2 to the algae test medium is enhanced by shaking, and hence also influences medium pH. The determination of algae growth by fluorescence measurement may result in artefacts (e.g. measurement of isolated chlorophyll may be a more reliable endpoint). For daphnids, the endpoints immobilisation and reproduction were considered. According to the test guidelines, the measurement of actual test concentration in the highest and lowest concentrations should be performed. The loss of substance should not be higher than 20%. Whether this is a meaningful level for ENMs is not clear. ENMs provoke physical effects on the surface of daphnids, which affects the movement of animals. In chronic tests, an interaction of ENMs with feed was also observed. Dr. Fernandes recommended taking shallow exposure vessels into account in order to increase the contact between ENMs and organisms. For *Daphnia*, semi-static and flow-through systems are also applicable.

For the sediment-water *Lumbriculus* test (TG 225), the sediment composition was amended (clay/peat content). The worms behaved normally, and were in a healthy condition in the changed sediment. The addition of ENMs was achieved by creating a sonicated stock solution and shaking it into the sediment.

In conclusion, it is important to report all test conditions clearly, giving specific regard to shaking, media composition, and preparation of stock suspensions.

Discussion and remarks

- The fluorescence measurement of chlorophyll extracts has been found to be the most reliable way of quantifying algal biomass for testing effects of ENMs on algae growth. However, background fluorescence of ENMs should be reduced as much as possible (as also described in Hartmann et al. 2012[14]).
- Results from *in vivo* chlorophyll measurements suggested using microtiter plates (instead of isolating chlorophyll). ENMs, however, may interfere with measurements.
- Test conditions should be described in as much detail as possible.
- Regarding interferences, reporting on results from control groups is essential.
- Assay should be tested well in advance for minimal interference with ENMs.
- More information on issues of shading and absorbance characteristics should be generated before the test of an ENM.
- Measurements of concentrations are more difficult at lower concentrations and may be distorted due to interactions between organisms and ENMs.
- Poly- or mono-dispersity may vary under different shaking conditions.
- Heteroaggregation of ENM-algae was reported.

[14] Hartmann NB, Engelbrekt C, Zhang J, Ulstrup J, Kusk KO, Baun A. 2012. Nanotoxicology. In press.

- One should outline what experimental variables may be included, instead of adding specific changes in the TG
- A shaking procedure is recommended for range finding
- Alternative testing group in the WPNM may be interested in that procedure. It has already been described in the ISO/TR 13014[15]
- *Daphnia* test: a standardization of medium is recommended, given that there is a high variance in results when using the media currently recommended in the guidelines.
- It is difficult to use these different results for regulatory decisions.
- How can the information on single tests and ENM be included into a general decision tree? Which information can the tree provide?
- How can the information be useful for risk assessment?

General discussion and recommendations

The discussion centered on how to include the topics discussed into the decision trees. The first tree is dedicated to **stock suspension**, the second to the **exposure media**, where organisms and - depending on type of test - food is present. In general, the tree guides the user as to decide which protocol to use and to clarify the need for potential changes in the test protocol. It was discussed, whether the NIST protocol can be used, but it was noted, that this protocol specifically refers to TiO_2 in sun screen, and hence does not represent a general approach. One suggestion made by the audience was to prepare 3 different stock solutions and compare ENM characteristics. Based on the outcome of these tests, a decision on which protocol would be most adapted could be made. Further points rose focused on the presence of organisms which influence media conditions, and on dissolved constituents which are relevant for several ENM. In addition, participants discussed the **inclusion of ENM coating in category approach**. It was suggested to start with the coating or surface property instead of the type of ENM. The final suggestion was to split the tree according to naked / coated material, and hence always start with the material. There was also a debate over whether NM grouping or NM categorising described the same approach. This remained open, however, several bases for grouping were collected: material-based, property-based (e.g. reactivity, ROS generation) or toxicity / endpoint based. For stock suspensions, the property-based grouping was not considered.

Differentiating between properties of ENM and characteristics of ENM ("2 m tall or aggressive") was put forward as a valuable suggestion. Characteristics were considered more important for toxicity testing and are influenced by test media. Further it was discussed how to deal with the **presence of organisms** in the media, as they influence its characteristics which are hard to measure with current methods. Again, the concentration issue was discussed, and how to deal with losses in the tests. If less than 20% of loss occurs, subsequent calculations are carried out with **nominal concentration**. If more than 20% of loss occurs, than **measured values** should be the basis for calculation. In this context, participants discussed whether loss should only be considered for water columns or also for sediment material (e.g. daphnids are also exposed to sediment). Loss in water columns was put forward as the main consideration, although there was some doubt as to the realistic nature of this measure of exposure. It was also pointed out that exposure / loss also depends on the type of test organism used.

There was some consensus among participants that mass is currently the best metric to reflect applicability and measurement. If other metrics are available, there should also be a robust method to measure them. Currently, however, **the availability of sensitive measurement techniques is the main limitation**.

[15] ISO 2012. ISO/TR 13014:2012 Nanotechnologies -- Guidance on physico-chemical characterization of engineered nanoscale materials for toxicologic assessment. International Organisation for Standardisation

When discussing the issue of the 'conflict' between scientific and regulatory interest, the conclusion was reached that the field of NM characterization and testing is currently full of uncertainties and high variability.

Concluding Remarks on Ecotoxicology (Compartment Water)

Chaired: Steve Diamond (US-EPA)

1) Media preparation (stock and exposure)
    a. Stock solution protocols exist (but are limited in number and scope).
    b. Few (if any) exposure media protocols exist.
    c. Existing protocols suggest the need for a decision tree approach.
    d. Where little information exists, some target range of agglomerate size or size distribution should be suggested.
    e. Potential to require testing of key variables.
    Results of exposure media development will determine the frequency of characterization.
    f. May be necessary to include organisms in developmental efforts
    g. Importance of feeding effects.
    h. Where specific methods cannot be prescribed, more development will be required.
2) Concentration targets
    a. Currently unacceptable losses of materials during tests may occur more frequently for NMs.
    b. This suggests that guidance should be provided on how frequently measurement should be made.
    c. Guidance should be provided on approaches to developing consistent and repeatable exposure media and conditions.
3) Other dose metrics
    a. Surface area has been addressed to a small extent.
    b. Particle/agglomerate count still experimental (but little or no knowledge).
4) Characterization
    a. Size/Agglomeration, surface area where possible.
    b. Settling/Sedimentation.
    c. Frequency dependant on variability.
5) Consider categories throughout guidance development
    a. Based on both material characteristics and biochemical activity.
6) Guidance development
    a. Use GSPD as guidance document (rather than guideline-by-guideline approach).
    b. Guidance would follow a decision tree approach.
7) Higher-level discussion need to address guidelines vs. guidance, MAD, etc.

Decision Trees

- Start with information-rich materials (e.g. silver, TiO_2)
    - Coating or / doping.
    - Crystal structure.
    - Morphology.
    - Zeta potential, settling, agglomerate state, effect of environmental variables.

    This approach provides for robust development of a decision tree framework and guides the acquisition of analogous data for low-information materials
- First tree addresses preparation of stock solutions; elements include:
    - Existing protocols.
    - Knowledge of critical factors (coating, crystal structure, morphology).
    - Includes characterization needs (methods, frequency).

- Second tree addresses dilution in exposure media; elements include:
  - As above.
  - Potential use of natural stabilizing agents.
  - Approaches for characterizing exposure system.

  The goal is to develop an exposure approach that maximizes consistency and repeatability so that in-assay characterization needs can be reduced
- Third tree addresses compartments tested / conduct of test
  - Media-specific issues and decisions (e.g., soil, sediment, water).
  - Frequency of measurement and characterization.
  - Nanomaterial specific endpoint observations.

The potential for category identification and development should be evaluated throughout.

Environmental Fate and Behaviour (Compartment Water)

Chair: Frank von der Kammer (University Vienna, A)
Rapporteur: Carmen Nickel (IUTA, GER)

Background: Issues discussed in this breakout group included bioaccumulation, biodegradation and the abiotic fate of nanomaterials in the aqueous phase. The topics of discussion included:

- providing technical guidance and identifying the need to update current test guidelines ;
- examining the need to develop additional test guidelines ;
- developing test guidelines as well as appropriate methods of sample preparation; and
- dosimetry and analytic methods .

Degradation and Transformation of ENM
Jed Costanza (EPA, US)

Jed Costanza gave a stimulus presentation on the degradation and transformation of ENM. As a means of illustration, he explored in particular the casee of nanosilver and carbon-based ENMs.

Nanosilver is registered in the US as a textile preservative. Mr. Costanza presented a tiered approach used as a risk assessment for nanosilver used in textiles. The data required for Tier 1 include physical and release characteristics as well as ecotoxicological and health effects. The aim in examining these characteristics is to test whether nanosilver is released. If it is, Tier 2 was conducted and data concerning physical characteristics, ecotoxicological and health effects as well as environmental fate were involved. Further tests to be conducted were described.

Mr. Costanza also highlighted the dissolution kinetics of nanosilver and the biodegradation of carbon-based ENM.

The conclusion of the discussion was that the establishment of robust physical-chemical characterization methods should be carried out before undertaking further tests.

Bioaccumulation in Aquatic Organisms – OECD TG 305
Eric Bleeker (RIVM, NL)

Eric Bleeker gave a stimulus presentation of the OECD Guideline 305 – bioaccumulation in fish. For chemicals, the bioconcentration factor (BCF) is determined as an estimate for the bioaccumulation potential of a substance. Two approaches are available for determining the BCF. In the kinetic approach, the ratio between the uptake and the elimination rate constants is calculated. In the steady state approach, on the other hand, the ratio between the concentration in the fish and in the water is calculated when these are in equilibrium (steady state). In 2012, a revision of the TG 305 was adopted which includes a "minimised" aqueous test design and a dietary exposure method.

The minimised method reduces the number of sampling points during the test, but not the duration of the test. Since in this test design it is not possible to determine whether a steady state was established, the BCF can only be based on the kinetic approach.

The dietary exposure method is based on a kinetic approach. The sampling of fish only takes place during the depuration phase. Based on the food intake and the concentration of the test substance in the food, the assimilation efficiency (alpha) can be calculated. The product of the food intake and the assimilation efficiency can be used as a proxy for the uptake rate of the test substance. In contrast with most tested chemicals, ENMs are poorly soluble in water by using a stabilised suspension can water exposure be mimicked. There are two main problems with this test approach. Firstly, there is a loss in exposure concentration due to (i) agglomeration during the tests and (ii) sorption, as well as a loss of the ENM at the surface of the fish, test vessels, etc. The other main problem with this test approach is that in contrast with most tested chemicals, the uptake of ENMs is not driven by passive diffusion and thus no equilibrium can be established between concentrations in the fish and the water phase (unless all substance is removed from the water phase). Since passive diffusion is assumed in the calculations for BCF values, the use of such calculations for ENMs is in the least questionable. Therefore, the dietary exposure appears to be a more suitable method for bioaccumulation testing of ENMs. Due to differences in mechanism, the uptake of ENM is likely to take longer, suggesting that the test duration must be adapted for ENM testing. It was also highlighted that bioaccumulation can also be dependent on the organisms: PAHs for example do not accumulating in fish, but they do accumulate in crustaceans and bivalves. It is therefore also advised to consider other test species (e.g. mussels or daphnids) for ENM accumulation testing. The extent to which TG 305 (or other TGs on bioaccumulation, e.g. TG 315) can also be applied to other test organisms needs further investigation. Alternatively, a new GD or TG may be needed.

General discussion, remarks and recommendations

Both stimulus presentations gave a very good starting point for the discussion during the breakout session dealing with the mode of action and properties of ENM, testing strategies for ENMs, available measurements and metrics, the applicability of TG 305 for the testing of ENMs as well as data interpretation. Future research needs were also identified and discussed.

Specific modes of action and properties for ENMs were discussed. In comparison with "traditional" / "conventional" chemicals it is expected that some ENMs can exhibit different environmental behaviours and other specific endpoints may become relevant. During the discussion it was emphasised that the **dissolution** as well as the **dispersability** of ENMs are key factors affecting further environmental behaviour and test performance. For example, if the ENM can be dissolved in the test media within a given timeframe, nanospecific testing principles should not be considered and testing methodologies for traditional chemicals can be applied. Since there are no available test guidelines on dissolution and TG105 is not appropriate for nanomaterials, the creation of a new TG was suggested.

If the ENM cannot be dispersed, some TGs are no longer applicable or another application of the test substance to the test system as well as other modifications need to be considered.

Additionally it was mentioned that the particle surface, mass, etc. can be estimated if the dissolution behaviour of the tested ENM is known, enabling the use of other dose metrics in expressing toxic concentrations.

Besides dissolution and dispersability, the **aggregation state** and **degradation rate** (abiotic and biotic) were identified as important parameters affecting the environmental behaviour of the ENMs. The aggregation state of an ENM is dependent on many different parameters such as the physicochemical characteristics of the suspension media, suspension preparation, the physicochemical characteristics of the ENM, the concentration of the ENM (critical coagulation concentration - CCC) and the concentration of other substances and particles in the suspension. The aggregation state is also highly dynamic in many cases, because it is determined by kinetic processes. An ENM aggregation state can thus only be stable in the kinetic sense, i.e. not perceivably changing within a subjectively set time limit.

When comparing ENM behaviour under environment and laboratory conditions, it was highlighted that the aggregation state as well as the dissolution and dispersability behaviour of ENMs are more variable under environmental conditions due to differences in parameters that affect the ENM aggregation. In the environment, for example, **heteroaggregation**, i.e. agglomeration of ENM with abundant naturally-occurring particles is more likely than homoaggregation, which affects the mobility or transformation of the added ENM as well as the bioavailability for some target organisms.

➔Therefore it was concluded that tests dealing with these issues must also be performed in **different media**, representative of environmental conditions, to reflect this difference.

In the course of this discussion the **grouping of natural conditions** was mentioned as a helpful basis for further tests. Due to the high variation of abiotic and biotic parameters in natural compartments, a definition of standardised conditions would be helpful. Therefore it was decided to conduct the mentioned tests in **different media**, with varying pH, ionic strength (IS) and NOM content, which reflects the most common natural conditions.

The biotic and abiotic degradation of chemicals in the environment can be analysed by using different OECD TGs. It was discussed whether these TGs can be used for ENM or if other TGs or adaptations are needed.

Generalizing biodegradation and abiotic effects to different ENMs is difficult. Whether a case-by-case approach is necessary was a topic for discussion, for which no clear conclusion was reached. It was nonetheless mentioned that biodegradation tests (microorganisms and their production of CO_2, biomass, etc.), are not feasible for most ENMs because of the low concentration of organic material. Furthermore; if organic-coated ENMs are tested, the standard biodegradation TG is not applicable due to the low concentration of carbon used for the coating. From this reason, it was concluded that specific TG are needed. For biotic degradation, it was mentioned that the bio-durability should also be examined[16]. For abiotic degradation, different processes such as hydrolysis, oxidation, sulfidization and different redox conditions should be considered.

A further point discussed focused on the fact that if one is solely concerned with surface coatings on nanoparticles, one should enquire as to whether available biodegradation information on just the coating

[16] In SG8 of the WPMN a project has started that takes a closer look at biodurability.

(as for a chemical) can be used to supplement the biodegradation of the organic surface modification on the nanoparticle.

➔ It was concluded that the TG dealing with the biodegradation is not directly applicable for ENMs and it was decided that a specific **TG** for the **biodegradation of ENMs** or different groups of ENMs is needed.

During the discussion, it was also indicated that the important degradation processes for ENMs need to be identified, harmonized and tested.

The mentioned endpoints, dissolution, dispersability, aggregation and degradation should be tested when dealing with ENMs. Hence they should be considered in the development of new Testing strategies. A **decision tree / tiered approach** were developed during the break out session:

Firstly, the **dispersability and dissolution** of the ENM should be tested. Secondly, the **dispersion stability and aggregation state** (aggregation rate constants, attachment efficiencies or critical coagulation concentration) should be analysed (Figure 1). The dispersability as well as dissolution of ENM were identified as important parameters affecting the fate and behaviour of ENM in the environment as well as affecting whether further test performance is required (as mentioned above).

It was decided that the dissolution as well as the dispersability should be tested as a first step before further environmental tests are performed.

To represent the main environmental conditions, the tests must be conducted under different conditions with varying parameters such as pH, ionic strength, hardness, and presence of natural organic matter (NOM) or proteins.

For the testing of the material dispersion, the preparation of the suspension (e.g. sonication of the ENM) must be described in order for different laboratories' methodologies to be harmonised. It was also mentioned that the supernatant of the dispersion should be characterized after 24 h to cover kinetics of aggregation processes.

For the testing of the dissolution, filtration should be used for the differentiation between the soluble and insoluble fraction. The test should be performed in a time-dependent manner to cover the kinetics of this process.

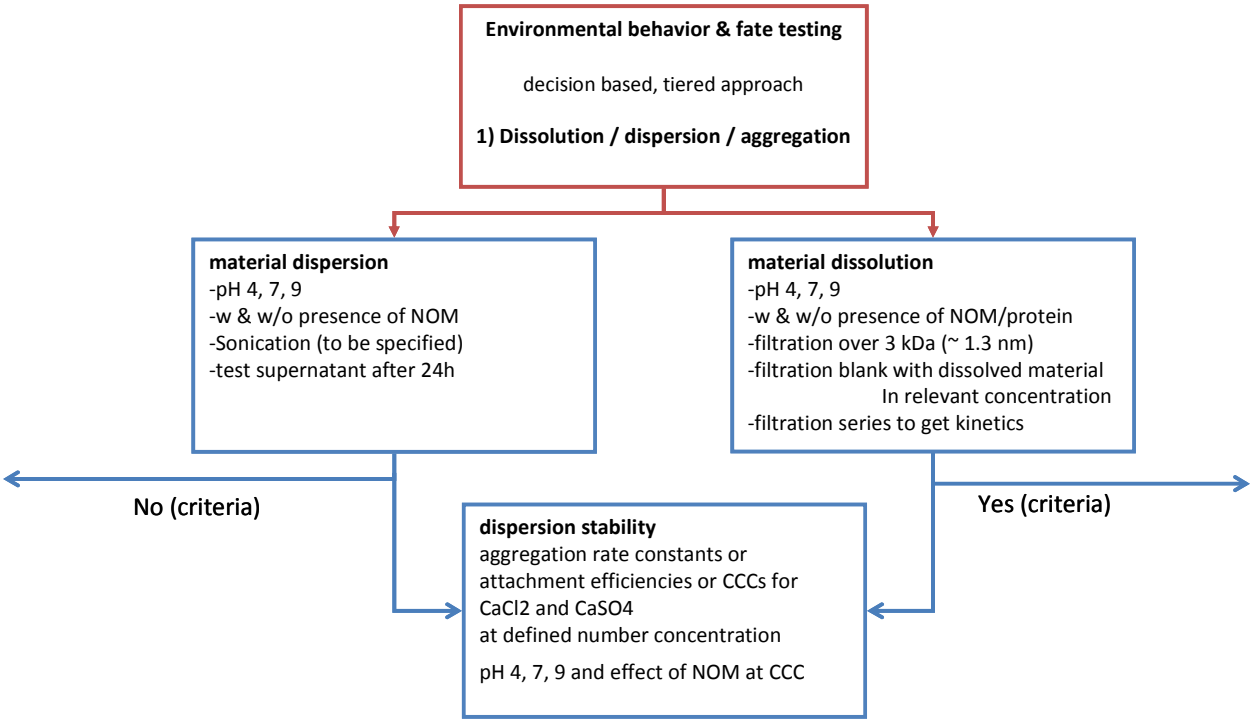

Figure 1: Draft of the first and second step of the decision tree for nanomaterial testing for fate and behaviour experiments. The decision tree has the aim to judge of the stability of ENM dispersion before conducting subsequent experiments. It will be further refined in the future.

During the discussion it was mentioned that in real environmental conditions, no pristine ENMs are expected. In addition, processes like biodegradation and abiotic degradation can change the physical and chemical form of ENMs and therefore their behaviour. In a third step, the **biodegradation** (Figure 2) and subsequently the **abiotic degradation** of the ENM should be tested (Figure 3).

The standard biodegradation tests are not applicable for most ENMs and specific TGs are needed. In the standard test, the degradation of organic C is determined mostly by TOC measurements. If no organic C is found the tested material, it is defined as not biological degradable. Most ENMs have no or show only a very low concentration of organic C (organic coated ENM). As such, it was decided that this measurement is not applicable to ENM.

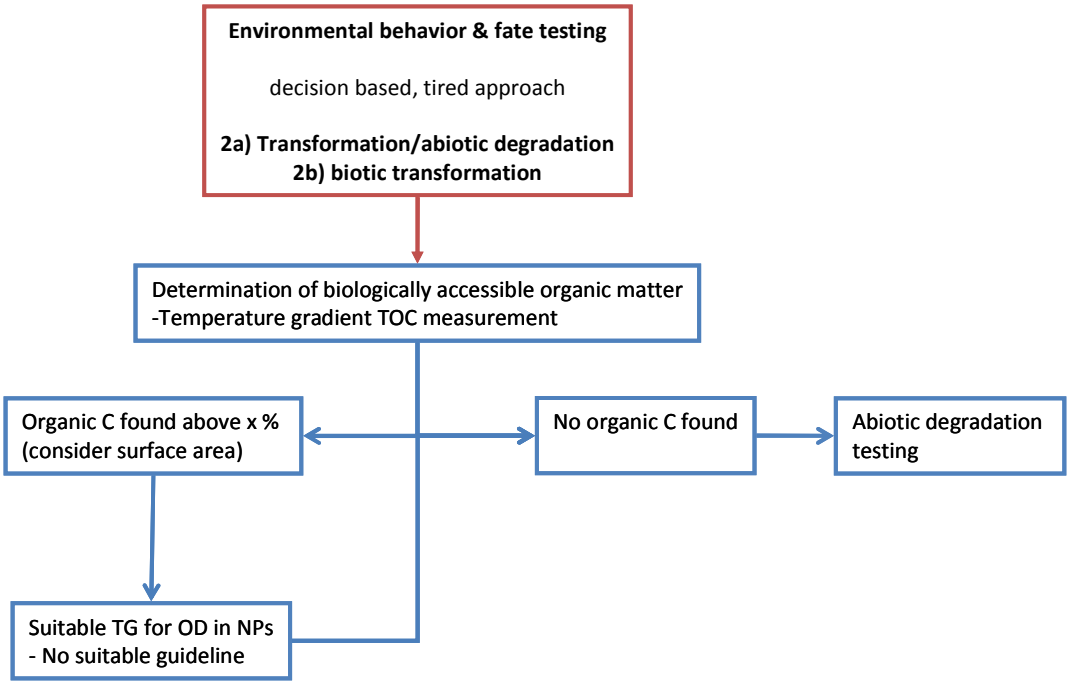

Figure 2: Draft of the third step of the decision tree for nanomaterial testing for fate and behavior experiments. The aim is to assess the biotic degradation of ENM. It will be further refined in the future.

As well as biotic degradation, abiotic degradation of ENMs should be determined to get information concerning the environmental behaviour of the ENM. The abiotic degradation tests should include for example the effect of UV light, anoxic and oxic conditions as well as biodurability. It should also be established whether existing guidelines can be applied to ENM.

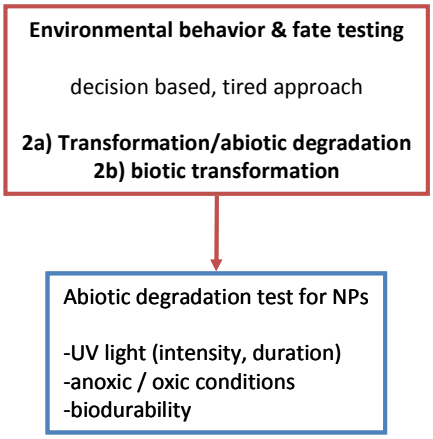

Figure 3: Fourth step of the decision tree for nanomaterial testing for fate and behaviour experiments. The aim is to guide through the abiotic degradation testing. It will be further refined in the future.

In the environment, no pristine ENMs are expected. The group decided that relevant conditions that lead to **alteration and transformation** of ENMs should be identified. These conditions will then be added as amendments to existing TGs or a separate new GD will be developed.

➔ The **harmonisation of the altering procedure** was identified as one important and challenging step. The following different circumstances for the abiotic degradation testing were specified during the discussion.

It was suggested that conditions like the abiotic physical and oxidative degradation as well as the biotic hydrolytic degradation are important parameters affecting the dispersability and therefore the fate and behaviour of ENM in the environment. Therefore this parameter should be tested in a first step in a time-dependent manner. During the discussion, it was also mentioned that reducing conditions and a sulfidic environment should also be added, in order to better represent the main environmental conditions (Figure 4).

The mentioned conditions are a starting point and can be updated if other important conditions are identified.

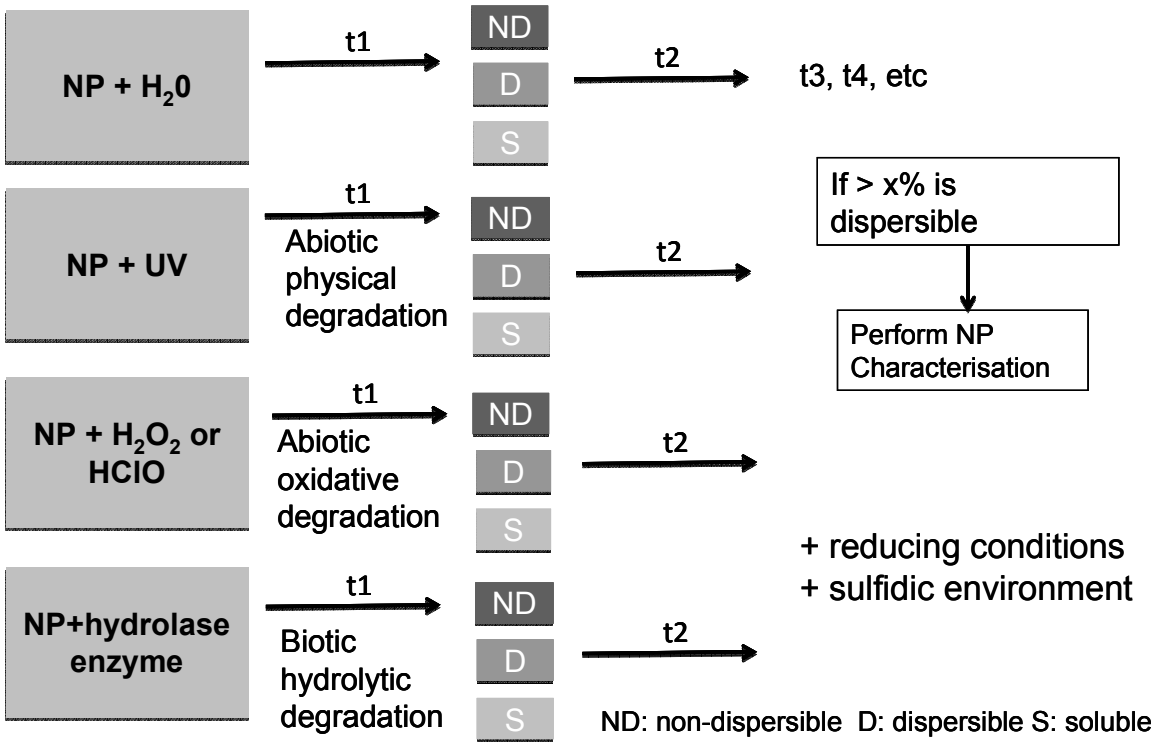

Figure 4: Examples of the aging parameters, for the abiotic degradation of nanomaterials

During the discussion, the **lack of sensitive detection methods** for the quantification of ENMs in the test system as well as in organisms (tissues) was noted as an important limitation. It was affirmed that more sensitive instruments are needed. This lack of methods is particularly apparent for carbon-based ENM, while some progress has been made to analyse metal- and metal oxide ENM in complex liquid matrices. Currently, it appears that the specific number concentration of ENM cannot be quantified. Therefore measuring the total concentration and the background concentration is the commonly used methodology. The detection/identification of some ENM located inside tissues or cells with electron microscopy should always be balanced against the total applied dose. It is not a quantitative determination of uptake: even a

few hundred particles represent minute fractions in terms of particle number or mass with respect to the dosed amount.

It was also mentioned that, based on the already listed endpoints for the physical chemical characterization of ENM in the GSPD, the specific metrics which are relevant to the fate and behaviour of ENM in the environment should be identified (e.g. particle number concentration, surface area concentration, etc.).

The applicability of the well-established bioaccumulation test guideline OECD TG305 for the testing of nanomaterials was discussed.

In contrast to traditional chemicals, no steady state / equilibrium for ENMs is reached during the tests. Therefore different applications of the test procedure are necessary if the TG305 is used for the testing of ENM.

First, the calculation of the bioconcentration factor (BCF) as a sufficient endpoint for ENMs was discussed. The BCF is associated with uptake of ENM from the water compartment. It was decided that the idea of the BCF is a very good concept also for ENMs but not in the same meaning as for traditional soluble chemicals, because no steady state is reached for ENMs. Therefore it was mentioned that the calculation of the BCF in tests with ENMs can be misleading and should be avoided. Furthermore, the BCF should be renamed to avoid confusion. Alternative terms for this endpoint could be internalisation rate or attachment efficiency. These terms wouldreflect its utilization for ENM and would specify that other endpoints are used instead of the BCF. The bioavailable fraction (BAF) or biomagnification factor (BMF) was mentioned as a promising endpoint.

The attachment efficiency was also suggested as an important point for the uptake mechanisms.

Furthermore, the partitioning of ENM and its quantification were discussed. For some ENM, no partitioning is currently expected and a partitioning of most ENMs compared to organic chemicals seems to be unlikely. Because of this, it was discussed that uptake tests could possibly provide more needed information. One hypothesis is that the mass transfer for most ENMs in tissues are quite negligible, but it is currently not possible to decide if this is true for all ENMs. This must be tested in further studies.

The internalisation rate was suggested as a promising endpoint. If the internalisation rate is used for the data interpretation, the fact that the internalisation rate is dose-dependent and that the test performance should be described in detail should be taken into account. The conclusion reached is that the internalisation rate should be complemented with a non-dose dependent endpoint.

The bioavailable fraction (BAF), which is associated with the uptake from food, was also suggested as a further endpoint. During the discussion, it was noted that the identification and quantification of the bioavailable fraction, which can be depend on different parameters (e.g. the crystalline structure shown for TiO_2 - rutile or anatase), cannot currently be guaranteed. It was therefore decided that the bioavailable fraction is currently not a sufficient endpoint.

One additional point to consider is that instead of the BCF, the biomagnification factor (BMF), which refers to the uptake through the food chain, should be calculated. Whether the BMF is applicable for ENM should be specified in further studies. If the BMF is used, the formula for the calculation must be reconsidered and if necessary adapted for ENM.

It was also noted that the detection of the mass transfer into biota is a challenge, if no marked ENMs were tested. Therefore the need for the development of more sensitive measurement techniques was highlighted.

In the course of this discussion, it was also noted that as the K_d, and also the K_{ow} are not applicable to ENMs since their underlying principles are different.

It was also mentioned that due to the lack of steady state, the defined test duration in the TG should be adapted to the testing of ENMs. No specifications were discussed: this has to be specified in further tests.

For the tests with ENMs, the defined starting conditions are often not the conditions which prevail at the end of a test. Therefore, a detailed description of the test procedure is essential for the comparability of the data with other studies.

The application of the ENM to the test system was also discussed. The application via the water phase and via the food was compared. Due to the experience during the tests with ENMs, it was decided that the dietary exposure of the ENM to the organisms is more suitable compared to water exposure. Through water exposure, a heterogeneous distribution of the applied ENM is expected, due to agglomeration processes and sedimentation or / and attachment of ENM to the organisms or test vessels. It should also be taken into account that with increasing particle size the availability for some organisms decreased. For example, for filtration organisms, like *Daphnia*, the uptake of ENMs is size-dependent.

The abiotic and biotic parameter in the media affected the organism-specific uptake rate of ENMs (hetero- and homoaggregation). This can lead to adapting the test procedure (e.g. application form or duration, test organisms). It was also mentioned that maintaining a homogeneous exposure over longer time periods is one of the biggest issues for environmental tests (sedimentation, aggregation, adsorption), but no further comments were made during the discussion.

With dietary exposure, a more homogeneous uptake of the ENM can be adjusted and the loss of the ENM during the application (sedimentation, attachment to the test vessel, adsorption to the organism) is minimized. Also, the agglomeration, sedimentation, attachment to surfaces and organisms are very likely processes in the natural environment that the ENM will undergo. Hence the dietary exposure route might be the more realistic exposure.

Therefore it was concluded that the dietary spiking is the preferred method for nanomaterial testing, which should be mentioned in the TG, as a worst case scenario.

In the course of this discussion, it was noted that it has to be taken into account that the spiking of the food can change the consistency / taste of the food, which can affected the feeding behaviour of the organisms. This is also true for conventional chemicals.

The added concentration to the test system was identified as an important parameter, affecting the uptake, fate and behavior of ENMs. It can also affect the type of the target organism (critical coagulation concentration – aggregation – sedimentation). For tests with direct application of ENMs to the water compartment, it was observed that most of the applied ENMs are adsorbed by the organisms or settle in the sediments and are not taken up. During the discussion it was hypothesized, but could not be clarified, that this effect is concentration-dependent and should be minimized if lower concentrations can be used.

However, depending on the type of ENM at the moment high concentrations of ENM have to be applied into the test system to enable their traceability in the different environmental compartments or target organisms

It was also discussed whether organisms other than zebrafish could be used, given that organism sensitivity to ENMs may differ. This was already shown for traditional chemicals like PAHs. It is also expected that based on the environmental behaviour and agglomeration state of the applied ENM, other organisms than fish are more exposed to the ENM (e.g. benthic organisms). For some ENMs it is

questionable whether zebrafish are the right target species and whether other organisms should (instead or additionally) be tested.

It was also mentioned that, for traditional chemicals, *in vitro* tests are used as pre-screening tests. It was decided that this should also be suitable for the testing of ENMs. The *in vitro* tests should be applied to reduce the number of animal experiments.

Needs for further studies were identified in the breakout session. It was discussed that bioaccumulation tests should also be conducted with other organisms, if fish are not the right target organism. The uptake of ENMs by other organisms, such as filtering organisms (mussels, crustaceans) must therefore be analysed.

The lack of sensitive measurements as one important limitation factor for the interpretation of data was also mentioned during the discussion. The development of sensitive measurements was therefore identified as an important topic for research.

The sensitive measurements are also needed for the differentiation between uptake (no information about transfer), intake (ingestion rate, digestibility rate) and attachment of applied ENMs.

In terms of the transferability of the biomagnification factor for ENMs, it was decided that further knowledge is needed to decide whether this concept is applicable to ENMs. Other endpoints may also be necessary and should be identified in further studies.

The transferability of threshold values for the classification of the persistence of traditional chemicals (regulatory view) for ENM could not be clarified during this discussion and further tests should be conducted.

Concluding Remarks on Fate and Behaviour (Compartment Water)

Presenter: Frank von der Kammer

In summary, it was concluded that:

➔ A decision tree / tiered approach for the testing of ENMs should be established:

- First step – dissolution and dispersability → new TG needed
- Second step - agglomeration state and dispersion stability → new TG needed
- Third step – biodegradation → a new TG must be developed.
- Fourth step – abiotic degradation → definition of important parameter.
- Definition of important parameters for the aging of ENMs.
- Identification of specific metrics which are relevant to the fate and behaviour of ENMs in the environment.
- Development of sensitive measurement techniques.

➔ The OECD TG 305 on the dietary approach is applicable to ENMs if some nano-relevant additions and changes were added to the TG as GD.

- The group decided that the bioconcentration factor (BCF) is not applicable if the tests are conducted with ENMs.
- Alternative endpoints were discussed (internalisation rate, attachment efficiency, bioavailable fraction) but no clear decision was made.
- The application of the ENM to the test system was defined - it was concluded that the dietary spiking should be used for the test with ENMs as a worst case scenario.
- It was decided that the test procedure needs to be described in detail.

➔ In vitro tests as pre-screening tests should be used.
➔The K_{ow} value is not suitable for predicting bioaccumulation and not an appropriate endpoint for the physicochemical characterization of ENMs.

Overall Conclusions on Compartment Water: Ecotoxicology and Environmental Fate and Behaviour

Chair: Gregg Goss (University of Alberta, CA)
Rapporteurs: Dana Kühnel (UFZ, GER) and Carmen Nickel (IUTA, GER)

The Chairs of the breakout session, Frank von der Kammer and Steve Diamond, summarised the results of the two breakout sessions (see Ecotoxicology – Compartment Soil and Sediments and Environmental Fate and Behaviours – Compartment Soil and Sediments.).
Following this summary, the important points identified were discussed with all participants.
- TG305
- Suspension preparation
- Measurements techniques and metrics
- Decision tree
- Application of the ENM to the test system
- Endpoints for environmental behaviour

With regards to TG 305, the K_{ow} (a parameter used for organic chemicals) was found to be non-applicable to ENMs. In accordance with this, the BCF is not applicable to ENMs either. It was discussed whether the BMF should be calculated, instead of the BCF. In principle, the BMF can be calculated but the risk of data misinterpretation is high, due to the fact that no steady state is reached and no partitioning is expected.

For the TG305 it was suggested that the ENM should be applied to the system via diet as a worst case scenario. The application form for ENM testing must be noted in the TG.

The sonication method for suspension preparation was discussed. If sonication is used for suspension preparation, measuring or calculating the inserted energy was recommended. In addition, favouring filtration instead of centrifugation for the preparation of suspensions was suggested, given that it is an easy method, available in every laboratory.

Other than mass, a list of important metrics to be used should be identified (e.g. surface area). Indeed, mass is considered inappropriate for ENMs, given that many nano-specific issues are not addressed: e.g. when measuring mass in soluble materials, all materials are lumped together without any consideration for their size and/or aggregation state. The EU definition can act as a basis for dose metrics, although there are several analytical problems with the detection of the number size distribution. One shortcoming of many studies is that particle counts are often mentioned, but that of primary particles, agglomerates, and aggregates are not available. The same kind of metrics for ecotoxicology and fate studies should be used and other metrics should be reported whenever possible.

Alternatively, advice is needed on how to measure mg/L for ENMs, as the TG refers to this parameter. In general, the availability of sensitive measurement methods was considered a major constraint here.

Regarding ENMs in aquatic media, two different issues should be considered: (1) a stock solution for one material, (2) interaction of ENMs with test media (e.g. different pHs). In addition, the question of how to deal with the presence of organisms in the media is problematic, given that they influence media characteristics and some may be hard to catch by measurement methods. Also, species' sensitivities may differ with different types of ENMs.

Exposure / loss of ENM from test solutions also depend on the type of test organism used. An ENM's attachment efficiency to surfaces should be identified as it is a process which may also account for losses. Measurement of exposure when ENMs are aggregated, agglomerated (OM), or adsorbed to animals is also a difficulty. The possibility of measuring surface area in suspension with BET-N_2 adsorption as well as NMR was discussed. Both methods give no reliable results e.g. it is unclear how agglomeration influences results.

Stable exposure systems for aquatic exposures are important. In this respect, ENM stability was considered more important than monodispersity. Furthermore, it was stated that different media produce different toxic outcomes - a fact that should not be used intentionally to reduce toxicity in regulatory testing.

Concerning biomagnification, differentiating between ENM taken up by organisms and ENM attached to an organism is not necessary, given that ENMs will be ingested by the next trophic organism and the whole body burden should be measured.

Regarding the "conflict between" scientific and regulatory interest, the GSPD addresses some of the uncertainties in characterization and testing for regulatory purposes. Nonetheless, further development is needed perhaps through mechanisms such as the 2013 physical chemical OECD workshop held jointly with ISO. Furthermore, advice on how to deal with unbound or bound ENMs (e.g. heteroaggregation with NOM) is needed. Current TGs are lacking in this respect. However, there was some recognition that not all environmentally mitigating factors and all natural processes could be included in TGs.

SESSION THREE: ENVIRONMENTAL TOXICITY AND FATE OF MANUFACTURED NANOMATERIALS – COMPARTMENT SOIL AND SEDIMENT[2]

Ecotoxicology – Compartment Soil and Sediments

Chair: *Willie Peijnenburg (RIVM, NL)*
Rapporteur: *Dana Kühnel (UFZ, GER)*

Background: Soil and sediments are of special importance for assessing the environmental impact of ENMs. Suggested exposure routes of manufactured ENMs to soil and sediment organisms are: sedimentation of unstable ENMs dispersed in water, application of biosolids to soils, landfill leachate, deposition of airborne ENMs, use of ENMs in agriculture and remediation etc. The particular aim of this session will be to discuss the TGs for earthworm and sediment worm reproduction (OECD 222 and OECD 225), as these tests are of special importance to assess the impact of ENMs in soil and sediment species. The second focus of the session will be the detection and quantification of ENMs in porous media.

Testing and Characterisation: OECD Test Guidelines 222 and 225
Kerstin Hund-Rinke (Fh-IME, GER)

As in water, the application of ENMs to soils and sediments is a major challenge, as Kerstin Hund-Rinke pointed out in her talk. The composition of soil and sediment is important for the behaviour of ENMs. In general, different media to be spiked are considered. For soil, either soil or feed can be spiked; for sediment, either the sediment or water can be spiked. In the following, the focus will be on the spiking procedure for sediment. Here, Dr. Hund-Rinke pointed out the different requirements for regulatory testing (aim: decision making) and academic testing (aim: improve understanding) with regard to test design. The main points addressed in the talk were:

- Spiking soil versus feed: avoidance, relevant exposure via food.
- Both spiking regimes led to comparable results, recommendation for spiking of soil.
- Dry vs. wet spiking (no chemical as carrier vs. preparation of dispersion).
- Limitations in test concentrations due to water holding capacity of soils when using wet spiking.
- Concentration-dependence with dry spiking, no concentration-dependence in wet spiking over a range of organisms.
- Recommendation to use dry spiking.
- No differences in EC_{50} for silver between dry and wet spiking.
- Same testing regime for inert and ion releasing materials?
- Natural soil should be preferred (compared to silica sand).
- One procedure for all the different ENMs (inert/ion releasing, coated/uncoated, hydrophobic/hydrophilic).

Concluding Remarks and Recommendations

Based on current results it is difficult to recommend one spiking procedure (either dry or wet spiking). Effects were observed after wet spiking at lower concentrations (also in bacteria and plants), hence wet application probably results in a higher bioavailability. The dry spiking is preferred by some participants because of its ease to prepare stock solutions and the exclusion of additional chemicals. Additionally, the dry spiking is thought to be a more harmonized procedure over a wide range of ENMs.

The spiking procedure for replicates was elaborated. Specifically, the question of whether it is more appropriate to spike every replicate separately or to prepare one stock and separate it into replicates was

examined. The repeated spiking procedure was not favoured, given that drying the soil is not recommended due to ENM aging factors. It was concluded that it is too early to come to a final recommendation on spiking procedure, due to a limited database and mechanistic understanding of the processes. The ENM application via food was seen as being critical, given that food is a difficult matrix leading to unknown interactions. Hence the soil application was favoured because of better knowledge of the dose applied. Regarding the variety of different ENMs and protocol amendments, it was mentioned that the recommendations given in GSPD are still valid.

Soil is the dominating factor in the test; hence the composition of soils is influencing the modifications of ENMs or the aging of materials. Differences between different soil types will occur, e.g. loamy soils have a higher sorption capacity. However, the reference soil (sandy soil) recommended in the guideline was found to be suitable for ENMs.

The characterization of ENMs in soil was considered as a black box, due to methodological problems associated to it on many levels. Firstly, the instrumentation to characterize ENMs in soil is lacking. Secondly, analysis of neither pore-water nor soil wash water is possible. Further, it is unclear how the ion concentration in the soil will influence aggregation. Finally, large differences in sediment and pore water ENM concentrations will occur and it was asked whether guidance for regulators will be necessary.

It was stated that the exposure in sediment systems is generally hard to assess. The fate in the systems is unclear, and a homogenous distribution of ENMs in soil / sediment is hard to prove. One suggestion was the application via food. However, the feeding rate of worms is unclear and hence the exposure is unclear as well. Furthermore, achieving homogeneous exposure for food spiking may be problematic. The measurement of internal concentrations or body burden was suggested. It was reported that juveniles are much more affected than adults, but the measurement of low ENM concentrations in these animals is difficult. Also, as exposure of worms occurs mainly via the pore water phase, the influence of exudates etc. remains unknown. Further points discussed include the application of ENM to sludge and plant testing (hydroponic vs. soil testing).

The participants discussed whether the endpoints used in the tests are suitable for ENMs. The use of probably more sensitive endpoints, e.g. enzyme regulation was suggested; however these endpoints must be robust enough to cover compensation by the organisms. Further, effects in soil were reported to be subject to seasonal changes.

Detection of ENM in Soil and Sediment
Geert Cornelis (University Gothenburg, SWE)

Dr. Geert Cornelis gave an introduction to the complex issue of speciation of ENMs in soil, based on the fact that pristine ENMs rarely occur, but are mostly linked to other substances. Additionally, aging processes play a role. In wastewater, ENMs may agglomerate with biosolids. The background concentration of e.g. metal salts or organics in the soil influences ENMs. All these processes influence bioavailability, a potential reduction of which may occur due to agglomeration. The available analytic techniques are too time-consuming for high-throughput. The main points addressed in the talk regarding this issue were:

- Total metal concentration: determination of a complex, careful digestion procedure (e.g. Ag complex formation with chloride)
- Bulk soil analysis: natural background concentration for some elements is high, so work with high concentration of ENM is necessary or the use (radio or stable isotopic) labelled ENM.

- For some ENM (e.g. Au), detection via SEM or ESEM is possible, EDX, EELS, also size distribution, number concentrations; time consuming!
- Separation techniques: separation of pore water, more techniques available for pore water analysis, recovery for some elements low.
- Centrifugation techniques: density of ENM has to be known.
- FFF laborious, but advantages (e.g. low size limit, data treatment…).
- Single particle-ICP-MS: slightly higher size limit than FFF and TEM and method requires development, but very low number concentrations are possible and fast method.
- Combination of FFF and sp-ICP-MS possible.

Concluding Remarks and Recommendations

Suitability of detection methods

The different detection limits of the methods were discussed (regarding particle size and amounts), as well as the different efforts regarding time and cost. In general, more validation and development of methods are required. The EELS method, suitable for speciation, is time-consuming, but it is not easy to relate to a specific spot in a soil sample. The XPS method is more suitable, faster than EELS, and allows the differentiation between metal and salt. XPS can also be used for orientation in the sample and to identify relevant fractions. In principle, a combination of both methods is possible. The EM methods were controversially discussed. SEM quantification of particles is possible, but very laborious, and agglomerates are difficult to count. EDX mapping is possible with a resolution of 3-5 nm, but is slow and costly. The electron contrast between carbon-based systems (organisms) and metal-based systems (soil & sediment) is different, and it will be much easier to track (metal-based) particles in carbon-based systems by EDX. Furthermore, EM is prone to artefacts: e.g. the evaporation of liquid during sample preparation may lead to agglomeration of ENM.

In addition, for some types of ENM (e.g. carbon based materials) EM is difficult. However, for other materials, EM will be the only suitable method. Furthermore, with regard to the detection methods, the different requirements for regulatory and academic purposes became evident. It was claimed that a good method should allow an insight into the presence and composition of the NM coating. To date, several preparation steps are systematically required before soil can be analysed with any method. Hence, either additional methods or improvements in existing methods for the detection of ENMs in soil are urgently needed. An understanding of complex interaction of ENMs with soil / sediment is also needed.

Bioavailability

One important step to assess the bioavailability is extraction. For ENMs, harshness of extraction depends on the type of material. Hence, extractable fractions / recovery strongly depend on choosing the right extraction protocol. The bioavailability determination also depends on ENM size as well as other material parameters and differs very much with time (e.g. Ag in sewage sludge). Checking the bioavailability and toxicity of different fractions after filtration is important. Also considered relevant, but well studied was the fate in environmental soil (from sludge to rivers).

Determining ENMs in pore water

The applicability of leachate or pore water isolation strongly depends on the type of soil. Also, ENM aggregation is influenced by dilution processes during pore water extraction, which will depend on presence of NOM in the soil. The influence of the soil particle composition on the interaction with ENM was discussed. Sequential filtration was suggested, in order to differentiate between background metal / nanoscaled material in the pore water. For regulatory purposes the measurement of total concentration is

also important. This is difficult to achieve by the procedure suggested, because the relation of pore water to total soil concentrations remains unclear.

Implications for guidance

The discussions lead to the conclusion that the methods presented are too premature to be used in new TGs, and further developments of EM and other techniques are required. In the guidance, however, both academic and regulatory needs should be considered. Furthermore, a tiered approach for soil testing was suggested. Again it was discussed whether the decision making practiced for chemicals can help for risk assessment. As for the water tests, the question remains as to the extent to which the tests are able and should replicate realistic environmental conditions.

Concluding Remarks on Ecotoxicology (Compartment Soil and Sediments)

Ecotoxicology

Tiered approach:
1. Dry or wet spiking.
2. Analytical part: concentrations in the test system, hopefully more development of suitable methods.

OECD TG 222 and TG 225 can be used for nanomaterial testing, on forehand relatively minor modifications needed, but keep open mind for inclusion of new insights

1. Recommendation to allow both wet and dry spiking.

Wet spiking: duplicate efforts possible aquatic testing: same stock/stem solutions

Issues to deal with:

1. Food spiking: relevance of not mixing food in soil?
2. Replication: true replication versus split up of samples.
3. Aging – modification of nanomaterials.
4. Application across different soil types – probably not a big issue with regard to guideline design.
5. Dose metrics – how to express effects. For now: total concentration – also for regulatory purposes.
6. Bioavailability: extracts – assessment bioavailable fraction.
7. Nano-sensitive endpoints: to be considered.
8. Applicability for other organisms.
9. Variability test results.
10. Applicability to simulate specific scenarios like sludge application.

With regard to issues 4-7: It has to be elucidated to what extend analytics methods may be applied to assists and improve the assessments.

Detection in soil and sediment

Tiered approach

1. Define regulatory needs.
2. Distinguish methods for stable – unstable particles.
3. Electron microscopy to characterize particles.

4. FFF + single particle ICP-MS to quantify.
5. Develop routine technique for regulatory testing: pore water extraction, filtration, (including future technical developments).
6. Apply operationally defined techniques – short cut once more experience available.

Discussion and remarks

- Drawbacks of methods.
- We do not know bioavailable fraction – no method to mimic.
- Limited number of techniques.
- Availability of advanced/ expensive equipment.

We can measure ←→ link to effects?

Summary / Recommendation

- No recommendation can be made for now.
- Methodology has to be developed further.
- Application of NMs is a challenge, also in terms of reproducibility and knowledge on transformation of NMs in soil.
- Spiking (feed, soil): both spiking regimes led to comparable results.
- Different procedures for inert, ion releasing coated NMs?
- Link concentration measurement to observed effects is difficult.

Environmental Fate and Behaviour – Compartment Soil and Sediments

Chair: Thomas Kuhlbusch (IUTA, GER)
Rapporteur: Carmen Nickel (IUTA, GER)

Background: The first issue of the breakout group will focus on the investigation of fate and behaviour of nanomaterials in soil and sediment. The applicability of the OECD TG 106 as well as 312 for the testing of ENM was discussed. The second issue discussed focused on the OECD test guidelines on bioaccumulation in soil and sediment species (OECD TG 315 and 317). The discussion will include challenges of sample preparation and analytic characterisation of nanomaterials in the respective test matrices.

Fate of Manufactured Nanoparticles in Soil Environments
Jason Kirby (CSIRO, AUS)

Jason Kirby gave a stimulus presentation about the fate and behaviour of different ENMs in soils. He pointed out that the colloid aggregation theory is also important for ENM and should be used. For example, it is known that colloids aggregate faster at high ionic strength solutions and that the importance of straining increases with aggregate growth. In addition, heteroaggregation is an important process in natural soils which can lead to retention of the ENM. In this case, a low mobility is therefore expected. This was presented for CeO_2 ENMs which were applied to natural soil columns. The retention and aggregation of ENM in soil columns depending on the biosolid concentration was also demonstrated. It was shown for Ag ENM that retention increased whereas no effect was observed for the tested CeO_2.

The OECD TG 106 can be used to measure the adsorption and desorption behaviours of a substance in soils. Its applicability for the testing of ENM was examined and it was concluded that it is not applicable to ENMs. No differentiation between adsorbed and non-adsorbed ENMs occurred during the tests, given that non-adsorbed but aggregated ENM will be also defined as an adsorbed fraction. Furthermore, no K_d values could be calculated, as no steady state was reached. However, the calculation of the likely retention factor (K_r) (Cornelis et al. 2010[17]) was favoured over K_d. The K_r values account for the potential dissolution process of ENPs in soils using ultrafiltration.

Remarks

Filtration for differentiation can have an effect on the results, given that also smaller particles could be retained. Therefore in pre-tests, different membranes and pore sizes should be tested and verified.

Bioaccumulation in sediment-dwelling benthic and terrestrial oligochaetes: OECD Test Guideline 315 and 317
Anne Schneider (RWTH, GER)

Anne Schneider gave a presentation on bioaccumulation testing and the applicability of TG 315 and TG 317. The methods for application, quantification and characterization, as well as caveats in bioavailable testing were presented.

The application of the ENM to the test system for the TG 317 can occur in two different ways: as dry powder (as already addressed in the guideline for poorly soluble substances), or as suspension of the ENM, the latter case not being addressed in the TG. The advantage of the suspension is that it can be characterised before it is added to the system. An institute-related study (Schneider et al. in prep.) and e.g. a study by Hund-Rinke et al. (2012)[18] demonstrate that both application forms show a good reproducible and homogeneous application of ENM. For the TG 315, three different ways for the ENM application can be used: sediment + ENM powder, sediment + ENM suspension, water phase + ENM suspension.

A significantly higher bioavailability of CNTs to *Lumbriculus variegates* was revealed after a seven day exposure to the water spiking experiment than after exposure to the sediment spiking experiment. Therefore, water spiking should also be considered as an application method (Maes et al. 2012[19]). Suspension application might not be suitable for all ENM, due to energy input during dispersion (e.g. coated ENM). Furthermore, the dry application method might not be suitable for some ENMs, e.g. CNT (homogeneity).

The quantification of ENMs in natural environments is still a challenge and analytical methods for ENMs are still under development.

[17] Cornelis et al. 2010. A method for determination of retention of silver and cerium oxide manufactured nanoparticles in soils. E*nviron. Chem. 7, 298-308.*

[18] Hund-Rinke K, Schlich K, Klawonn T (2012): Influence of application techniques on the ecotoxicological effects of nanomaterials in soil. Environmental Sciences Europe 24-30.

[19] Maes, H. M.; Rhiem, S.; Stibany, F.; Daniels, B.; Björn, D.; Simon, A.; Gieffers, S.; Riding, M.; Semple, K. T.; Jones, K. C.; Martin, F. L.; Baumgartner, W.; Hollert, H.; Schäffer, A., Bioavailability of carbon nanotubes to aquatic organisms of different trophic levels and the consequences of CNT-cell interactions to vital functions - extended abstract. In ABSTRACT BOOK of the 6th SETAC World Congress/SETAC Europe 22nd Annual Meeting, 2012

Remarks

The spiking of the sediment with an ENM suspension was favoured by the group. ENMs can be characterised in the suspension and a homogeneous application can be conducted. If the ENMs are applied to the water phase, the most likely scenario is that aggregation occurs and that no homogenous distribution in the sediment can therefore be reached.

Concluding Remarks on Environmental Fate and Behaviour (Compartment Soil and Sediment)

Both presentations gave a very good starting point for discussion during the breakout session dealing with the applicability of TG 106, 312 and TG 315 and 317 for the testing of ENMs. Furthermore, future research needs were identified and discussed. In the course of the discussion on the applicability of the TGs, specific physical chemical properties of ENMs (which were important before the tests were conducted) were identified and discussed.

Similarly to the results of the water breakout session, the dissolution as well as the dispersability (Figure 1) of the ENM was identified as important parameters which should be specified before the fate tests are conducted. For example, tests on mobility in soil should not be undertaken if no stable dispersion of the ENM can be produced under environmental conditions.

➔ Therefore a decision tree was developed (Figure 5), with the identification of the dissolution and dispersability behaviour as first step.

The results of these tests will affect the rest of the experimental procedure. Some test, for instance, should not be conducted if the ENM cannot be dispersed. Furthermore, no "specific" nano test needs to be conducted if the ENM can be dissolved. The TG which was applied for traditional chemicals can therefore be used.

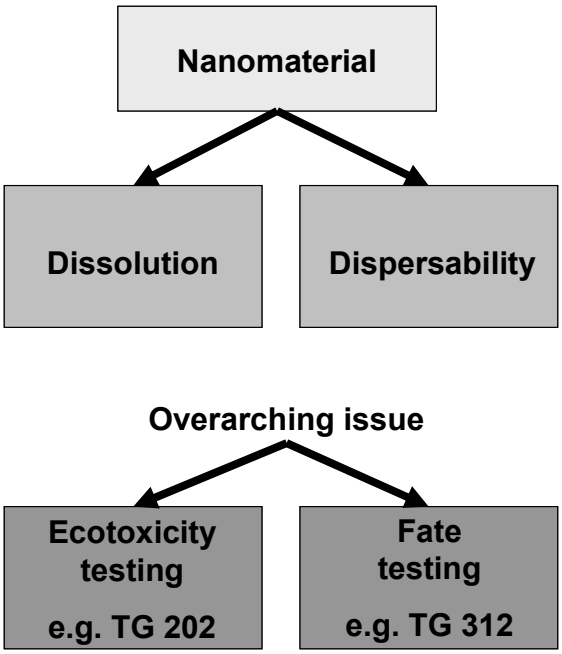

Figure 5: Draft decision tree which should be established before the OECD TG is conducted. The aim is to judge on the dissolution and dispersability of ENM before further tests are conducted. It will be further refined in future.

During the breakout session, the fate and behaviour of ENMs in soil and sediments as well as the applicability of different TG were discussed. Furthermore, information regarding the fate and behaviour of natural colloids can also be informative of the fate and behaviour of ENMs in soils. This information should be taken into account.

It was also noted that the mobility of ENMs in unsaturated soils is expected to be very limited because of ENM attachment to air-water or air-solid surfaces. However, it was also mentioned that the soil transport normally takes a long time span (years). Data for this long time span are not available for ENMs. In a worst-case scenario it was hypothesized that probably immobile ENMs can be modified and / or mobilized after aging. This has to be confirmed in further studies. During the discussion of the mobility of ENMs in soil and sediment compartment, it was mentioned that NOM plays an important role and could affect the ENM stability and mobility in the system. Depending on the type of NOM, ENMs can be stabilised or destabilised after NOM adsorption. Whereas soil NOM often results in a stabilising effect for ENMs, surface water NOM often destabilises the ENM (aggregation) in the system.

➔ It was concluded that the type of NOM is an important parameter affecting the fate and behaviour of ENMs. The type of NOM should be specified in the reporting of the test.

It was also mentioned that with increasing naturalness of the test system, the mixture of different substances and charges in soil compartments increases. This in turn hampers the predictability of transport behaviour as well as the identification of specific effects of different parameters for the transport behaviour.

Additionally, information about the most expected pathways should be obtained before the tests are conducted, in order to get information about the entry pathway of the ENM to the compartment. This can be important for the subsequent test performance (ENM application).

Whether sediments needed a specific TG was also discussed. The conclusion reached was that no specific GL for the testing of sediments is needed, given that sediment specifications were already covered by the existing GL.

It was also noted that the application specification of the TG often excludes the testing of ENMs, although it could include it. This has to be changed or an amendment should be made for each specific TG.

Moreover the available measurement techniques and metrics for the different TG were discussed. It was mentioned that it is important to specify the particle size distribution, mass concentration, surface area or other endpoints in the suspension before it is applied to the system as well as in the test system and after the tests. No sensitive instruments able to detect other endpoints (such as mass of non-marked ENMs in environmental systems) are currently available. Nonetheless some promising tools are available (such as FFF or spICP-MS - both techniques having to be validated).

➔ It was concluded that general guidance for measurement techniques, describing their applicability as well as their limitations, should be developed for all TGs. This GD should also specify how the measurement results should be treated and how the data can be interpreted.

The applicability of different TGs was discussed during this session beginning with TG 106 (Adsorption - Desorption Using a Batch Equilibrium Method).

It was concluded that the OECD TG 106 is not applicable to ENM and a new TG should be developed.

Whether a new TG should be developed or whether a chapter for a pre-screening test should be added to TG 312 was a controversial issue. The majority of the group argued for the development of a new TG.

The new TG should be developed as pre-screening test for OECD TG 312.

It should define a benchmark value for the mobile fraction, and provide a ranking of important materials and conditions for the OECD TG 312.

The following points should be specified in the new TG:

- Shaking time
- Which filter is used
- Liquid to solid ratio
- Batch or flow conditions
- Electrolyte (type and concentration)
- Number concentration of the particles (collusion possibility)
- Measurable
- Application of the ENM

➔ It was concluded that the OECD TG 106 is not applicable to ENM and a new TG should be developed.

Furthermore the applicability of TG 312 (leaching in soil columns) was discussed.

During this discussion, the dissolution and dispersability behaviour of the ENM in the media were highlighted as important characteristics, affecting its fate and behaviour in the environment. The dissolution and dispersability behaviour should be tested in the beginning of the experiments, as a first step before the "main" experiments are conducted - Figure 5 (first step of the decision tree comparable to the water compartment - Figure 1).

During the discussion, there was some allusion to the fact that the determination of the size distribution (or of other nanospecific endpoints) of the applied and eluted ENMs should be measured.

The application of the ENM (dry powder or wet via suspension) was also discussed in this session. Advantages for both application forms were presented. For wet spiking, the advantage is the possibility of characterising the starting conditions (such as size, size distribution, agglomeration state, zeta potential, surface reactivity). With the application of a suspension it should be easier to guarantee a homogeneous distribution of the ENM in the soil column and the use of a suspension is likely to increase the reproducibility of the test conditions. Furthermore it was mentioned that the main entry pathway (at the moment) of ENM to soil and sediment compartments occurs via sewage treatment plant sludge (Gottschalk et al. 2009[20]). The application of an ENM suspension simulated this pathway. The disadvantage of the wet spiking is the effect of the suspension procedure, which can change the basic conditions of the ENM. This is not the case if the ENMs are applied with dry spiking. The disadvantage of dry spiking is the difficulty to guarantee a homogeneous distribution of the applied material or reproducible conditions.

Taking all these arguments into account, it was concluded that the application of dispersion is more preferable than dry spiking, and that the preparation steps should be described in detail for the data interpretation.

[20] Gottschalk, F.; Sonderer, T.; Scholz, R.W.; Nowack, B. (2009): Modeled Environmental Concentrations of Engineered Nanomaterials (TiO_2, ZnO, Ag, CNT, Fullerenes) for Different Regions. *Environmental Science & Technology* 43 (24), 9216-9222

For a better transferability of the results of the tests to natural conditions, the tests should be conducted in different soil types, to represent different natural conditions. A definition of 3 – 5 "average" soil types, with different pore sizes, NOM content, charge etc., should be tested.

Guidance on the various soil types can be provided by the report from a "soil meeting" held in 1995, which aimed to define a selection of soil and sediments for the testing of traditional chemicals. The information and decisions which were made during this meeting can also be very helpful, and should be used as a starting point on deciding on which soil or sediment should be used for ENM testing. During the discussion of this topic, it was mentioned that the testing in soil types representing extreme conditions (e.g. clean quartz sand and soil types with high clay and NOM contents) can be helpful for the identification of parameters which affect the transport behaviour of ENMs in natural soils. However, given the differences in the two environments, results derived from tests in extreme soils cannot be extrapolated to what happens under average natural conditions. No clear consensus was found on whether the tests should be conducted in either average or extreme soil types, or whether they should be carried out in both.

➔ It was concluded that the **OECD TG 312 is generally applicable** to the testing of ENMs if a preamble or an additional GD is developed with specification for the testing of ENM.

Furthermore, the applicability of the OECD TG 315 (Bioaccumulation in Sediment dwelling Benthic Oligochaetes) and OECD TG 317 (Bioaccumulation in Terrestrial Oligochaetes) was debated. During this session the mentioned endpoints in the TG, the application of the substances, the organism preparation and the data interpretation were discussed in more detail.

The applicability of the mentioned endpoints of the TG for the testing of ENM was discussed. It was concluded that the bioaccumulation factor (BAF) is also an appropriate endpoint for the testing of ENM.

The application of the ENM to the test system can affect the outcome of the tests. As it was already mentioned during the water breakout fate session, maintaining a homogeneous exposure over longer time periods is one of the biggest issues for environmental tests. Different ways of spiking (dry and wet) were therefore discussed during this session.

The advantage of wet spiking, as it was already discussed for TG 312 is the increased reproducibility of the test conditions and the possibility for the characterization of starting conditions of the suspension (e.g. size, surface reactivity or zeta potential). The disadvantage of wet spiking is the effect of the suspension procedure. It can change the basic conditions of the ENM or change the coating or functionalisation if these types of ENMs are used. This does not occur for dry spiking. The disadvantage of dry spiking is the difficulty to guarantee a homogeneous distribution of the applied material or reproducible conditions as well as the lack of the ENM characterization.

For the bioaccumulation test of benthic oligochaetes (TG 315), four different spiking procedures were discussed: wet spiking of the water phase, wet spiking of the sediment, dry spiking of the water phase, and dry spiking of the sediment.

For dry spiking of the water phase as well as the dry spiking of the sediment it was concordantly concluded that dry spiking of the test system should be avoided, due to the lack of characterization, and the difficulty to guarantee a homogeneous distribution of the applied material. The first step of the dry spiking of the sediment is to dry the sediment, spike it with the materials and wet it again. This procedure destroys the specific conditions in this compartment. Furthermore the dry spiking does not represent the most expected entry pathway.

The wet spiking of the water phase or the wet spiking of the sediment was discussed more intensively.

If the ENM suspension was applied to the water phase, the main entry pathway of the ENM to the systems is simulated. The disadvantage is the inhomogeneous distribution of the applied materials which can lead to hotspots with high concentrations. These are avoided by wet spiking of the sediment, which also makes it easier to generate a homogeneous distribution of the ENM. It was concluded that this procedure should be favoured for TG 315.

The application method should be added to the TG.

For the bioaccumulation test in terrestrial oligochaetes (TG 317), dry and wet spiking of the test system was discussed. Based on the afore-mentioned advantages and disadvantages of the spiking methods, it was determined that wet spiking of the substrate should be favoured for this TG. Nonetheless, dry spiking can also be used for some specific ENMs (e.g. coated, functionalised), if necessary. The spiking method selected as well as justification for its use must be described in detail in the test report.

Iit was also mentioned that the treatment of the test organisms (e.g. purged and unpurged worms) affected the results of the test. How the organisms were treated therefore has to be reported in detail. The deportation period as well as the availability of food can also affect the results (excretion of the incorporated ENM).

The importance and relevance of different endpoints to be measured were discussed. The endpoints differ depending on the question: if the effects of the ENM to worms are significant, uptake and partitioning to the tissue must be quantified. For the food chain and biomagnification, the uptake or partitioning as well as the attachment of the ENM to the organisms are not relevant given that the whole body burden will be taken up by the next trophic level.

➔ It was concluded that, depending on the question, the total body burden as well as the tissues (meaning the purged and unpurged worms) should be analysed for the quantification.

➔ The OECD TG 315 and 317 are in general applicable to the testing of ENM, provided that some specifications for the testing of ENM are considered.

For TG 315, it was concluded that wet spiking is mostly preferable. For TG 317 dry as well as wet spiking was concluded to be feasible.

Additionally a GD clarifying how the data of the experiments can be interpreted should be developed.

In summary it was concluded that:

➔ The OECD TG 106 is not applicable to ENMs. A new TG including a pre-screening test for the TG 312 should be developed. New, such as the retention determination of ENM in soils by screening techniques[21], were discussed.

➔ The OECD TG 312 is applicable to the testing of ENMs if some additions and changes are added to the TG.

- Decision tree / tiered approach (dissolution and dispersability) were conducted as a first step before the tests.
- The application of dispersion is more preferable than dry spiking.
- The test should be conducted in more than one soil: 3 to 5 soil types should be defined in the TG for the testing of ENMs.The important parameter affecting the fate and behaviour of ENMs in soils should at least be specified.

➔ The OECD TG 317 is applicable to the testing of ENMs if some additions and changes are added to the TG.

- The application of the ENM must be reported in detail. Wet and dry spiking are feasible.
- A GD that clarifies how the data of the experiments can be interpreted should be developed.

➔ The OECD TG 315 is applicable to the testing of ENM if some additions and changes are added to the TG.

- Wet spiking of the sediment was favoured in the discussion.
- A GD that clarifies how the data of the experiments can be interpreted should be developed.

➔ It was concluded that the type of NOM is an important parameter affecting the fate and behaviour of ENMs. The type of NOM should be specified in the reporting of the test.

Compartment Soil and Sediments

Chair: Jonathan Veinot (University of Alberta, CA)
Rapporteurs: Carmen Nickel (IUTA, GER) and Dana Kühnel (UFZ, GER)

The Chairs of the breakout session, Thomas Kuhlbusch and Willie Peijnenburg, summarised the results of the two breakout sessions (see Ecotoxicology – Compartment Soil and Sediments and Breakout group – Environmental Fate and Behaviour – Compartment Soil and Sediments)

Afterwards, the important points identified were discussed with all participants:

- Measurement techniques
- Decision tree
- Application of the ENM to the test system
- Test strategies – soil types
- ENM Aging
- Bioavailability

[21] Cornelis et al. 2010. A method for determination of retention of silver and cerium oxide manufactured nanoparticles in soils. E*nviron. Chem. 7, 298-308*

The different available measurement techniques were introduced and their applicability for the quantification and identification of ENMs in environmental media was discussed. It was mentioned that spICP-MS and FFF as well as SEM or TEM are currently promising techniques although. most of these techniques are expensive. Other cheaper and cost efficient methods should be developed in the near future. Thereupon a differentiation between cost efficient methods and cheap methods was demanded, given that if only an "expensive" method can be used (e.g. SEM), it is cost efficient. It was also noted that for the different TGs, the required techniques should be identified.

➔ The conclusion reached was that the detection methods between different research areas should be harmonized.

➔ Furthermore, a new GD should be developed. This GD would specify how and for which questions the different measurement techniques can be applied and how the results can be interpreted.

In the fate breakout session, a decision tree was established (Figure 5), starting with tests for the dissolution and dispersability behaviour of the ENM. These tests should be performed before the OECD TGs are conducted. The establishment of a decision tree for soils and sediments was also deemed pertinent in the water - fate breakout group. The first step was also considered relevant for soil and sediments. The results of these tests will affect the subsequent experimental procedure. Furthermore, some tests should not be conducted if the ENM cannot be dispersed. Additionally, no "specific" nano test should be conducted if the ENM can be dissolved. The same TGs which were applied for traditional chemicals may therefore be used.

Participants also noted that the current level of knowledge is too low for specific decisions, hence

➔ It was concluded that the decision tree as a first step should be developed as GD.

The application of the ENM to the test system was discussed in both breakout sessions and different results were determined.

For the ecotoxicology breakout session, the spiking procedure for the test outcome had no effect. For other wet spiking tests, no 100% effect was observed. Identification of the LC and EC values can therefore be problematic. No decision for each of the application methods for ecotoxicology tests was made.

During the fate breakout session, the application of the ENM to the tests system was also discussed. In comparison to the ecotoxicology session, wet spiking, which allows for lower concentrations to be used and for starting conditions to be characterised, should be favoured. Given that no information about the processes in the compartments can be obtained, starting conditions can be more harmonized. For some substances, the application of the dry ENM should also be feasible. In this case, the ENM should be premixed with the test media. This option of premixing is already mentioned in the TG (e.g. for pesticides with low solubility). It was also mentioned that wet spiking vs. dry spiking may increase bioavailability.

➔ It was concluded that the application method should always be described in detail for ecotoxicology as well as fate and behaviour tests.

➔ For harmonisation between ecotoxicology and fate studies, the same application method should be used.

➔ For the fate & behaviour tests, wet application should be favoured over dry spiking, although the latter should not be ignored. If no dispersion could be generated, the dry spiking should be used.

➔ Dry spiking should not be used for sediments: considered to be more realistic, wet sediment mixing should be favoured.

The strategies, performance and the original conditions for the tests were discussed in detail. The type of the soil to be used for the tests was particularly debated.

Given the heterogeneity of the natural conditions, more than one soil should be tested for the fate experiments. 3 or 5 standard soils, with a wide range of natural occurring circumstance and with variation in grain size, charge, NOM should be identified and specified. Information concerning different soil types is available from a 1995 meeting (which discussed exactly this topic in the course of the testing of traditional chemicals).

The selection of the used soil type is also important for ecotoxicology tests, given that the type of soil may affect the bioavailability of the ENM.

The question of whether "average natural soils" or standard soils (such as the OECD standard soil) should be used for the testing was discussed.

The OECD standard soil (sandy soil (70%), < 1% NOM, peat has to be added, Fe and Ca, pH 5.5-7) is a highly artificial substrate. It shows a high variation, due to the addition of peat. This was demonstrated during a round robin test, which compared the water holding capacity in different laboratories. Due to its composition, the OECD standard soil represents a worst-case scenario,.

Accordingly, other natural sandy soils should possibly be used as a starting point, given that sandy soils are applicable in nearly all OECD tests and standardization in these soils appears to be easier than in peat. The variability of sandy soils as well as their comparison between different regions and countries should be examined in order to get an idea of the comparability of the different regional sandy soils.

One point mentioned during the discussion is that soil type is maybe not the parameter to be changed. It may be easier to identify other parameters to be characterized for the testing of ENM – "nanorelevant" characteristics.

No definitive statement was made on whether the soil tested should be purely natural or whether standard soil should be included as well.

➔ The conclusion was reached that an amendment to the TG, specifying the reporting of the required soil characteristics, must be developed

For sediments, standardized sediment should be used for benchmarking (starting with a broad basis and narrowing down on applicable sediments).

Note: These arguments are also relevant for other chemicals and should maybe be discussed outside of the Nano-Session. No consensus was found on this issue, given that some mechanical processes may be different due to the fact that some specifications apply to ENMs but not to chemicals.

The transformation and the aging of ENMs under natural conditions were discussed as important processes which have to be considered in environmental tests. Currently, insufficient information on these processes is available (e.g. there is only limited information concerning the effect of some kinds of NOM, IS, etc.).

➔ Further tests should be conducted addressing this topic, before specific recommendations can be made.

It was noted that specific factors for aging are mentioned in TGs for metals.

The identification of the bioavailable fraction of the ENM was also mentioned as an important piece of information for environmental tests. Substances in the pore water of soil and sediments are expected to be important for bioavailability, given that this is the first fraction which is available to the soil and sediment organisms.

Similarly than for water compartments, kinetic studies are important for soil and sediment compartments, given that no steady state is expected for ENMs. The entry pathway of ENMs to the compartments should also be identified before the "main" tests are conducted, given that this is important information for bioavailability. In addition, sensitive measurement techniques are missing for the identification and quantification of the bioavailable fraction in the different environmental compartments (e.g. ion release for some ENMs, differentiation between ingested or adsorbed ENMs).

➔ It was concluded that more research is needed. The development of sufficient measurement techniques as well as environmental tests should be conducted in the future.

SESSION FOUR. CONCLUSIONS AND RECOMMENDATIONS[2]

Chairs: Phil Sayre (EPA, US) and Klaus Steinhäuser (UBA, GER)
Rapporteurs: Carmen Nickel (IUTA, GER) and Dana Kühnel (UFZ, GER)

Dr. Phil Sayre and Dr. Klaus Steinhäuser gave a final presentation which summaries the recommendations and decisions which were made during the breakout session. The list of recommendations can be found below.

Identifying Risk Assessment Needs

Yasir Sultan (SG6 RA of ENM, Environment Canada, CA)

Dr. Yasir Sultan presented an overview of risk assessment priorities as part of Steering Group 6 of the WPMN. The purpose of the presentation was to align research and regulatory needs. It provided an overview of Steering Group 6, some uncertainties in current risk assessments as they relate to ENM, and the prioritization exercise led by this steering group to better focus resources and strengthen risk assessments. Moving forward, this group will focus on projects relating to utilization of physical-chemical properties in risk assessments and how they can provide information on endpoints, including environmental fate and effects.

Yasir Sultan also presented an example of a new project, initiated by steering group 6 and led from Canada, on how to deal with nanoparticle dissolution/solubility in risk assessments. Participation was sought from the workshop attendees along with ideas on how to further refine the project. Follow-up discussions were focused on 'when a nanoparticle is not a nano-issue anymore, i.e., when the nanoparticle degraded sufficiently to become an ionic issue'.

Discussion

- Advancement of read across from ENM to ENM: Category concept.
- Reporting requirements for ENMs: can they help for Risk Assessment?

- Differentiating bulk or nanoscale materials? Not easy, companies do not report nano form.

Conclusions and Recommendations

Ecotoxicology

- Most OECD TGs for toxicity testing in soil/sediment and water are generally applicable
- No recommendations regarding amendments of OECD TGs were given, mostly due to limited knowledge on a multitude of factors.
- Specific recommendations can only be given when a better understanding of the complex interactions between ENM and water / soil & sediment is developed.

Aquatic Ecotoxicology

- The inclusion of decision trees into the GSPD was suggested, with 3 tiers covering the following issues:
  - Tier 1 Stock / stem suspension preparation:
    - It was suggested to use the same stock suspensions for both aquatic ecotoxicology and environmental fate testing (TiO_2/NIST approach, MARINA protocol as basis).
    - The aim is to anchor stock characteristics before dilution for comparability of results amongst tests and minimisation of test artefacts.
    - The preparation method should lessen unintended ENM damage; and should be repeatable and achievable with standard equipment.
    - The stock suspension should be as stable and as monodisperse as possible.
    - Stable suspension refers to retaining a certain size range for a certain time (ideally the duration of a test). No specific time points were defined during the discussion.
    - Protocols cover individual nanomaterials, where a solid dataset on the influence of dispersion methods on ENM characteristics already exists (TiO_2, CNTs, Silver).
    - It was suggested to undertake pilot runs to determine applied energy needs, stability with or without stabilizers (NOM, etc.).
  - Tier 2 Preparation of exposure solution:
    - From the existing knowledge, it is likely that the properties of ENM will change upon the addition of more complex exposure media (e.g. presence of proteins, sugars, salts).
    - A point discussed from various perspectives during the meeting was whether the acceptance of more than 20% loss during the test (as practiced to chemicals) is also applicable to ENM. However, no final recommendation on this point was achieved.
    - There may be a need for a test solution replacement / renewal (vs. flow-through or agitation).
    - Considering alternative dose metrics for varying exposure (nominal concentration as worst case; geometric mean etc.).
  - Tier 3 conducting the test:
    - Media and particle specific decisions.
    - Dosimetry - the following metrics may need further consideration: measurement of particle number, mass, & surface area in test waters during the test (already in GSPD), measure relevant ENM ion release; surface area metric.
    - Results from high exposure concentrations should be considered in the context of more realistic (lower) environmental exposures.
    - It was suggested to run pilot tests to determine material loss (with or without organisms present).
    - Consider testing with ENMs which reflect most likely transformations after their introduction into environment (species resulting from ligand binding).

- The category or grouping approach was suggested in order to identify ENMs for which the same protocol can be used for testing.
- Different starting points for grouping were identified: based on material properties and characteristics, MoA etc.
- Additionally, sections of the GSPD will be updated over time, when new knowledge has accumulated.

- Discussion was mostly focused on algal tests were specific points were identified:
  - The suggestion was made to test the assay in advance for lack of interference due to the particle presence.
    - Particles were reported to confound measurement of algal cell counts/biomass (surrogate) quantification by interfering with the cell counting method.
    - Further, particles may alter availability of photon energy (increase or decrease in growth by interference with photosynthesis, shading).
    - Photoreactivity should be considered (ex, ROS generation).
    - Material may affect the availability of solved nutrients in the test media (iron sequestration, etc.).

Soil and Sediment Ecotoxicology

- It is recommended continuing to move forward with both dry and wet spiking of soils and sediments in order to identify the most suitable procedure.
- The use of the same stock solutions as in aquatic toxicity tests is recommended; advantages to both approaches are enumerated.
- Guidance on detection techniques in soil and sediment is needed.
- The amount of ENM accumulating in organisms is likely to be key for regulatory policies.
- Understanding the state of ENMs in soils/sediments is critical to inform overall results, guidance (vs. new protocol). Regulatory needs should be considered.

Test Guidelines for Fate and Behaviour Testing

- All presented OECD TGs except TG 106 can applied to the testing of ENMs, provided that some amendments or changes are taken into account. Specific adaptations for ENM testing of ENMs should be added to the TGs as GDs.
- Dissolution, dispersability, agglomeration, degradation and transformation are identified as important pieces of information to be known before further fate tests in water compartments are conducted. Therefore, a decision tree / tiered approach should be established as prior tests before the further ecotoxicology or fate tests are conducted. The decision tree must be added to the specific TG as a GD.
- It was also discussed that TG105 is not appropriate for nanomaterials and a new TG should be created to address the dissolution behaviour of ENM.
- Decision tree:
  - First step – dissolution and dispersability → new TG necessary.
  - Second step – agglomeration state and dispersion stability → new TG necessary.
  - Third step – bio-degradation → a new TG must be developed.
  - Fourth step – abiotic degradation → definition of important parameter.
- Furthermore a GD for ENM pre-treatment scenarios (most probably transformation processes) must be developed for the harmonisation of aging and transformation processes.

→ *Guidance for dissolution, dispersion, and agglomeration of ENMs as a first step – priority (Development of new TG).*

- A decision tree / tiered approach was also developed before soil / sediment testing. Here, the dissolution and dispersability step (comparable to the first step of the water decision tree) should be added to the TG as a GD, as a harmonized procedure applying to several guidelines.
- A GD for nanorelevant aspects of OECD TG 312 should include:
  - Soil Selection: During the meeting, no clear statement was made with regards to which soil type should be tested – only average natural soils or standard soils were mentioned. This has to be specified in further studies. It was nonetheless concluded that the test should be conducted in more than one soil type.
  - Application of the ENM (wet spiking favoured).
  - Data interpretation must be developed.
- Instead of the existing OECD TG 106, a new adsorption test should be developed as a pre-test for TG 312. New approaches were discussed, such as the retention determination of ENM in soils by screening techniques[22]
- For OECD TG 305, a GD including ENM specific modifications should be developed:
  - The nanospecific endpoint could not be identified but some alternative endpoints were discussed - internalisation rate, attachment efficiency, bioavailable fraction. Further research is needed.
  - The group decided that the **bioconcentration factor (BCF) is not applicable** if the tests are conducted with ENM.
  - Dietary spiking should be used for ENM testing reflecting a worst case situation.
  - The test procedure must be described in detail.

→ *GD for OECD TG 305 (dietary uptake, BCF, BMF, etc.).*

- A GD including all nanorelevant aspects for the applicability of the OECD TG 317 for ENM testing must be developed, including the application of the ENM to the test system (wet and dry spiking feasible) and data interpretation.
- A GD focusing on all nanorelevant aspects for the applicability of the OECD TG 315 for ENM testing should be developed, including the application of the ENM to the test system (wet spiking of the sediment should be favoured) and data interpretation.

Overall Comments

- For a better comparability of results from ecotoxicity and fate tests, the same test conditions (such as the application of the ENM, physic.-chemic- parameter of the suspension and test media) should be used. At the very least, the same stem suspensions should be used.
- The importance of testing transformed and aged ENMs instead or as an addition to pristine materials was mentioned. The conclusion reached was that environmental tests should also be conducted with aged ENMs. The aged ENM should reflect the most likely transformation processes after its introduction into the environment compartments. Pre-treatment scenarios must therefore be identified and harmonised.
- During the environmental tests, a loss of the applied ENM is expected (adsorption to test vessels, organisms, settling). An adjustment should be made for the acceptable loss of ENM during the tests (20% acceptable?).
- The importance of NOM for the fate and transport of ENMs in the environment was mentioned. As such, the type of NOM used for the testing or which is already available in the system must be

[22] Cornelis et al. 2010. A method for determination of retention of silver and cerium oxide manufactured nanoparticles in soils. E*nviron. Chem. 7, 298-308*

specified given that different types of NOM may have different effects, e.g. stabilising or destabilising.

- Depending on the environmental behaviour of an ENM, zebrafish may not be the right target organism and other organisms should be tested. It was therefore discussed whether a new TG for the bioaccumulation of ENMs in mussels or daphnids should be developed based on the TG305. The applicability of the OECD TG 305 to other organisms must be tested in further studies.

Needs for Further Research in the Near Future

In the final session of the meeting, the future needs in regulatory environmental testing were discussed and recommendations were elaborated within the expert community. The main issues included (i) method development for detection, identification and quantification of nanomaterials in both environmental and test media, (ii) the characterisation and understanding of particle behaviour in these media, (iii) data gaps in toxicity testing and (iv) category approaches to group nanomaterials.

Ecotoxicology

- Robust physical-chemical characterisation for biological assays is needed, requiring progress in ENM detection methods, such as:
  - Total Metal determination.
  - EM, EDX, EELS, ICS-MS, X-ray powder diffraction, XBS: develop understanding on potential artefacts and tiered approach in general.
  - Separation Techniques (filtration, centrifugation; interpretation of results).
- Improvement of understanding on homo- and heteroaggregation processes.
- Improvement of understanding of interferences of ENM with assays.

Aquatic Toxicity

- Data gaps were identified for chronic toxicity (growth, reproduction, energetics, new materials), environmentally realistic concentrations, toxicokinetics (ADME).
- Furthermore, the knowledge on effects of pristine vs. aged ENMs should be improved.
- The interaction of ENMs with sense organs of animals should be studied in more detail.

Soil & sediment

- Robust measurement methods for determination of ENM concentrations in soils / sediments are needed (mass measurements feasible for some parent ENMs). Soil and sediment pore water concentrations are also important although technical problems remain to be solved. In addition, only a few on-line measurement methods are available.
- Several key points should be clarified:
  - Assessment of the bioavailable fraction, e.g. by developing proxies obtained by extraction methods.
  - Preferable spiking techniques: spiking of food, or spiking of sludge to add to soils.
  - Consideration of different types of ENMs and implications for spiking and detection.
  - Consideration of different soil and sediment types/aging.
  - Are we looking at the most appropriate endpoints?
  - Are the Guidelines applicable to other organisms?
- More understanding of aggregation processes, soluble species, transformed species and their effects on bioavailability is needed.

Measurement Techniques

- In all sessions, the need for more sensitive measurement techniques for the identification and quantification of ENMs under environmentally realistic concentrations or in different compartments and/or tissues or cells was identified. Furthermore, the need for measurement techniques for specific metrics such as surface area, particle number in environmental media, bioavailable fraction was specified.

Fate and behaviour

- During the discussion, it was decided that information about dissolution, dispersability and transformation processes in environmental media are very important for the assessment of the fate and behaviour of ENMs. More research in these areas is needed.
- It was also concluded that besides the testing of pristine ENMs, tests should also be conducted with aged or transformed ENMs which represent the more environmental relevant fraction. More information concerning degradation processes (biodegradation and abiotic degradation processes) and transformation processes is therefore needed.
- Some important parameters used for traditional chemicals (such as K_{ow}, BCF or BMF) cannot be directly used for the characterisation of ENMs. Other "nanorelevant" endpoints should be identified, such as uptake rate, internalisation rate, and attachment efficiency. The endpoint to be used has to be determined in further studies.
- No clear statement could be made with regards to which type of soil should be tested or if only natural average soils (rather than artificial standard soils) should be used. It was therefore decided that soil parameters affecting the fate and behaviour of ENMs should be identified. With these identified parameters, a grouping of relevant soil types can be implemented.
- The exposure pathway of an ENM to the natural compartments was already mentioned as an important process which may affect the environmental fate and behaviour as well as the further test design. Application via sewage sludge was for example identified as one important entry pathway. Further research addressing these entry pathways and tests under varying conditions are needed.
- Other than the need for environmental testing, further modelling studies focusing on aged ENMs and on the main entry pathways are needed. .
- The lack of long-term studies was also mentioned and further research needs were identified.
- In view of reducing the number of animal studies, the increased use of in-vitro tests as a pre-screening test for some TG was agreed. Further studies are needed in the areas of (i) the identification of the right models, and (ii) examination of the applicability to ENMS of in vitro tests used for traditional chemicals.

ANNEX I – AGENDA

ORGANISATION FOR ECONOMIC CO-OPERATION AND DEVELOPMENT

ORGANISATION DE COOPÉRATION ET DE DÉVELOPPEMENT ÉCONOMIQUES

OECD SPONSORSHIP PROGRAMME FOR THE TESTING OF MANUFACTURED NANOMATERIALS

OECD Expert Meeting on Ecotoxicology and Environmental Fate

To be held at German Federal Press Office in Berlin, 29th – 31th January 2013
(Starting at 9h00 on the first day)

Background:
The OECD Working Party on Manufactured Nanomaterials has initiated a series of expert meetings to improve the applicability of the OECD Test Guidelines to nanomaterials. A preliminary report was published in 2009[1] and based on the available results from the OECD Sponsorship Programme on the Testing of Manufactured Nanomaterials (hereafter OECD Testing Programme) the methodology will be reviewed. Based on results from the Testing Programme, the focus of this expert meeting is on eco-toxicity and environmental fate and its objective is to provide recommendations to the OECD WPMN on the need for:

1. Updating current (OECD) environmental fate and ecotoxicology Test Guidelines or development of new ones. If updates are necessary, discuss what kind of changes or developments would be needed and which further steps need to be taken;
2. Identify specific areas to further develop/ update on the "*Guidance on Sample Preparation and Dosimetry*" or if specific additional sections in the area of environmental fate and ecotoxicology testing and assessment need to be developed; and;
3. Specific guidance or a guidance document (GD) for environmental fate and ecotoxicology testing of nanomaterials or adaptation of existing guidance documents.

Tuesday 29 January 2013

[1] ENV/JM/MONO(2009)21. Preliminary Review of OECD Test Guidelines for their Applicability to Manufactured Nanomaterials.

8h30	**Registration at German Federal Press Office, Berlin**	
9h00	**Welcome Remarks by President of Federal Environment Agency**	
9h15	**Welcome by OECD and general introduction into the agenda**	
Session 1: General Aspects of Environmental toxicity and fate of Manufactured Nanomaterials (chaired by Susanne Walter-Rhode UBA (GER))		
9:30	Sample preparation and dosimetry – a key issue: Reviewing the Guidance Document on Sample Preparation and dosimetry – Phil Sayre US-EPA	
9:50	The OECD test Guideline Program (eco-tox & fate) – Appropriateness of TGs / Needs for Testing MN - Jukka Ahtiainen TUKES (FIN)	
10h10	**Coffee break**	
10:40	Keynote lecture: Characterization of NM in the environmental compartments - knowledge, challenges, perspectives - James Ranville Colorado School of Mines (US)	
11:05	Keynote lecture: exposure routes of NM in the environment – Martin Scheringer ETH (CH)	
11:30	Discussion	
12:00	**Lunch**	
13:00	Keynote lecture : Ecotoxicology of NM – knowledge, challenges, perspectives - Richard Handy University of Plymouth (UK)	
13:25	Keynote lecture : Environmental fate of NM – knowledge, challenges, perspectives - Graeme Batley CSIRO (AUS)	
13:50	Discussion	
14:20	**Coffee break**	
14:50	Introduction into the break out groups	
Session 2: Environmental toxicity and fate of Manufactured Nanomaterials - compartment water -		
15:00-17:00	**Break Out Group on Ecotoxicology** Dispersion protocols for aquatic ecotoxicology Stimulus presentation (10 min.): Nanna Hartmann JRC(EC) Chair: Steve Diamond US-EPA Rapporteur: Dana Kühnel UFZ (GER)	**Break Out Group on Fate & Behavior** Degradation/Transformation of NM Stimulus presentation (10 min.): Jed Costanza EPA (US) Chair: Frank v.d. Kammer University Vienne (AT) Rapporteur: Carmen Nickel IUTA (GER)
17:00	Service information - End of day 1	
19:00	**Dinner to be offered and hosted by Germany**	

Wednesday 30 January 2013		
9:00	**Welcome and Agenda of day 2**	
9:10-11:00	**Break Out Group on Ecotoxicology (cont)** OECD201/211 Stimulus presentation (10 min.): Teresa Fernandes Heriot-Watt University (UK) Chair: Steve Diamond US-EPA Rapporteur: Dana Kühnel UFZ (GER)	**Break Out Group on Fate & Behavior (cont)** Bioaccumulation OECD 305 Stimulus presentation (10 min.): Eric Bleeker RIVM (NL) Chair: Frank v.d. Kammer University Vienna (A) Rapporteur: Carmen Nickel IUTA (GER)
11:00	**Coffee break**	

11:30	Reports from the Breakout groups	
12:00	**General discussion: – compartment water – Proposal of revisions on OECD guidelines and guidance document on sample preparation; Conclusion on Session 2** Chair: Gregg Goss University of Alberta (CA)	
13:30	**Lunch**	
Session 3: Environmental toxicity and fate of Manufactured Nanomaterials - compartment soil & sediment -		
14:30 - 16:30	**Break Out Group on Ecotoxicology** OECD 222/225 Stimulus presentation (10 min.): Kerstin Hund-Rinke Fh-IME (GER) Chair: Willie Peijnenburg RIVM (NL) Rapporteur: Dana Kühnel UFZ (GER)	**Break Out Group on Fate & Behavior** Fate and behavior in soil Stimulus presentation (10 min.): Jason Kirby CSIRO (AUS) Chair: Thomas Kuhlbusch IUTA (GER) Rapporteur: Carmen Nickel IUTA (GER)
16:30	**Coffee break**	
17:00	Service information - End of day 2	

Thursday 31 January 3013		
9:00	**Welcome and Agenda of day 3**	
9:10 – 11:00	**Break Out Group on Ecotoxicology (cont)** Detection in soil and sediment Stimulus presentation (10 min.): Geert Cornelis University Gothenburg (SWE) Chair: Willie Peijnenburg RIVM (NL) Rapporteur: Dana Kühnel UFZ (GER)	**Break Out Group on Fate & Behavior (cont.)** Bioaccumulation OECD 315/317 Stimulus presentation (10 min.): Hanna Maes RWTH Aachen (GER) Chair: Thomas Kuhlbusch IUTA (GER) Rapporteur: Carmen Nickel IUTA (GER)
11:00	**Coffee break**	
11:30	Reports from the Breakout groups	
12:00 – 13:30	**General discussion: – compartment soil & sediment – Proposal of revisions on OECD guidelines and guidance document on sample preparation; Conclusion on Session 3** Chair: Jonathan Veinot University of Alberta (CA)	
13:30	**Lunch**	
Session 4: Final plenary (co-chaired by Phil Sayre, US EPA and Klaus-G. Steinhäuser UBA (GER))		
14:30	Identifying RA needs: Important Issues on Risk Assessment of Manufactured Nanomaterials Yasir Sultan Environment Canada (CA)	
15:00	Recommendation on future needs in regulatory environmental testing for Phase 2 of the OECD Sponsorship Programme - Conclusions	
16:00	Any other business - Closure	
END OF THE MEETING		

ANNEX II – LIST OF PARTICIPANTS

Participants list for WPMN Expert Meeting on Environmental Fate & Ecotoxicology

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ANNEX III – WELCOME ADDRESS BY JOCHEN FLASBARTH

Umwelt Bundes Amt
Für Mensch und Umwelt

ORGANISATION FOR ECONOMIC CO-OPERATION AND DEVELOPMENT

ORGANISATION DE COOPÉRATION ET DE DÉVELOPPEMENT ÉCONOMIQUES

Welcome Address

of
Jochen Flasbarth,
President of the Federal Environment Agency,
at the OECD Expert Meeting on Ecotoxicology and Environmental Fate of Nanomaterials
29 – 31 January 2013
in Berlin

Ladies and Gentlemen,

It is a pleasure for me to welcome you all here at the Federal Press Office in Berlin, the venue of the OECD Expert Meeting on Ecotoxicology and Environmental Fate of Nanomaterials. Thank you all for taking part in this conference which is aimed at giving important input to the progress of the Sponsorship Programme of the OECD Working Party on Manufactured Nanomaterials. The present conference is one of the major scientific events for our agency in 2013.

[UBA]

Some words on our agency: The Federal Environment Agency (UBA) is Germany's central federal authority on environmental matters giving scientific advice to the federal government.

The daily work includes environmental research, the enforcement of environmental laws and providing information to the general public about environmental protection issues.

Identifying tomorrow's problems today is one of the main goals of UBA. UBA plays its role as an early warning system which detects potential future adverse impacts on mankind and the environment in a timely fashion, assesses associated risks, and offers proposals for practicable solutions. To that end, experts at the Agency carry out research in in-house laboratories in addition to commissioning research

projects to scientific institutions in Germany and abroad. UBA is committed to contribute to international processes related to environmental policy. Various members of our staff are actively involved in working groups in the EU, OECD and on a global basis. As part of these missions members of UBA's staff take part in the OECD Working Party on Manufactured Nanomaterials (WPMN) and its steering groups and actively contribute to its Sponsorship Programme.

Nanotechnology is rapidly growing. On the global market level the annual production of nanomaterials is estimated at around 11 million tons, which results in a market value of roughly 20 billion €. Products optimised by nanotechnology are forecast to grow from a volume of 200 billion € in 2009 to 2000 billion € by 2015 in the EU. The direct employment in nanotechnology in the EU is estimated at 300 000 to 400 000 jobs, with an increasing tendency[23].
Meanwhile, nanotechnology has reached nearly every economic sector. Areas of applications include for example electronics, catalysis, energy storage and medicine. Moreover, many manufactured nanomaterials are already used in a number of commercial products such as household articles, textiles and cosmetics. Next to classical nanomaterials like amorphous silica or Carbon Black, which have been produced in large quantities for decades, additional manufactured nanomaterials gained in importance on the global market. These include semiconducting materials, quantum dots, carbon nanotubes and nanosilver, which are used in diverse applications. In addition, many novel nanomaterials exhibit modified surfaces which enable special technical functionality and offer possibilities for innovative applications.

OECD is an important player. One of the diverse working areas of the OECD is chemical safety. The aim of its work on Environment, Health and Safety of chemicals is to protect human health and the environment, while avoiding duplication of efforts; and ensuring efficiency and reduction of trade barriers. For 40 years now the Chemical Safety Programme has accomplished important achievements related to harmonized testing and assessment of chemicals. With the Chemical Safety Programme the OECD has attained a global key role in the safe use of chemicals. For mutual acceptance of data OECD provides test guidelines for chemicals and other tools for risk assessment. As a member state to the OECD Germany contributes to the development and constant update of these tools. UBA in particular provides input to the different groups of the Chemical Safety Programme with the active contribution of experts in the field of environmental risk assessment.
One of the most interesting and most dynamic groups within the OECD is the Working Party on Manufactured Nanomaterials (WPMN) which is dealing with environmental and health related issues of nanotechnology.

The OECD's decision to establish WPMN was meaningful. The WPMN was established 7 years ago with the aim to promote international co-operation and harmonisation of nanopolicy between EU, USA and other industrialized countries including industry, NGOs and accession countries like South Africa and Thailand. The "heart" of the WPMN is the so-called "Sponsorship Programme for the Testing of Manufactured Nanomaterials". In a common effort of member countries, but also non-member countries and other stakeholders, 13 manufactured nanomaterials of commercial relevance have been tested for 59 endpoints in an explorative programme. These endpoints include effects measurements and observations regarding identification, physical-chemical properties, environmental fate and toxicology, mammalian toxicology and material safety. Furthermore existing test methods are reviewed for their adequacy to assess the safety of manufactured nanomaterials. In this context potential adaptations and amendments are discussed. Research on the environmental and health risks of nanomaterials increased extraordinarily in the last years. However, published scientific results are often extremely contradictory. The comparability of data is hampered by missing harmonized protocols regarding test preparation and test performance.

[23] Data from the Second Regulatory Review on Nanomaterials

The Sponsorship Programme, and in particular the Guidance Notes for Sample Preparation and Dosimetry prepared within the WPMN, are of special value to tackle this problem. At present 35 publications of the WPMN are providing information on the activities and the outcome reached so far by the diverse activities at OECD related to human health and environmental safety of manufactured nanomaterials. An important goal is the reduction of the gap between the scientific and technical progress of a new technology and approaches to regulate risk-related issues of this technology. Based on the lessons learned from green biotechnology such international co-operations involving all kinds of stakeholders like the WPMN are necessary to avoid mistakes happened in the past.

Irrespective of the potential environmental risks, Nanotechnology offers great chances for a sustainable development. Technical processes may be improved; products with novel features become available. High energy and resource efficiency are possible. For example, while conventional light bulbs convert only 5 per cent of electrical energy input into light, the conversion rate with nano-based Organic Light Emitting Diodes (OLED) is up to 50 per cent or even more. Another example: Constructional elements for vehicles and aircraft made of nano-optimized plastics can cut fuel consumption by weight reduction. The OECD Working Party on Manufactured Nanomaterials also deals with the environmentally sustainable use of manufactured nanomaterials. In this context the main emphasis of WPMN is concentrated on nano-enabled applications with the potential to address major environmental challenges such as climate change, pollution of water, soil, and air, and natural resource depletion. At the same time, it is investigated which potential negative impacts such new technical approaches may pose on environment and health. Furthermore it is the aim to enhance the knowledge of life cycle aspects of manufactured nanomaterials and nano-enabled applications. In the long term, it is the objective of WPMN to broaden its initiative focusing on maximising environmental benefits and minimizing risks of nano-enabled applications. This objective will be pursued together with OECD partners in compliance with along the line of the “Green Growth Strategy”. “Green Growth” describes a path of economic development which uses natural resources in a sustainable manner.

Nanotechnology is a challenge for politicians, risk assessors and scientists. Nanomaterials are chemicals. As such they are covered by well-proven chemicals legislation and their methods to assess and manage risks. However, there is the need to modify and adapt current chemicals legislation since nanomaterials exhibit special features compared to materials of the same chemical identity but larger size. These special features comprise for example higher surface reactivity, quantum effects or chemical and catalytic properties. The special characteristics of materials in nano-size may alter their behavior in biological tissues and environmental media which in turn might has influence on their persistence, bioavailability and toxic potential.
German Federal agencies including UBA developed a proposal how to implement nanomaterials in the European Chemical Legislation REACH. Nanomaterials are not regarded as substances on their own. However, a separate risk assessment is necessary. Therefore, a regulation of nanomaterials under REACH has to meet specific requirements. This includes a differentiated consideration of the bulkform and nanoform as well as different nanoforms of the same substance. Nanoforms should undergo a separate risk assessment. An adequate handling of surface treated nanomaterials has to be defined. Tonnage bands and information requirements need to be adjusted. Special characteristics concerning toxico-kinetics and environmental fate, together with the existing uncertainties and special features with regard to mode of action, necessitate requirements which go beyond those implemented in REACH to date. In order to cope with these nano-specific characteristics and uncertainties it is necessary to apply the precautionary principle and to amend REACH.

Nanotechnology is not only a challenge for natural scientists and technical experts. The success of a new technology also depends on its perception in the broad public. Fair and early communication about the

opportunities and risks of nanotechnology is crucial for the way society deals with this technology. In 2006 the German Federal Government set up a NanoCommission which served as the central platform for a national stakeholder dialogue. The so-called “NanoDialogue” is continued by carrying out workshops with stakeholders on various issues in order to support the responsible use of nanotechnology via a strengthened communication between stakeholders from civil society, industry, science and politics.

In 2011, at the final Conference of the second nanocommission the German Federal Environment Minister called for a nanoproduct register on a European basis. A nanoproduct register is necessary for transparency and traceability and thus social acceptance. In the opinion of UBA such a register could enable public authorities to set priorities in enforcement and monitoring and, in the case of adverse effects, ensure traceability. For actors in the supply chain a product register creates transparency. Last year, UBA has published key points of a proposal for a European register for products containing nanomaterials.
Within this proposal substances and mixtures that comprise or contain nanomaterials are subject to notification, furthermore, articles that intentionally or unintentionally release nanomaterials. Elaboration of such a product register should also have the objective of avoiding duplicate obligations and maintaining a reasonable cost-benefit ratio. At the same time, there are substance-related regulatory bases and also product-related regulatory bases in European legislation. Both legal bases should be utilized for a product register, since both contain appropriate points of departure. Our idea of a register comprises a "public" part that is generally accessible and a confidential part to which only authorities have access.
It should be considered, that a product register substitutes neither further developments in the elaboration of the REACH Regulation nor necessary, nano-specific provisions in substance-, product- and environment-related legislation, which is currently being discussed; it is merely a component thereof.

This conference is an important step to cope with the issues to develop nanotechnology safely. It is a significant contribution to the objectives of the WPMN: To diminish uncertainties regarding risks related to nanotechnology as well as to provide tools to calculate the safety of nanomaterials. This is of particular importance for both regulators as well as for producers.
For you as participants, this meeting offers the possibility to share your knowledge, discuss the challenges and exchange your experience of testing nanomaterials for their ecotoxicity and environmental behavior with experts from all over the world.
I wish you every success for the conference. Special thanks go to the OECD secretariat for their support in organising, as well as to the delegates from the U.S., The Netherlands, Finland, and the Joint Research Centre of EU for their scientific input in preparation of the meeting.