GUIDANCE

Guidance on information requirements and chemical safety assessment

Appendix R7-1 Recommendations for nanomaterials applicable to Chapter R7b Endpoint specific guidance

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DOCUMENT HISTORY

Version	Changes	Date
Version 1	First edition	April 2012
Version 2	• New advisory note (section 1.1) on testing for ecotoxicity and fate, to provide overall advice for conducting ecotoxicity and environmental fate testing for nanomaterials • Update of section 1.2.1 on aquatic pelagic toxicity, to clarify that high insolubility cannot be used as a waiver and to include further recommendations on the text to be performed for this endpoint • Update of section 1.2.2. on Toxicity for sediments organisms to provide advice on spiking methods and include applicability of available OECD guidelines • Update of section 1.2.3 on degradation/ biodegradation to clarify that waivers for hydrolysis and degradation simulation testing are not applicable as sole evidence, provide advice on photocatalytic degradation and general advice on performing the tests Please note that the numbering of the sections has changed, the section numbers above refer to the updated numbering of the guidance	Xxxx 2017

PREFACE

The three appendices concerning information requirements (appendices to R7a, R7b and R7c) have been developed in order to provide advice to registrants for use when preparing registration dossiers for nanomaterials[1].

In the absence of any specific recommendation, either because the endpoint is not relevant for nanomaterials (e.g. flash point or surface tension), or the guidance already provided is considered to be equally applicable to nanomaterials or because more research is needed before developing advice, no additional guidance for the endpoint has been included in this appendix.

This appendix intends to provide advice specific to nanomaterials and does not preclude the applicability of the general principles given in Chapter R.7a (i.e. the parent guidance). The parent Guidance applies when no specific information for nanomaterials has been given in this appendix.

[1] See Recommendation on the definition of nanomaterial adopted by the European Commission

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1 RECOMMENDATIONS FOR ECOTOXICOLOGICAL ENDPOINTS for NANOMATERIALS:

1.1 General Advice on how to perform nanomaterials ecotoxicity and fate testing

This section is applicable for ecotoxicological and fate testing and provides general advice regardless of the test compartment or endpoint. Endpoint specific guidance is provided under corresponding endpoint specific sections.

This section summarises the advice (sampling, preparation for testing, testing itself and reporting the results) provided in the documents listed below and in the publications by Petersen et al. [1] and Rasmussen et al. [2].

- OECD No.36 : Guidance on Sample Preparation and Dosimetry for the Safety Testing of Manufactured Nanomaterials [3]
- OECD No.40: Ecotoxicology and Environmental Fate of Manufactured Nanomaterials: Test Guidelines. Expert Meeting Report [4]
- OECD No.40 (1): Addendum to Ecotoxicology and Environmental Fate of Manufactured Nanomaterials: Test Guidelines. Expert Meeting Report [5]
- OECD No.62: Considerations for Using Dissolution as a Function of Surface Chemistry to Evaluate Environmental Behaviour of Nanomaterials in Risk Assessments. A Preliminary Case Study Using Silver Nanoparticles [6]
- OECD No.64: Series on the Safety of Manufactured Nanomaterials- No.64- Approaches on Nano Grouping/ Equivalence/ Read-Across Concepts Based on Physical-Chemical Properties (Gera-Pc) for Regulatory Regimes [7]

The considerations detailed in the main points below should be reported (when relevant for the endpoint) in the technical dossier together with the test results.

Prerequisites

The following issues should be considered when planning the test:

- Define representative controls for the test (e.g. for algae tests and metal oxide nanomaterials, use of metal salt solutions as benchmarks)

- Dissolution rate and potential ion release (see for example adaptation of OECD TG 29 [8], as per OECD No. 62 [6] with dissolution criteria high, moderate, low or negligible). Please take into account that testing "the smallest representative particle" and using the data to predict the behaviour and subsequent effects of another nanomaterial might not be enough to take into account the specificities of the nanomaterials.

- Agglomeration behaviour, degradation and transformation (using the OECD TG on agglomeration behaviour and GD under development, OECD No. 40)

- Justification of the selected exposure regimes (e.g. test duration, static or flow through, exposure route, etc.).

The exposure media and conditions of the test should be consistent and repeatable (as explained in the section on sample preparation *of Appendix R7-1 Recommendations for nanomaterials applicable to Chapter R7a - Endpoint specific guidance* [9].

- Define the frequency of the measurements of concentration of the test material to detect any losses during the test.

- When considering assessing the results of a test based on nominal concentrations, a justification will be required for the test to be considered as acceptable.

- When performing a test, besides the use of mass metric, other nano-specific measurements have to be considered and performed giving the measurement techniques used for the determination of the measured values (see for instance [10], [11]), if available.

Preparations before testing

The following considerations need to be taken into account when preparing the test:

- Stock solution:
  - Direct preparation vs. use of dispersion protocols should be justified and reported.
  - The level of purity needed for the stock solution (purified material vs commercial preparations) should be considered.
  - Dispersion stability in stock solution ([1], [11])

- Test media and possible interactions with the test material:
  - Selection of the dispersion protocol appropriate for the test media and the test material. The dispersion method should not change the characteristics of the test material (See for instance [11]).
  - Consider the agglomeration behaviour of the nanomaterial in the test media and its potential effects on exposure (See OECD No. 36 [3] and OECD No. 40 [4] and addendum [5]). Apply the test guidelines and guidance (once available) for the Agglomeration Behaviour and Dissolution Rate of Nanomaterials in the Aquatic Media (See also [12], [13]).
  - Consider particle stability in the test medium. This means performing the test as required by the guideline but without the test organism(s) to clarify the interactions between the test material and the test media. Potential interactions (See for instance Petersen et al. 2015, [10]) of the test material with the test media may be:
    - complexation with the nutrients;
    - interaction with dissolved organic matter (DOM);
    - precipitation or sedimentation of the test material.

The OECD Guidance on Aquatic (and Sediment) Toxicology Testing of Nanomaterials will provide further advice on these issues once available.

1.2 Specific advice for endpoints

The parent R7b Endpoint specific guidance section R7.8 includes sections for aquatic pelagic toxicity, toxicity to sediment organisms and activated sludge. The approaches and methods described for these endpoints in the parent guidance are in principle applicable also for nanomaterials.

Nevertheless, the recommendations set out in *Appendix R.7-1 to Chapter R.7a* [9], section 2.1.1 need to be taken into consideration, especially with regard to methods of suspension, method of nanomaterials introduction, storage and stability of test material, chemical composition of the test media, characterisation of stock dispersions, characterization of samples (prepared from stock dispersions prior to administration/testing and if possible during and/or at the end of the test) and different measurement protocols.

If it is proven that the nanomaterials are readily dissolvable or fast/high dissolving forms, they would be assessed as traditional chemicals (as explained in OECD No.62 [6], [14]). In that case, for ecotoxicological and fate endpoints, the advice provided in the parent guidance will apply. The only nanospecific tests would be the physico-chemical ones including data on dissolution rate.

1.2.1 Aquatic pelagic toxicity

When performing aquatic toxicity testing for nanomaterials, the advice provided in this section should be followed instead of that in Section 7.8.1 of the parent guidance. The following steps are recommended:

- Sample preparation (section 2.1.1 in Appendix R7-1 Chapter R7a)
- General advice on how to perform nanomaterial ecotoxicity and fate testing (see section 2.1)
- Applicability of the test guidelines
- Waiving based on high insolubility
- Preference for long-term testing
- Endpoint-specific recommendations

In addition to the general advice given above, the following specific advice for aqueous experiments must be followed, implemented and reported:

- No use of synthetic dispersants to prepare the stock solution or solution for aquatic toxicity testing, unless they are compounds of the registered substance (product formulation), then the bioassay should be conducted with the as-produced material [1]
- Provide the media characteristics (e.g. pH, Ionic Strength, NOM, humic acid).
- Testing to be carried out with accompanying analytics to monitor the exposure concentration (for example: sedimentation and settling rate [10], [15], [1]).

The OECD TGs and their recognised equivalent for algae, aquatic invertebrates and fish are considered generally applicable for nanomaterials ([2]). Therefore, contrary to the parent guidance R7b Section 7.8.2., the adaptation to waive aquatic toxicity tests based on substance being highly insoluble in water cannot be used without proper and scientifically robust justification (as highlighted in Appendix R.7-1 to Chapter R.7a, section 2.2.1.). Furthermore, the dissolution rate should be considered instead of solubility for nanomaterials. Based on the results of the dissolution rate test, the following options are possible:

- The nanomaterial does dissolve quickly and has a high dissolution (as per OECD No.62 [6]). In these cases, there are no further specific nanomaterial considerations to be taken into account, and the parent guidance can be followed.
- The nanomaterial does not dissolve fast or conforms to moderate or lower dissolution criteria. The registrant is advised to perform directly only long-term toxicity testing instead of testing for short-term toxicity[2] (on *Daphnia* or Fish).

[2] Short term testing does not generally provide reliable results because the short test duration leads to low bioavailability and therefore limited exposure.

- Long-term toxicity testing (including Algae) must be considered for nanomaterials, as already specified in the ITS in the parent guidance Section R.7.8.2.

1.2.1.1 Test guidelines specificities for aquatic toxicity

When aquatic toxicity tests are performed, some additional parameters and testing specifications are recommended for nanomaterials that will need to be reported, as specified (per endpoint) below:

- For Fish testing (OECD TG 210 [16]):
  - enhanced toxicity due to photoactive or catalytic properties of the nanomaterials
  - mechanical effects, e.g. blocking of respiratory organs, decrease of ventilation rate, gill pathologies and blocking of digestive tract
  - activity levels of relevant antioxidant enzymes such as catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase (GPX), and glutathione-S-transferase (GST).
  - fish mucus secretion
  - fish brain pathology,
  - animal behaviour, and
  - histopathology of fish

- For Daphnids testing (OECD TG 202 [17] and OECD TG 211 [18]):
  - nutrient depletion effect (for long-term evaluation)
  - sex-ratio for Daphnia (number of males and females as per OECD TG 211)
  - any behavioural observations
  - mechanical effects of the nanomaterial (e.g. adherence to the organism, blocking of respiratory organs or digestive tract, [15], [19]), and
  - enhanced toxicity due to photoactive or catalytic properties of the nanomaterials

- For Algae testing (OECD TG 201):
  - quantification of effects on colour or shading, using protocols such as the ones developed by [19] and [20].
  - mechanical effects of the nanomaterial (e.g. adherence to the organism)
  - the type of agitation used in the test plan (stirring/shaking) for preventing/slowing down sedimentation
  - fluorescence measurement of chlorophyll extracts (considered as the most reliable way of measuring algal biomass for testing effects of NMs on algae growth (OECD No. 40 [4], OECD Nr. 40(1) [5], [11]) or pigments quantification [20].
  - autofluorescence of the tested NM to avoid misinterpretation of chlorophyll extracts based on adsorption/interaction with nanomaterials, and
  - enhanced toxicity due to photoactivity or catalytic properties,

For activated sludge inhibition:

- longer test duration, if possible
- In the parent Guidance R7b Section R.7.8.17, Information requirements for toxicity to STP microorganisms, it is stated that STP toxicity testing is not needed if there are mitigating factors such as a very low solubility that would limit the exposure. This adaptation is generally not acceptable for nanomaterials and as explained above, for aquatic toxicity testing.

1.2.2 Toxicity for sediment organisms

Situations in which the equilibrium partitioning method (EPM) can be applied in estimating toxicity to sediment organisms are presented in parent guidance Sections R. 7.8.9.1 and R.7.8.10.1, covering use of non–testing data on toxicity to sediment organisms. Regarding nanomaterials, estimates based on results from "equilibrium partitioning methods" are limited to the distribution of a substance in molecular form. In the case of nanomaterials, the partitioning method may underestimate exposure in soil and sediment environments and overestimate the exposure in water.

There are no estimation methods available for particle distribution, so this has to be dealt with on a case-by-case basis. With regard to nanomaterials, the recommendations set out in the OECD Guidance Manual for testing [21] and updated Guidance Notes on Sample Preparation and Dosimetry for nanomaterials [3] need to be taken into consideration, including the further advice from Appendix R.7-1 to Chapter R.7a, section 2.1.1 and the ones aforementioned in this chapter section 2.1; especially in regard to methods of suspension, method of nanomaterials introduction, storage and stability of test material, chemical composition of the test media, characterisation of stock dispersions, as well as characterization of samples (prepared from stock dispersions) prior to administration/testing and possibly during and at least at the end of the test. Many of the considerations for aquatic toxicity testing for nanomaterials, as detailed above in section 2.2.1, are also relevant to sediment tests, with the notable exception that there is no need to remove insoluble test material according to standard assay protocol [1].

Nanomaterial suspensions are not stable in natural waters (e.g. agglomeration, sedimentation). Therefore hazard assessment in the sediment compartment may in many cases provide more reliable information than the pelagic aquatic hazard assessment ([1] and [2]).

Some added complications are that nanomaterial interactions with sediments can significantly alter their properties. Additionally, the methods for quantifying nanomaterial characteristics in sediments (e.g. concentration) are very limited. Current sediment toxicity standard methods acknowledge significant uncertainty regarding test substance homogeneity, exposure, bioavailability and synergisms. Nevertheless, the consistency of sediment toxicity bioassays can still be generally improved by implementing standards for preparation and experimental set-up as indicated above (section 2.1 and 2.2). For instance, the use of a standardized (e.g., OECD) freshwater sediment in nanomaterial spiking studies would reduce variability in bioassay results relative to the use of field-collected sediments because sediment-specific factors (e.g., organic carbon concentration) that can influence toxicity assay results are controlled. Thus, for nanomaterial sediment toxicity testing it is recommended to apply sediment spiking.

Two types of spiking methods for nanomaterials have been applied in sediment toxicity testing:

(1) direct addition of dispersed nanomaterials to the sediment followed by homogenization and

(2) indirect addition of nanomaterials to the overlying water, followed by subsequent settling of the nanomaterials to the surficial sediment.

The test material will be better dispersed if the spiking is done with an already dispersed solution rather than with dry material (type method 2)[3]. This is related to general difficulties regarding homogenizing chemicals into sediments. If a nanomaterial is added to sediment in powder form (undispersed), it is likely that substantial clumping of particles within the sediment occurs, resulting in greater heterogeneity and therefore greater variability among bioassay test replicates.

Equilibration time between performing the test and sediment spiking depends on the type of nanomaterial and knowledge on its behaviour parameters such as agglomeration, aggregation and sedimentation. For example, if one uses an equilibration time of 48 hours, the test will be considered a worst-case scenario with the highest bioavailability, as no pseudo-equilibrium stage will be reached in such a short time [1].

Technical challenges in nanomaterials characterization methods may limit the detection of nanoparticles and the determination of particle characteristics in sediment. Certain measurements may still be performed, such as using ICP-MS to determine the total elemental concentration of metal and metal-oxide nanomaterials. It is practical to take samples for such measurements from the whole sediment, sediment porewater, and overlying water at test initiation and termination, as recommended in current OECD sediment testing guidance. However, nanomaterial-specific modifications of porewater separation methods may be needed in order to yield accurate results [1]. Such methods could be applied to measuring nanomaterials in the different phases of the test and would allow a better distinction of the source/type of toxicity, depending on where the nanomaterial distributes.

1.2.2.1 Test guidelines for sediment toxicity

The following OECD TGs are considered generally applicable for nanomaterials: OECD TG 225 (Sediment Lumbriculus Assay [22]) and OECD TG 218 [23] and 219 [24] (Sediment-Water Chironomid Toxicity Using Spiked Sediment and Sediment-Water Chironomid Toxicity Using Spiked Water respectively).

Whatever the test method and the method of spiking chosen, the equilibration time before performing the testing, the sampling method and the analysis technique and frequency have to be reported.

1.2.3 Degradation / Biodegradation / Transformation

Degradation is a process that can result in the loss or transformation of a substance in the environment. Environmental compartments to be considered in risk assessment are water, sediment, and soil. In addition, degradation and transformation of a substance in sewage treatment plant plays a key role in fate and exposure assessment.

The degradation process can be abiotic or biotic. Biodegradation is a biological process in which organic substances are decomposed by microorganisms. A pre-requisite for biodegradation is that the test material is based on organic carbon chemistry (for bulk chemicals as well as for nanomaterials). This leads to the conclusion that biodegradation is not relevant for inorganic substances, including inorganic nanomaterials such as Ag, TiO_2, CeO_2, nZVI, ZnO, CuO and QDs [25]. In addition, many of the carbon-based nanomaterials such as

[3] According OECD Guidance 40, it is recommended to use the same aqueous solution for the sediment and the aquatic toxicity testing.

carbon nanotubes (CNTs) and Carbon black are considered to be of inorganic nature. There is however evidence on biotic degradation of carbon-based nanomaterials, single-walled carbon nanotubes (SWCNT), multiwalled carbon nanotubes (MWCNTs) and fullerenes (C60) by oxidative enzymes ([26]; [27]; [28]). On the other hand, for MWCNTs there are results indicating no degradation by oxidative enzymes alone but up to 7 % mineralisation by a mixed bacterial culture at 39 C resulting several degradation products [29]. Even if the extent of biodegradation of carbon-based nanomaterials in natural environmental conditions is considered limited, the above-described studies indicate that potential for biological degradation in relevant environmental conditions remains to be established ([25]). Thus performing a degradation study on carbon-based nanomaterials always needs to be considered. If a carbon-based nanomaterial is considered persistent without testing, this needs to be justified.

Considering the above, for inorganic nanomaterials and nanomaterials of inorganic nature (e.g. carbon-based ones), the assessment of ready biodegradability is not relevant. However, the potential for release of degradation/transformation products is recommended to be taken into account in the degradation assessment.

If the nanomaterial is coated or functionalized with organic and potentially biodegradable materials, then biodegradation tests are relevant and would need to be performed for the coatings alone or for the coated nanomaterials. If the test is performed with the coated nanomaterial, the amount of carbon needs to be high enough to allow reliable detection of the e.g. released carbon dioxide or consumed oxygen. In addition, potential effects of surface modifications on degradation/transformation may need to be considered, as it has been shown that surface modifications may have an effect on the degradation/transformation properties of nanomaterials, e.g. MWCNTs in [30]).

In the parent guidance R7b section 7.3.3, abiotic processes such as hydrolysis, oxidation and photolysis are considered the main transformation routes for chemicals in water, soil and sediment. Hydrolysis might be relevant to consider also for some nanomaterials and/or coatings. The oxidation-reduction process does play a key role in the behaviour of some nanomaterials such as Ag, CuO and ZnO. Measurement of redox potential is important for nanomaterials that can participate in electron transfer and uptake. This phenomenon is important also in relation to interaction with environmental media ([31]; [32]). Photochemical transformation is relevant for some nanomaterials as it may lead to changes in the nanomaterial's surface properties, or degradation of the coating or degradation of the nanoparticle itself ([25], OECD No. 63 [33] and OECD No. 65 [34]). These changes may lead to altered behaviour and hazard and are therefore important to be considered in degradation/transformation assessment. It is recommended to consider also alternative means of clarifying the environmental fate of the nanomaterial. The following key transformation processes influencing environmental fate and behaviour have been considered relevant for nanomaterials (in [25]; [35], [32] and [36]):

- Oxidation-reduction
- Photochemical degradation
- Adsorption - desorption
- Sedimentation as a removal mechanism from the water phase
- Biotransformation
- Speciation – complexation
- Loss of coating

The processes listed above take into account processes on the level of an individual particle (e.g. photochemical transformation), interactions between particles (e.g. sedimentation), and interactions of particles with solid surfaces and with other substances (e.g. adsorption).

Water solubility and the octanol-water partitioning tests may not be appropriate for nanomaterials, as explained in the Appendix R.7-1, Chapter R.7a, sections 2.1.1 and 2.2.2.

Therefore, the above-mentioned transformation processes are recommended to be considered in the testing strategy for nanomaterials degradation. This approach is also supported by Rasmussen et al. [2] proposing a fate decision tree logic and testing strategy taking into account the dissolution rate and agglomeration behaviour when testing the nanomaterials.

1.2.3.1 Test guidelines for degradation / biodegradation

Abiotic degradation

The chemical structure of the nanomaterial and whether it contains functional groups which could be subject to hydrolysis dictate whether a hydrolysis test is necessary or appropriate. If the nanoparticle is coated or functionalised, then abiotic degradation, e.g. hydrolysis of the substance, must be considered.

OECD TG 316 (Phototransformation of Chemicals in Water – Direct Photolysis), though not specifically validated for nanomaterials, may be applied to assessing the photocatalytic degradation or photolysis of nanomaterials ([25], OECD 63 [33] and OECD 65 [34]).

Biodegradation

Concerning information on degradation/biodegradation (Section R.7.9.3 of parent guidance R7b section R7.9), it should be noted that the OECD biodegradability test methods have been developed and validated principally for the assessment of organic compounds. Many nanomaterials are inorganic and even most carbon-based NM are of inorganic nature, and therefore the biodegradation test methods currently recommended in the parent guidance are inadequate for predicting their long-term fate in the environment.

The OECD TGs for ready biodegradability and simulation tests in water, soil and sediment listed in the parent guidance are in principle applicable for testing the degradation of an organic nanomaterial, coated/functionalised nanomaterial, organic coating or functionalisation agent. If the degradation of a coating or functionalisation agent is tested on its own, the potential differences in the degradation/transformation potential compared to when bound to the particle should be taken into account. The guidance provided in OECD No. 36 [3], and OECD No. 40 [4] and in this Appendix section 2.1.1 Chapter R7a of R7-1 on sample preparation, dispersion and dissolution should be followed.

Determination of sorption is also critical for assessing amounts of nanomaterials released to surface waters, and to soils and sediments ([37], [38], [39]; [40]). Some biodegradation test guidelines could be applied for nanomaterials to provide information on distribution of the nanomaterials, acknowledging that, nanomaterials do not sorb to sludge according to the equilibrium kinetics that apply to traditional chemicals [2].

The OECD TG 303A "Aerobic Sewage Treatment Simulation Test" although not designed for nanomaterials has been found to be useful , in particular for assessing the distribution of nanoscale TiO_2 particles in sewage treatment plants [41] with the following proposals for modifications:

- The dosing of nanoscale suspensions should be made separately from that of the organic synthetic wastewater in order to avoid any agglomeration of the particles.
- The use of synthetic drinking water for preparation of the test suspension instead of tap water to allow better comparability of test results.
- The test should be performed under nitrifying conditions to also assess the impact of nanomaterials on the nitrifying microorganisms, besides the effects on the organic carbon degrading microorganisms in the activated sludge.
- The determination of the filterable solids in the effluents of the laboratory sewage treatment plant (LSTP), nature and partitioning of the nanoscale particles in the effluent (filtration/centrifugation) is recommended.
- The calculation of an overall mass balance

In parallel to these alternative protocols and guidelines, a new test guideline is under development in OECD that could be used to estimate the particle attachment and removal efficiency from nanomaterials in the wastewater treatment.

Alternative methods

Alternative protocols can provide information on the abiotic degradation/transformation of nanomaterials when very low or negligible degradation is observed in degradation measurements.

- Oxidation-reduction
- Photochemical degradation
- Dissolution (see section 2.2.1 in appendix R7-1 to chapter R7a [9])
- Adsorption - desorption (currently no standard method available, see section 2.2.4 in appendix R7-1 to chapter R7a [9])
- Agglomeration (see section 2.2.1 and 2.2.2 in appendix R7-1 to chapter R7a [9])
- Aggregation (see section 2.2.1 and 2.2.2 in appendix R7-1 to chapter R7a [9])
- Sedimentation
- Biotransformation
- Speciation – complexation

This type of information is recommended to be used as part of the WoE on degradation assessment of nanomaterials to strengthen the conclusion on (bio)degradability/transformation and fate ([3], [4], [25]).

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