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Appendix R7-1 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 sections.	May 2017

PREFACE

Three appendices concerning information requirements (appendices to the IR&CSA Guidance Chapters R7a, R7b and R7c) have been developed in order to provide advice to registrants for use when preparing REACH registration dossiers that cover "nanoforms"[1].

The advice provided in this document focuses on specific recommendations for testing materials that are nanomaterials[2]. Part of the advice provided is not strictly nano-specific (e.g. may for instance also be applicable to other particulate materials). However, when such advice has been included, it is because it is considered that the issue covered is especially relevant for nanomaterials and should be part of the nano-specific guidance.

In the absence of availability of any suitable specific recommendation (either because the endpoint is not relevant for nanomaterials, because the guidance already provided is considered to be equally applicable to nanomaterials as to non-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.7b (i.e. the parent guidance). Moreover, when no advice has been given in this appendix for a specific endpoint the advice provided in the parent Guidance should be followed.

Please note that this document (and its parent guidance) provides specific guidance on meeting the information requirements set out in Annexes VI to XI to the REACH Regulation.

General information for meeting the information requirements such as collection and evaluation of available information, and adaptation of information requirements is available in Chapter R.2 to R.5 of the Guidance on IR&CSA).

Moreover, when considering the use of data already available *Appendix R.6-1: for nanomaterials applicable to the Guidance on QSARs and Grouping* of Chemicals [1] may be useful as it provides an approach on how to justify the use of hazard data between nanoforms (and the non-nanoform) of the same substance.

[1] Please see How to prepare registration dossiers that cover nanoforms: best practices [57]

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

This section summarises the advice (on sampling, preparation for testing, testing itself and reporting the results) provided in the documents listed below and in the publications by Petersen *et al*. [2] and Rasmussen *et al*. [3].

- OECD No.36 : Guidance on Sample Preparation and Dosimetry for the Safety Testing of Manufactured Nanomaterials [4];
- OECD No.40: Ecotoxicology and Environmental Fate of Manufactured Nanomaterials: Test Guidelines. Expert Meeting Report [5];
- OECD No.40 (1): Addendum to Ecotoxicology and Environmental Fate of Manufactured Nanomaterials: Test Guidelines. Expert Meeting Report [6];
- 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 [7];
- OECD No.64: Approaches on Nano Grouping/ Equivalence/ Read-Across Concepts Based on Physical-Chemical Properties (Gera-Pc) for Regulatory Regimes [8].

The guidance detailed below should be taken into account when results on nanoform(s) are reported (when relevant for the endpoint) in the technical dossier.

Prerequisites

It is advised to consider the following issues when nanomaterials are tested:

- Define representative controls for the test (e.g. for metal oxide nanomaterials, metal salt solutions as benchmarks)
- Dissolution rate and potential ion release (see section 2.2.2.1 in Appendix R.7-1 to Chapter R.7a of the IR&CSA Guidance for dissolution criteria: high, moderate, low or negligible).
- Agglomeration behaviour, degradation and transformation (using the OECD TG on agglomeration behaviour in aquatic media and the corresponding guidance document with a decision tree under development, OECD No. 40 [5])
- 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 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 decrease in concentration or transformation during the test.

- Quantify the concentration changes due to e.g. aggregation and sedimentation or transformation with relevant metrics to provide reliable exposure concentrations during the testing

- When performing a test, besides the use of mass metric, other nano-specific measurands (e.g. specific surface area, volume) have to be considered. The measurement techniques and metrics used should be provided, when these measurements have been performed (see for instance [10], [11]).

Preparations before testing

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

- Stock dispersion[3]:
    - Dispersion preparation used for the stock dispersion should be reported
    - Direct application of the stock dispersion vs. preparation of further dilution steps should be reported.
    - The level of purity[4] needed for the test material stock dispersion should be considered.
    - Dispersion stability in stock dispersion ([2], [11])
- Test media and possible interactions with the test material:
    - Selection of the dispersion protocol appropriate for the test media and the test material (as mentioned above). The dispersion method should not change the characteristics of the test material (See for instance [11]).
    - The agglomeration behaviour and dissolution of the nanomaterial in the specific test media used and its potential effects on exposure (See OECD No. 36 [4] and OECD No. 40 [5] and addendum [6]), where relevant. 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 [10]) of the test material with the test media may be:
        - complexation with the nutrients;
        - interaction with dissolved or natural organic matter (DOM/NOM);
        - Surface affinity;
        - 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.

[3] The dry spiking method is discussed in section 2.1.1.

[4] In this context "purity" may refer to chemical purity and also to the absence of biological contamination

1.1.1 Non-testing data

Although the use of non-testing approaches such as (Q)SAR approaches in addressing data gaps for nanomaterials is still limited, non-testing methods are recommended if and when they provide relevant and reliable information and are applicable as they significantly reduce the amount of testing required. However, the use of non-testing approaches for nanomaterials in deriving an assessment of hazard for the environment must be thoroughly and scientifically justified. Further non testing approaches are explained in Appendix R.6-1: Recommendations for nanomaterials applicable to the Guidance on QSARs and Grouping which provides an approach on how to justify the use of hazard data between nanoforms (and the non-nanoform) of the same substance. When considering read-across and/or grouping between nanoforms of different substances the advice provided in the ECHA IR&CSA Guidance Chapter R.6 on QSARs and Grouping of the Chemicals [10] together with the advice provided in its nanospecific appendix [1] could be considered.

1.2 Specific advice for endpoints

The parent R7b Endpoint specific guidance section R7.8 includes sections on 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 also applicable 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 dispersion preparation, method of nanomaterials introduction, storage and stability of test material, chemical composition of the relevant 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 under investigation are quickly and highly dissolved, they would be assessed in the same way as traditional chemicals (See section 2.2.1 in *Appendix R7-1 Chapter R7a*). 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 in the specific test media.

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. It is recommended that the following points are taken into account:

- 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 1.1)
- Applicability of the test guidelines
- Specific considerations for waiving based on high insolubility, as per REACH column 2 adaptations
- Preference for long-term testing
- Endpoint-specific recommendations

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

- Use of synthetic dispersants is not recommended to prepare the stock dispersion or

solution for aquatic toxicity testing, unless they are constituents of the registered substance (product formulation), in which case the bioassay should be conducted with the as-produced material [2]

- Provide the media characteristics (e.g. pH, ionic strength, natural organic matter (NOM) content, humic acid content).
- Testing to be carried out with accompanying analytics to monitor the exposure concentration (for example: sedimentation rate [2], [10], [14]).

The OECD TGs and their recognised equivalents for algae, aquatic invertebrates and fish are considered generally applicable for nanomaterials [3]. However, contrary to the parent guidance R7b Section 7.8.2., this adaptation is generally not acceptable for nanomaterials because the adaptation to waive aquatic toxicity tests based on a 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.). As explained above, low solubility does not automatically result in limited exposure of nanomaterials in the aquatic environment. Furthermore, in most cases, 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 is dissolved and has a high dissolution rate in relevant media (in OECD No.62 [7]). However, "fast dissolution" should be assessed with respect to the test duration, e.g. a material can be considered as dissolving fast for a long-term fish test but not for the activated sludge inhibition study. In case high solubility and fast dissolution can be demonstrated, there are no further considerations specific to nanomaterials to be taken into account, and the parent guidance can be followed.
- The nanomaterial does not dissolve fast e.g. conforms to moderate or lower dissolution rate criteria. Thus, the registrant is advised to preferably perform long-term toxicity testing instead of testing for short-term toxicity[5] depending on the type of testing and experimental set-up applied (in particular for *Daphnia* and Fish long term testing is advised)[6]. For these testing considerations the integrated testing strategy (ITS) proposed in the parent guidance (section R.7.8.2) can be followed.
- If acute toxicity testing is chosen, the conditions and test settings must be assessed in order to prove that the exposure concentration is adequate and duration is long enough to capture potential toxic effects. If further testing is needed the ITS from the parent guidance should be followed (section R 7.8.2).
- Long-term toxicity testing (including Algae) must otherwise be considered for nanomaterials, as already specified if they have poor water solubility (as outlined in the parent guidance Section R.7.8.2) and if their dissolution rate is negligible, low or moderate (See Appendix R.7-1 to Chapter R.7a, section 2.2.1) [9].

[5] If the dissolution rate is slow, short term testing will not provide reliable results due to limited exposure. For nanomaterials that do not dissolve 'quickly' a chronic test is more appropriate to capture effects after dissolution than an acute test. Kinetics of uptake and biodistribution are the key factors in this respect, not only for the dissolved material, but also for the nanoparticles themselves.

[6] In cases where nanoparticles dissolve over time in media, acute toxicity tests may be conducted using not only a freshly prepared suspension in test medium, but also an aged suspension where nanoparticles are added to the media 1-3 days prior to testing, depending on the shelf life of the media [10]. This aging step may increase or decrease toxicity, which regardless provides important weight of evidence on toxicity. Furthermore, it allows the processes of aggregation and dissolution of nanoparticles in aqueous suspensions to stabilize prior to exposure. However, testing solely with aged particles does not fulfil the information requirement for short-term aquatic toxicity.

- In case the nanomaterial behavioural properties (e.g. dissolution rate negligible, aggregation or agglomeration) lead to reduced aquatic and relevant sediment exposure, then a testing strategy favouring sediment toxicity tests can be considered.
- In any cases where long term toxicity tests are chosen as the aquatic toxicity tests to be performed, a testing proposal must be submitted by the registrant for both invertebrate and vertebrate testing as per the REACH information requirements of Article 40 in Annex IX sections 9.1.5 and 9.1.6.

1.2.1.1 Test guidelines specifics for aquatic toxicity

When aquatic toxicity tests are performed for nanomaterials, some additional parameters and testing specifications could be considered (and further reported, if applied), as specified (per endpoint) below. Please note that not all the parameters listed below are part of the respective guidelines. When that is the case suitable references are provided.

- For Fish testing (OECD TG 210 [15]):
  - mechanical effects, e.g. blocking of respiratory organs, decrease of ventilation rate, gill pathologies and blocking of digestive tract, [16], [17],
  - activity levels of relevant antioxidant enzymes such as catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase (GPX), and glutathione-S-transferase (GST), [18], [19], [20]
  - fish mucus secretion [16],
  - fish brain pathology [17],
  - animal behaviour [15],
  - histopathology of fish [17],
  - the potential effects of photoactivity or catalytic properties of the nanomaterial on toxicity [19], [20], when relevant (for instance a depigmentation or other stress indicators)
- For Daphnids testing (OECD TG 202 [21] and OECD TG 211 [22]):
  - the role of nutrient depletion effect (for long-term evaluation) should be considered in relation to the test setup to avoid potential artefacts in the interpretation of the results
  - sex-ratio for Daphnia (number of males and females as per OECD TG 211 [22])
  - any behavioural observations [21], [22], [23]
  - mechanical effects of the nanomaterial (e.g. adherence to the organism, blocking of oxygen diffusion or digestive tract, [14], [24]), and
  - the potential effects of photoactivity or catalytic properties of the nanomaterial on toxicity [25], [26], [27]when relevant
- For Algae testing (OECD TG 201 [28]):
  - quantification of effects on colour or shading, using protocols such as the ones developed by [24] and [29].

- mechanical effects of the nanomaterial (e.g. adherence to the organism)
- the type of agitation used in the test setup (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 nanomaterials on algae growth (OECD No. 40 [5], OECD No. 40(1) [6], [30]) or pigments quantification [29].
- autofluorescence of the tested nanomaterial to avoid misinterpretation of chlorophyll extracts based on adsorption/interaction with nanomaterials [30], and for instance testing under different light regimes, additional endpoints to improve reliability of the results
    - Example; in addition to the algal growth rate inhibition or carbon-assimilation another endpoint for more subtle effects to the individual algal cells, such as membrane damage and oxidative stress[7].
- when relevant, the potential effects of photoactivity or catalytic properties of the nanomaterial on toxicity,

For activated sludge inhibition:

- 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 high insolubility that would limit exposure. This adaptation is generally, not acceptable for activated sludge toxicity testing of nanomaterials or, as explained above, for aquatic toxicity testing of nanomaterials in general.

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" (i.e. those based on thermodynamic equilibrium) are limited to the distribution of a substance in molecular form (excluding ionic forms as explained in the parent guidance). In the case of nanoparticles, partitioning methods are not recommended, as they may underestimate exposure in soil and sediment environments and overestimate exposure in water.

There are no estimation methods available for particle distribution in sediment, 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 [31] and updated Guidance Notes on Sample Preparation and Dosimetry for nanomaterials [4] 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 above mentioned in this chapter section 1.1 and 1.2. Especially important to be addressed are recommendations with regard to methods of suspension, method of nanomaterials introduction, storage and stability of test material, chemical composition of the test media,

[7] A possibility for nanomaterials with fast acting toxic mechanisms or substantial dissolution in media is to perform a short-term 2h 14C-assimilation test, potentially combined with an ageing step. Carbon assimilation is probably less influenced by shading than growth rate. Also, less interference with scintillation counting is expected, compared to the spectrophotometric determination of algal pigments often used in growth rate inhibition tests. Ultimately, the use of single endpoint testing is sensitive to artefacts and misinterpretations, especially where the testing prerequisite of solubility and stability is violated, and there is little knowledge on the toxic mode of action. (Sørensen 2016)

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 1.2.1.1, are also relevant to sediment tests [2].

Nanomaterial suspensions are often not stable in natural waters (e.g. due to agglomeration and sedimentation) and will have a long residence time [32]. Consequently, there is often relevant exposure to the sediment compartment. Hazard assessment in the sediment compartment can in many cases provide more relevant information than the pelagic aquatic hazard assessment ([2], [3]). In case the nanomaterial behavioural properties and uses lead to reduced aquatic and relevant sediment exposure, as described above in this document and under R7a section 2.2.1.2., then an alternative testing strategy including sediment toxicity tests can be considered.

Some added complications can arise because nanomaterial interactions with sediments can significantly alter their properties. This is also the case for metals and metal oxides for which these aspects have been discussed in *Appendix R7.13-2 to the Guidance on IR&CSA* [33]. Additionally, the number of methods for quantifying nanomaterial characteristics in sediments (e.g. concentration) is very limited. Current standard sediment toxicity methods acknowledge the 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 of the sample and experimental set-up as indicated above (section 1.1 and 1.2). 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.

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

(1) direct addition to the sediment of dry nanomaterials (dry spiking) or dispersed nanomaterials (wet spiking) 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 surface sediment.

The test material will be better dispersed in sediment if the spiking is done with an already dispersed solution rather than with dry material[8]. 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 will occur, resulting in greater heterogeneity and therefore greater variability among bioassay test replicates. When the test substance is mixed with the sediment (direct sediment spiking) it is recommended to use dispersed nanomaterial preparation instead of dry stock test material.

Indirect spiking of overlying water also has challenges. Indirect spiking is followed by settling of the nanomaterials to the sediment and will result in non-homogeneous distribution of the nanoparticles in the sediment (gradient from surface to deeper layers) and therefore increases the heterogeneity of the subsamples. This should be acknowledged when indirect spiking is applied and variability of the exposure in each sub-sample should be minimised. The optimal spiking method depends on both the test material and the test method. It will depend on the

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

physicochemical properties of the nanomaterial, the target concentration, the medium, and the bioassay method selected, and preliminary data gathered prior to the test.

Further to the spiking method, 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 could be considered a worst-case scenario with the highest bioavailability, as no pseudo-equilibrium stage will be reached in such a short time [2], unless it is proven otherwise.

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. As an example, the use of ICP-MS may be combined with separation techniques (e.g. field-flow-fractionation) enabling single particle measurements and more detailed information on the metal/metal-oxide nanoparticles. It is practical to take samples for such measurements from the whole sediment, sediment pore water, and overlying water at least at test initiation and termination, as recommended in current OECD sediment testing guidance. However, nanomaterial-specific modifications of pore water separation methods may be needed in order to yield accurate results [2]. Such methods could be applied to measuring nanomaterials in the different compartments 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 reviewed and considered generally applicable for nanomaterials: OECD TG 225 (Sediment Lumbriculus Assay [34]) and OECD TG 218 [35] and 219 [36] (Sediment-Water Chironomid Toxicity Using Spiked Sediment and Sediment-Water Chironomid Toxicity Using Spiked Water respectively). In addition OECD TG 233 Sediment-Water Chironomid Life-Cycle Toxicity Test Using Spiked Water or Spiked Sediment could also be applied for nanomaterials.

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.

Furthermore, the reporting of information on the preparation sampling and experimental setup need to be provided as explained in *Appendix R.7-1 to Chapter R.7a* [9], section 2.1.1. In addition the parameters specified in this appendix from sections 1.1 and 1.2 on aquatic pelagic testing need also to be followed (such as pH, ionic strength, natural organic matter (NOM) and humic acid content). All this information would also need to be reported together with the methods of analysis and test results as explained above and in section 1.1.

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 plants plays a key role in fate and exposure assessment. If the degradation/transformation rate is fast this should be taken into account in hazard, exposure and risk assessment.

1.2.3.1 Biodegradation

The degradation process can be biotic or abiotic. Biodegradation is a biological process in which organic substances are decomposed by microorganisms. A baseline for biodegradation in the context of the available biodegradation test guidelines 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 the concept of biodegradability as applied to organic substances has limited or no meaning for inorganic substances, including inorganic nanomaterials e.g. Ag, TiO_2, CeO_2, nano zerovalent iron (nZVI), ZnO, CuO and quantum dots (QDs) [37]. In addition, many of the carbon-based nanomaterials such as carbon nanotubes (CNTs) and carbon black are considered to show inorganic characteristics. There is however evidence of biotic degradation of carbon-based nanomaterials, single-walled carbon nanotubes (SWCNT), multi-walled carbon nanotubes (MWCNTs) and fullerenes (C60) by oxidative enzymes ([38], [39], [40]). 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 in several degradation products [41]. Even if the extent of biodegradation of carbon-based nanomaterials in natural environmental conditions is considered limited, the above-described studies indicate that a potential for biological degradation in relevant environmental conditions remains to be established [37]. Thus for carbon-based nanomaterials it is recommended that performing a degradation study always be considered. If a carbon-based nanomaterial is considered non-degradable without testing, this needs to be justified.

Ready biodegradation testing is most likely not relevant for inorganic nanomaterials which do not contain carbon, at least in terms of ultimate biodegradation parameters (O_2 consumption, CO_2 production, and DOC removal). Regarding carbon-based nanomaterials of inorganic nature, even though their degradation potential may be limited, it is at least theoretically possible that ultimate biodegradation based on O_2 consumption or CO_2 production could be detected in ready biodegradation tests. In addition, there can be issues with the applicability of the test methods to nanomaterials, e.g. due to stringent test conditions. Therefore, for carbon-based nanomaterials of inorganic nature, ready biodegradability testing may be less relevant compared to organic substances. However, despite these limitations, even when the pass level for ready biodegradability is not met, a ready biodegradation test or other screening level biodegradation test might give valuable information on the extent of degradation. Furthermore the potential for release of degradation/transformation products is recommended to be taken into account in any degradation assessment of nanomaterials, including those of inorganic nature.

1.2.3.2 Abiotic degradation

In the parent guidance R7b section 7.9.3.1, abiotic processes such as hydrolysis, oxidation and photolysis are considered important 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 plays 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 interactions with environmental media ([42], [43]). 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 ([37], OECD No. 63 [44] and OECD No. 65 [45]). These changes may lead to altered behaviour and hazard and are therefore important to be considered in degradation/transformation assessment. It is recommended to also consider alternative means, some of which are described below, to clarify the environmental fate of the nanomaterial.

1.2.3.3 Transformation

Transformation of the nanomaterial may be chemical, biologically mediated or an interaction with macromolecules in the test media or in the environment. Where nanomaterials have a high surface to volume ratio, transformation is highly relevant to their fate. Methods useful to study the transformation of nanomaterials in relevant environments are still scarce; standard protocols are not available and many methods are still under development. Therefore, in many cases, it may not be possible to give clear recommendations on the applicable test methods. However, in the absence of standardised and/or quantitative methods, a qualitative assessment may provide valuable information on the fate of nanomaterials. Transformation processes considered relevant for nanomaterials are described below (not exclusive).

Reduction and oxidation are the main chemical transformation processes. Nanomaterials may undergo oxidation and reduction in all environmental compartments. Light-catalysed redox reactions may also be important transformation processes affecting e.g. oxidation state and generation of reactive oxygen species (ROS). Dissolution and sulfidation may also be considered as chemical transformation processes relevant for nanomaterials. In biologically mediated transformation, chemical transformations are mediated by living organisms in living tissue (intra end extracellular) and environmental media via redox-labile enzymes, cytochromes, and intracellular ROS production (hydroxyl radicals or H_2O_2). For example it has been demonstrated that biological oxidation can result in carboxylation of CNTs or formation of an insoluble metal oxide shell. Interactions with macromolecules e.g. proteins, polysaccharides and NOM, may alter the behaviour of nanomaterials as they may be adsorbed to the surface of the nanoparticle forming a "corona" around the nanoparticle. This corona may then change for instance the size, mobility and surface characteristics of the nanoparticles leading to different behaviour and biological responses compared to particles without the corona. For example the dissolution rate, entry into cells, accumulation and ROS production of the nanomaterial might be affected [46].

The following key transformation processes influencing environmental fate and behaviour have been considered relevant for nanomaterials (in [37], [43], [46] and [47]):

- Oxidation-reduction
- Photochemical degradation
- Biotransformation
- Speciation – complexation
- Loss of coating
- Adsorption/desorption of (other) substances
- Corona formation

The processes listed above take into account processes at the level of an individual particle (e.g. photochemical transformation), interactions between particles (e.g. corona formation), and interactions of particles with solid surfaces and with other substances (e.g. adsorption/desorption). When quantitative analysis of these parameters is not possible due to lack of applicable methods, qualitative assessment may also provide valuable information in fate assessment of nanomaterials. The parent guidance highlights the challenges and case specificity in using the information on photochemical degradation in classification and chemicals safety assessment [48].

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. This approach is also supported by Rasmussen *et al*.

[3] who propose a fate decision tree logic and testing strategy that takes into account the dissolution rate and agglomeration behaviour when testing nanomaterials.

1.2.3.4 Surface chemistry in degradation/transformation testing

If the nanomaterial is coated or functionalized with organic and potentially biodegradable material(s), then biodegradation test would need to be performed for the coating(s) alone or for the coated nanomaterials. If the test is performed with the coated nanomaterial, when the amount of the coating material is substantially lower than that of the core particle, interpretation of the results may be challenging. The amount of carbon needs to be high enough to allow reliable detection of biodegradation (measured e.g. as 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 [49]. In case the coating is degraded/transformed, the observed changes and their potential effects on the behaviour, fate and toxicity need to be considered within the endpoint specific testing regimes. For instance, knowledge on the degradation / transformation of the coating may influence the testing strategy. Depending on whether the coating of the nanomaterial is stable or not, it may be more relevant to perform hazard testing on the coated nanomaterial, the non-coated or both. (See for instance *Part D of the Guidance on IR&CSA*)

1.2.3.5 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 ([37], OECD 63 [44] and OECD 65 [45]).

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 many carbon-based nanomaterials are of inorganic nature, and therefore the biodegradation test methods currently recommended in the parent guidance may be inadequate for predicting the long-term fate of nanomaterials 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, organic coated/functionalised nanomaterial, organic coating or functionalisation agent. If the degradation of an organic 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 documents No. 36 [4] and No. 40 [5], in this appendix and in Appendix R7-1 to Chapter R7a on sample preparation, dispersion and dissolution should be followed before proceeding with fate testing.

Determination of sorption (see section 2.2.4 Appendix R7-1 to Chapter R7a) is also critical for assessing amounts of nanomaterials released to surface waters, and to soils and sediments (

[50], [51], [52], [53]). Some biodegradation test guidelines could be applied for nanomaterials to provide information on distribution of the nanomaterial, acknowledging that nanomaterials may not sorb onto solid phases (e.g. in soil, sediment or sludge) according to the equilibrium kinetics that apply to traditional chemicals [3].

OECD TG 303A "Aerobic Sewage Treatment Simulation Test" has been found to be useful, in particular for assessing the distribution of nanoparticles in sewage treatment plants (see e.g. [54]) 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. (Unless it is the intention of the study to investigate such processes).
- 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 should be provided with the test results.

A new test guideline is under development by the OECD that could be used to estimate the particle attachment of nanomaterials and their removal efficiency during wastewater treatment.

Other 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 (e.g. OECD TG 316)
- 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])
- Biotransformation
- Speciation – complexation

Applicability of available methods is dependent of the type of nanomaterial, many methods are still under developments and standard methods are not available. However, this type of information, even if qualitative, is recommended to be used as part of the Weight of Evidence in degradation assessment of nanomaterials to strengthen the conclusion on (bio)degradability/transformation and fate ([4], [5], [37]). One of the intentions of the use of these alternative methods and data is to feed-in more realistic estimations of the levels and nature of environmental (and human) exposure to the nanomaterial, as well as to allow appropriate testing of the form of the nanomaterial to which exposure predominantly occurs.

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