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Case studies putting the decision-making framework for the grouping and testing of nanomaterials (DF4nanoGrouping) into practice

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ABSTRACT

Case studies covering carbonaceous nanomaterials, metal oxide and metal sulphate nanomaterials, amorphous silica and organic pigments were performed to assess the *Decision-making framework for the grouping and testing of nanomaterials* (DF4nanoGrouping). The usefulness of the DF4nanoGrouping for nanomaterial hazard assessment was confirmed. In two tiers that rely exclusively on non-animal test methods followed by a third tier, if necessary, in which data from rat short-term inhalation studies are evaluated, nanomaterials are assigned to one of four main groups (MGs). The DF4nanoGrouping proved efficient in sorting out nanomaterials that could undergo hazard assessment without further testing. These are soluble nanomaterials (MG1) whose further hazard assessment should rely on read-across to the dissolved materials, high aspect-ratio nanomaterials (MG2) which could be assessed according to their potential fibre toxicity and passive nanomaterials (MG3) that only elicit effects under pulmonary overload conditions. Thereby, the DF4nanoGrouping allows identifying active nanomaterials (MG4) that merit in-depth investigations, and it provides a solid rationale for their sub-grouping to specify the further information needs. Finally, the evaluated case study materials may be used as source

Abbreviations: AA, Atomic adsorption; AAN, Average agglomerate number; ALF, Artificial lysosomal fluid; AMA, (*in vitro*) Alveolar macrophage assay; AOP, Adverse outcome pathway; AUC, Analytical ultracentrifugation; BAuA, *German Federal Institute for Occupational Safety and Health*; BET, (method of) Brunauer–Emmett–Teller; Cat, Category; CPH, Centrophenoxine; DF4nanoGrouping, Decision-making framework for the grouping of nanomaterials; DLS, Dynamic light scattering; DMEM, Dulbecco's modified Eagle medium; dnp, Determination not possible for technical reasons; DOPG, 1,2- Dioleolyl-sn-glycero-3-phosphocholin; DPP, Diketopyrrololpyrrol; DPPG, 1,2-Dipalmitoyl-sn-glycero-3-phosphatidylcholin; ECETOC, European Centre for the Ecotoxicology and Toxicology of Chemicals; ECHA, European Chemicals Agency; EDAX, Energy dispersive analysis of x-rays; EPA, Environmental Protection Agency; ESR, Electron spin resonance; FCS, Foetal calf serum; FFF, Field-flow-fractionation; FPG, Formamidopyrimidine DNA glycosylase; FRAS, Ferric reducing ability of serum; FTIR, Fourier-transformed infrared; GBP, Respirable granular biodurable particles; GHS, Globally harmonized system; HAR NM, High aspect ratio nanomaterial; HPRT, Hypoxanthine-guanine phosphoribosyltransferase; IATA, Integrated approach for testing and assessment; ICP-AES, inductively coupled plasma – atomic emission spectrometry; ICP-MS, Inductively coupled plasma – mass spectrometry; IEP, Iso-electric point; JRC, Joint Research Centre; LDH, Lactate dehydrogenase; LMM, Low molar mass; LO(A)EL, Lowest observed (adverse) effect level; MEM, Minimum essential medium; MG, Main group; MNvit, *In vitro* micronucleus test; MNviv, *In vivo* micronucleus test; MPS, Mononuclear phagocyte system; MTT, C,N-diphenyl-N′-4,5-dimethyl thiazol-2-yl tetrazolium bromide; MWCNT, Multi-walled carbon nanotube; N/A, Not available; NAA, Neutron activation analysis; NM, Nanomaterial; NMR, Nuclear magnetic resonance; NOAEC, No observed adverse effect concentration; OEL, Occupational exposure limit; PBS, Phosphate buffered saline; PEG, Polyethylene glycol; PSF, Phagolysosomal simulant fluid; REACH, Registration, Evaluation, Authorisation and Restriction of Chemicals; RIVM, Netherlands National Institute for Public Health and the Environment; SEM, Scanning electron microscopy; SIMS, Secondary ion mass spectrometry; SSA, Specific surface area; STIS, Short-term inhalation study; TEM, Transmission electron microscopy; TG, Test guideline; UBA, *German Environmental Protection Agency*; wt%, Weight percentage; XPS, X-ray photoelectron spectroscopy; XRD, X-ray diffraction.

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Biopersistence and biodistribution
Cellular effects
Apical toxic effects

nanomaterials in future read-across applications. Overall, the DF4nanoGrouping is a hazard assessment strategy that strictly uses animals as a last resort.

Information box: definitions of terms

Benchmark material: A (nano-)material, which has been tested and evaluated according to standard criteria and to which new materials may reliably be compared for grouping purposes (Kuempel et al., 2012).

(Certified) reference material: A material that has undergone a process for validation or round robin assessment as 'reference material', thereby having fulfilled specific predefined requirements for, e.g., its homogeneity and stability (Stefaniak et al., 2013).

Functionality: A (nano)material's activity affecting its environment, such as dissolution rate in biological media, surface reactivity, and dispersibility (*cf.* system-dependent properties).

Intrinsic (material) properties: Characteristics of the material that are determined independently of the biological environment or test system. Accordingly, intrinsic material properties include chemical composition and impurities, primary particle size, surface area, water solubility and shape or aspect ratio.

Mode-of-action: Mechanisms by which materials may elicit cellular or apical toxic effects. To date, only a limited number of such mechanisms have been discerned for nanomaterials (*cf.* Arts et al. (2015) for further information on different modes-of action).

Nanoform: As defined by the EU Commission's NANO SUPPORT Project (2012), the term 'nanoform' is used for REACH registration dossiers *that (seem to) also address other forms (e.g. bulk). Thus, a nanoform registered 'alone' (not along with non-nanoforms) would be a nanomaterial.*

Nanomaterial: In line with the EU definition (EU Commission, 2011), 'nanomaterial' is an overarching term to describe materials containing particles with external dimensions in the size range 1–100 nm.

Substance: The EU Regulation on the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH; EP and Council of the EU, 2006) defines a substance *a chemical element and its compounds in the natural state or obtained by any manufacturing process, including any additive necessary to preserve its stability and any impurity deriving from the process used, but excluding any solvent which may be separated without affecting the stability of the substance or changing its composition.* Accordingly, in the present article, 'substance' is used as an overarching term encompassing nanosized and non-nanosized substances in all forms regardless of their state of dissolution.

System-dependent properties: Characteristics that are linked to the material's functionality in its environment, such as surface reactivity, dissolution in biological media, and dispersibility. The outcome of measurements of system-dependent properties is affected by the given surroundings, i.e. the choice of the test system (culture media, supplements, dispersing agents, etc.) or of the product application. System-dependent properties constitute biophysical interactions of the particles with their environment. Accordingly, 'systems' may be, e.g., matrices in which a nanomaterial is embedded in a product, exposure media (aerosols, suspensions, etc.), or biological systems that the nanomaterial comes into contact with.

1. Introduction

In the context of the EU chemicals regulation REACH (Registration, Evaluation, Authorisation, and Restriction of Chemicals; EP and Council of the EU, 2006), grouping is defined as the process of uniting substances into a common group *if they are structurally similar with physico-chemical, toxicological, ecotoxicological and/or environmental fate properties that are likely to be similar or to follow a regular pattern* (ECHA, 2013). Within a group, each individual substance may not need to be tested. Instead, endpoint-specific effects of an unknown substance may be derived from the endpoint-specific effects of further substances within the group (ECHA, 2013). For substances in general, technical guidance documents on grouping are available, e.g. from the European Chemicals Agency (ECHA, 2008, 2012a, 2012b, 2013, 2014) or from the Organization for Economic Cooperation and Development (OECD, 2014). By contrast, to date there are no specific regulatory frameworks for the grouping of nanomaterials (NMs; *cf.* Information box for definitions of key terms). However, this topic is addressed in different publications, and preliminary guidance is provided in the context of substance-related legislation or the occupational setting (Arts et al., 2014).

The *International Standardisation Organisation* (ISO) suggests addressing the following questions in determining the potential hazard of a NM: Does its water solubility exceed 100 mg/L; does it contain biopersistent fibres or fibre-like structures; are there hazard indications for the NM, or is there a hazard band for the bulk material or an analogous material (ISO, 2014)? The *United States Environmental Protection Agency* (EPA) has proposed to exclude NMs which dissociate completely in water from the foreseen rule on the reporting and recordkeeping of nanoscale materials under the Toxic Substances Control Act (EPA, 2015). The *German Environmental Protection Agency* (UBA; Umweltbundesamt) suggests assigning nanotubes into a distinct group and proposes a preliminary long-term lowest-observed-effect-level (LOEL) of 0.1 mg/m^3 to distinguish 'inert' NMs from NMs with specific toxicity (UBA, 2014). Walser and Studer (2015) from the *Swiss Federal Office for Public Health* call for the establishment of predefined test strategies for different groups of NMs based upon their specific modes-of-action, which may lead via specific adverse outcome pathways (AOPs) to apical toxic effects. A report from the *Dutch National Institute for Public Health and the Environment* (RIVM; Sellers et al., 2015) highlights the scientific relevance to perform NM testing in tiers of increasing complexity of the endpoints addressed. As proposed in the RIVM report, Tier 1 serves to obtain additional physico-chemical data to fulfil REACH endpoints (exceeding the basic data that should be available by default) or to support grouping or read-across. In Tier 2, the behaviour of the NM is

characterized in its given environment and *in vitro* toxicity is assessed; and Tier 3 of the scheme suggested by the RIVM encompasses *in vivo* testing, *'if necessary to characterize the (eco) toxicity of a NM'* (p. 124; Sellers et al., 2015).

In a comprehensive literature review (Arts et al., 2014), the European Centre for Ecotoxicology and Toxicology of Chemicals (ECETOC) 'Nano Task Force' evaluated existing approaches for the grouping of NMs available in the published literature or in guidance documents from different jurisdictions. It came to the conclusion that, whereas a NM's apical toxic effect is eventually directed by its intrinsic material properties (*cf.* Information box), the exact correlation between the two is not yet established. Therefore, the grouping of NMs should not rely on intrinsic material properties alone. Instead, grouping should address all relevant aspects of a NM's life cycle and biological pathways, i.e. intrinsic material and system-dependent properties (*cf.* Information box), biopersistence, uptake and biodistribution, cellular and apical toxic effects.

As Arts et al. (2014) revealed, nearly all available approaches for the grouping of NMs involve some form of grouping by intrinsic material properties or system-dependent properties. However, none of the evaluated approaches consistently addressed all of the mentioned relevant aspects of a NM's life cycle and biological pathways.

Therefore, in the second part of its work, the ECETOC Nano Task Force developed a comprehensive DF4nanoGrouping *Decision-making framework for the grouping and testing of nanomaterials* presented in detail in Arts et al. (2015). Overall, the DF4nanoGrouping aims to group NMs by their specific mode-of-action (*cf.* Information box) that results in an apical toxic effect. Since the direct correlations of intrinsic material properties and NM apical toxic effects are not yet understood, the DF4nanoGrouping uses the 'functionality' of NMs for grouping rather than relying on intrinsic material properties alone. Such functionalities include system-dependent material properties, such as dissolution and dispersibility in biologically relevant media, and *in vitro* cellular effects, further taking into account relevant release and exposure scenarios.

By founding the grouping concept on 'functionalities' of NMs instead of restricting it to assessments of structural similarities, as laid down in e.g. the ECHA's or the OECD's guidance documents on grouping of substances (ECHA, 2013; OECD, 2014), the DF4nanoGrouping pursues a novel approach that addresses all toxicologically relevant aspects of a NM's life cycle and biological pathways.

Specifically, the DF4nanoGrouping allows assigning NMs to one of the four following main groups (MGs), to sub-group active NMs and to determine and refine specific information needs for hazard and risk assessment (Arts et al., 2015):

- MG1: Soluble NMs: Non-biopersistent NMs, for which the chemical composition is important for hazard assessment than the as-produced nanostructure.
- MG2: Biopersistent HAR NMs that are rigid and fulfil the WHO criteria for respirable fibres.
- MG3: Passive NMs: Biopersistent, non-fibrous (neither MG1 or MG2) NMs that (a) do not exhibit specific bio-interactions (low surface reactivity); (b) do not possess toxic potential (chemical composition devoid of active components; no specific cellular effects); and (c) are not mobile (agglomeration in biological fluids). *In vivo*, the 'passive state' of NMs is confirmed in that they do not elicit apical toxic effects and are not biodistributed from the site of contact or outside the mononuclear phagocyte system (MPS). Examples for such passive, inert NMs are respirable granular biodurable particles (GBPs). At high concentrations, they may elicit effects on account of their particulate nature, especially by dust inhalation, just as non-nanosized particles may also do. NMs that are not released from their matrix in products are also assigned to MG3.
- MG4: Active NMs: Biopersistent, non-fibrous NMs with a hazard potential (i.e. 'activity') that is determined using multiple characteristics including intrinsic material properties and biophysical interactions. Arts et al. (2015) proposed assigning NMs to MG4 by chemical composition, dissolution in biological media, surface reactivity, dispersibility, or cellular effects. *In vivo*, 'active' NMs are expected to elicit apical toxic effects at lower concentrations. Additionally and importantly, *in vivo* data may be used to sub-group 'active NMs' since their local toxic potency or potential to induce systemic effects may differ considerably.

As this overview of the four MGs reveals (*cf.* Arts et al. (2015) for details), the DF4nanoGrouping has incorporated relevant elements from existing approaches for the grouping of NMs. Overall, the DF4nanoGrouping is structured into three tiers that cover all relevant aspects of a NM's life cycle and biological pathways, i.e. intrinsic material properties (Tier 1), system-dependent properties and cellular effects (Tier 2), and apical toxic effects as well as *in vivo* biopersistence, uptake and biodistribution (Tier 3). Intended use (including manufacture), release and route of exposure may be applied as 'qualifiers' within the DF4nanoGrouping to determine if, e.g. NMs cannot be released from a product matrix, which may justify the waiving of testing.

The value of the *Decision-making framework for the grouping and testing of nanomaterials* for hazard assessment has to be substantiated by putting it into practice. Therefore, the case studies presented in this article, summarizing the outcome of the third and final part of the work of the ECETOC Nano Task Force, pursue the following goals:

- Exemplify how the DF4nanoGrouping *Decision-making framework for the grouping and testing of nanomaterials* may be used;
- Evaluate the appropriateness of the four MGs of the DF4nanoGrouping, specifically:
  - evaluate the appropriateness and significance of each individual grouping criterion for NM grouping;
  - determine if the position of each grouping criterion within the tiers of the DF4nanoGrouping requires adaptation;
  - determine if threshold values of specific grouping criteria triggering NM assignment to a given MG require amendment;
  - evaluate the appropriateness of benchmark materials (*cf.* Information box, also for distinction between 'benchmark material' and '(certified) reference material') that serve to assign NMs into the specific MGs (and sub-groups if applicable).
- Evaluate the usefulness of the DF4nanoGrouping:
  - to support the application of read-across techniques in filling data gaps for specific substances within a group by using data from other substances of the same group;
  - to determine information needs;
  - to justify the waiving of unnecessary testing; in the context of regulatory hazard and risk assessment and specifically in fulfilling the REACH requirements for the registration of substances.

The case studies demonstrate how the DF4nanoGrouping may be applied for the hazard assessment of NMs. Tiers 1 and 2 of the DF4nanoGrouping (serving to group NMs by intrinsic material properties and system-dependent properties) are basic tiers that are generally applicable for NM hazard assessment. The present case studies focus on the inhalation route of exposure, i.e. the predominant route of NM uptake, which was also the focus of the

first two parts of the ECETOC Nano Task Force's work. Thereby, the focus of the case studies lies on potential human health effects in the respiratory tract as the primary target organ upon inhalation as well as in secondary organ systems that might be affected if NMs become systemically available after deposition in the lung. Nevertheless, the general approach of the DF4nanoGrouping is equally applicable to other routes of exposure, and further grouping criteria may be included into its tiers as necessary, e.g., for ecotoxicological assessment. Finally, in acknowledgement that for many NMs a broad spectrum of intended uses is foreseeable, the case studies aim at revealing how the decision-making framework may generally be applied irrespective of intended use (or specific release scenarios).

2. Design of the DF4nanoGrouping case studies

2.1. Selection of case study materials

The following 24 materials were selected for four specific case studies. (Of note, NM-x numberings (e.g. 'ZnO NM-110') refer to the respective codes of the representative NMs from the OECD Sponsorship Program for the Testing of Manufactured NMs (http://www.oecd.org/science/nanosafety/and https://ec.europa.eu/jrc/en/scientific-tool/jrc-nanomaterials-repository).

1. Carbonaceous NMs (5 materials)
   - Multiwalled carbon nanotubes MWCNT NM-400 (Nanocyl® NC7000)
   - MWCNT NM-402 (Graphistrength™)
   - Graphene
   - Graphite nanoplatelets (GraphEx®)
   - Low surface area carbon black
2. Metal oxides and metal sulphates (8 materials)
   - $BaSO_4$ NM-220
   - CeO_2 NM-211
   - CeO_2 NM-212
   - 10 nm-CuO
   - 15 nm-Fe_2O_3 (hematite)
   - TiO_2 NM-105
   - ZnO NM-110
   - ZnO NM-111
3. Amorphous silica NMs (7 materials)
   - SiO_2 NM-200 (equivalent: Zeosil® 45, SIPERNAT® 22S)
   - SiO_2 NM-203 (equivalent: Cab-O-Sil® M5, AEROSIL® 200)
   - Levasil® 200 (in the following: aSiO_2-susp)
   - aSiO_2-susp with four different surface functionalizations, i.e. acrylate, amino, polyethylene glycol (PEG), and phosphate, respectively
4. Organic pigments (4 materials)
   - Diketopyrrololpyrrol (DPP) orange (bulk)
   - DPP orange (nano)
   - Pigment red 254-2 (nano)
   - Pigment blue 15:1 (Cu-phthalocyanin)

In addition to the 24 materials in the 4 case studies, C_{60} fullerene and non-nanosized crystalline quartz DQ12 were analysed. C_{60} fullerene was evaluated using only data from peer-reviewed literature that had not been co-authored by any ECETOC Nano Task Force member. These data for C_{60} fullerene were collected from a variety of different unrelated sources (*cf.* 2.2 Data collection). Non-nanosized crystalline quartz DQ12 is known to elicit pronounced effects in the lung upon inhalation exposure.

Each case study (except for the organic pigments) included DF4nanoGrouping benchmark materials as they had been specified by Arts et al. (2015), i.e. for the carbonaceous NMs MWCNT NM-400; for the metal oxides and metal sulphates $BaSO_4$ NM-220, CeO_2 NM-211 and NM-212, TiO_2 NM-105, ZnO NM-110 and NM-111; and for the silica NMs SiO_2 NM-200 and NM-203.

2.2. Data collection

Data for the case studies were collected from the following sources:

- **Study reports** from members of the ECETOC Nano Task Force, many of which had been published (e.g. Arts et al., 2007; Ma-Hock et al., 2009a, 2013; Van Ravenzwaay et al., 2009; DeLorme et al., 2012, 2015; Keller et al., 2014; Landsiedel et al., 2010, 2014a; Schuler et al., 2013; Wohlleben et al., 2013). No new *in vivo* studies were performed for the case studies.
- **Reports from joint research projects and actions** at which members of the ECETOC Nano Task Force had participated, specifically the German Federal Ministry for Education and Research funded projects NanoCare (Kuhlbusch et al., 2009; Kroll et al., 2011) and nanoGEM (Hahn et al., 2014; Izak-Nau and Voetz, 2014; Landsiedel et al., 2014b), the EU joint action NANOGENOTOX (NANOGENOTOX, 2013a, 2013b), and the EU 7th research framework programme-funded SUN project (*cf.* http://nanopartikel.info/projekte/abgeschlossene-projekte/nanocare; www.nanoGEM.de; www.nanogenotox.eu; www.sun-fp7.eu, respectively).
- **Dossiers of the OECD** Working Party on Manufactured NMs Sponsorship Program on the testing of NMs (OECD, 2015a,b,c,d; http://www.oecd.org/chemicalsafety/nanosafety/dossiers-and-endpoints-testing-programme-manufactured-nanomaterials.htm; and related documents: Singh et al. (2011, 2014));
- **Peer-reviewed publications** (or equivalent types of documents) were only used if the identities of the tested materials and the test methods used for physico-chemical characterization and toxicity assessment were unequivocally described.

2.3. Grouping criteria

For all case studies, data for the following grouping criteria of the three tiers of the DF4nanoGrouping were collected. Additionally, available information related to the DF4nanoGrouping qualifiers and supplementary criteria (that are not essential for NM assignment to one of the four MGs but that may relate to a NM's mode-of-action) were gathered (Table 1).

- DF4nanoGrouping Tier 1 – intrinsic material properties: Water solubility, particle size and shape (aspect ratio) and composition (including surface functionalization, and noting the presence of material components or impurities that have been assigned Globally Harmonized System categories (GHS, *cf.* Section 2.4). As described in Arts et al. (2015), these Tier 1 criteria may also be used in a 'Tier 0' preceding the DF4nanoGrouping to define if a material is in fact a NM. However, such a 'Tier 0' serves the purpose to recognize mere material similarities. By contrast, the DF4nanoGrouping serves the purpose to recognize similarities in respect to hazards and risks. NMs may have differing nanoforms that nevertheless have similar hazards.
- DF4nanoGrouping Tier 2 – system-dependent properties and *in vitro* effects: Dissolution in biological media, surface reactivity, dispersibility, cellular effects and *in vitro* genotoxicity;
- DF4nanoGrouping Tier 3 – *in vivo* effects: Apical toxic effects, toxic potency, *in vivo* genotoxicity, reversibility of effects, (primary and secondary) organ burden and clearance, biodistribution and biopersistence;

Table 1
DF4nanoGrouping: Grouping criteria, threshold values, relation to main group assignment as published in Arts et al. (2015).

DF4nano-grouping Tier	Grouping criterion	Threshold value for grouping	Main group (MG) assignment or indication	Preferred test methods and further explanations
Tier 1 Intrinsic material properties	Water solubility	>100 mg/L [a]	Assignment to MG1	Water solubility was recorded at pH values ≥ 4. If water solubility data were available from different methods, preference was given to ICP methods over, e.g. spectrophotometric methods. If available water solubility data were expressed as dissolved percentage, values > 10% were assessed as indicating high solubility even though the corresponding mass-per-unit-volume value might not be equivalent to >100 mg/L. For particles with high dispersibility in water, water solubility values may have high variability due to traces of particles in the supernatant in which the ions are determined.
	Particle size and shape	Aspect ratio >3:1, length >5 μm, diameter <3 μm [b]	Indication for MG2	TEM/SEM; primary particles are measured by TEM as constituent particles of aggregates and agglomerates (ISO, 2015)
	Composition; including impurities	≥0.1% of component with GHS classification for systemic effects	Indication for MG4	Inorganic nanomaterials: XRD, AA, NAA, ICP methods, EDAX Organic nano-materials: NMR, FTIR *cf.* Section 2.4 for determination of 'activity' potential of material components or impurities by GHS classification
Tier 2 System-dependent properties *In vitro* effects	Dissolution in biological fluids	>100 mg/L [a]	Globular NMs: >100 mg/L: Indication for MG1 Fibres: <100 mg/L: Indication for MG2	For the inhalation route of exposure, data on the dissolution in DMEM + FCS, PBS, PSF, ALF or Gamble's solution (i.e. simulated lung fluid) were considered relevant. Incubation time: 24-h in DMEM + FCS, 24-h, 72-h or 28-d in Gamble's solution (as specified together with the respective data), 72-h in ALF, 28-d in PBS or PSF (all: at 37 °C); followed by centrifugal separation and (for inorganic materials), ICP-MS, SEM or TEM, supported by SAD, if necessary; or (for organic materials): AUC using an UVvis detector
	Surface reactivity	≥10% of Mn_2O_3 reactivity, which is equal to: ≥0.19 μUFRAS/m^2*h	Assignment to MG4	FRAS assay; comparing results to Mn_2O_3 surface reactivity (i.e. 1.921 μUFRAS/m^2*h). As laid down in Arts et al. (2015), the ≥10% of Mn_2O_3 surface reactivity threshold was used for substance assignment to MG4. Additionally, surface reactivity values between <10% and >1% of Mn_2O_3 surface reactivity were recorded as indicating intermediate surface reactivity and values ≤ 1% as indicating 'non-oxidative' surface reactivity. Alternatively: ESR (CPH spin traps) with a threshold value of >10–20 relative to D_2O (^2H_2O). Assuming a 30% variability of the methodology, only ESR measurements >1.3 are considered relevant, and also this value should only serve as a guiding principle, and not as an absolute value.
	Dispersibility	AAN <3 or diameter <100 nm	Assignment to MG2 or MG4, as applicable	AUC, FFF; in case of differing AANs in different fluids, data obtained in DMEM + FCS, i.e. a complex medium containing a spectrum of physiologically relevant ingredients, or in PSF were considered decisive. Since the 'AAN <3' cut-off value serves to recognize NMs that prevail as individual nanoparticles, the DF4nanoGrouping criterion dispersibility does not take into account the materials' effective density, which is relevant when assessing the behaviour of larger nanoparticle agglomerates (Pal et al., 2015). Data obtained in DMEM-F12 supplemented with bovine serum albumin were not used since this dispersing agent generally elicits high nanomaterial dispersibility (Sauer et al., 2014b).
	Cellular effects	Effect at ≤10 μg/cm^2 [c]	Assignment to MG4	*In vitro* alveolar macrophage assay Alternatively: MTT or LDH assays with specified test protocols
Tier 3 *In vivo* screening	Toxic potency	STIS NOAEC; four ranges: I: <0.1 mg/m^3 [d] II: <1 mg/m^3 III: <10 mg/m^3 IV: ≥10 mg/m^3	Ranges I-III: Confirmation of MG2 or MG4; sub-grouping of MG4; Range IV: Confirmation of MG3	STIS for inhalation exposure
	Biopersistence	t_{50} < 40 days	Confirmation of MG1	Extrapolated from the STIS organ burdens immediately after the end of exposure and upon completion of the 14–28-day post-exposure observation period. Of note, these extrapolated values only provide an indication of the respective pulmonary half-times. However, they should not be considered precise values, since they were calculated from

				two values (i.e. the time points immediately after exposure and at the end of the observation period) and do not, e.g., distinguish between different stages of substance clearance (i.e. the initial fast clearance that is followed by a phase of slower clearance). Even though the $t_{50} \geq 40$ day value published in BAuA (2014) relates to data from pulmonary instillation studies, this threshold value may also be used to evaluate biopersistence upon inhalation, since the pulmonary t_{50} upon inhalation is identical to the one upon instillation.
Qualifier	Dustiness	None assigned	Indication of a substance's emission potential	Rotating drum method
Supplementary criteria	Surface area	None assigned	Not primary grouping criteria	BET or Hg porosimetry
	Surface chemistry	None assigned	Not primary grouping criteria	XPS, SIMS
	Surface charge	Positive: $\zeta > 10$ mV	Joint evaluation with 'dispersibility' Pos. surface charge: Indication for MG4	Zeta sizer (zeta potential in water at pH 7.4) Electrophoretic mobility (pH titration method)
	Hydrophobicity	None assigned	Joint evaluation with 'dispersibility'	Water contact angle on pressed powder: 0°−90° (hydrophilic), 90°−180° (hydrophobic) Lipid affinity using different lipids, such as DPPG or DOPG

Abbreviations: AA: Atomic adsorption; AAN: Average agglomeration number; ALF: Artificial lysosomal fluid; AUC: Analytical ultracentrifugation; BET: (Method of) Brunauer, Emmett and Teller; CPH: Centrophenoxine; DMEM + FCS: Dulbecco's modified Eagle's Medium supplemented with 10% foetal calf serum; DOPG: 1,2- Dioleolyl-sn-glycero- 3-phosphocholin; DPPG: 1,2-Dipalmitoyl-sn-glycero-3-phosphatidylcholin; EDAX: Energy dispersive analysis of x-rays; ESR: Electron spin resonance; FFF: Field-flow-fractionation; FRAS: Ferric reducing ability of serum; FTIR: Fourier-transformed infrared; GHS: Globally harmonized system; ICP(-MS): Inductively coupled plasma (- mass spectrometry); LDH: Lactate dehydrogenase; MG: Main group; MTT: C,N-diphenyl-N′-4,5-dimethyl thiazol-2-yl tetrazolium bromide; NAA: Neutron activation analysis; NMR: Nuclear magnetic resonance; NOAEC: No observed adverse effect concentration; PBS: Phosphate buffered saline; PSF: Phagolysosomal fluid; SAD: Selected area diffraction; SEM: Scanning emission microscopy; SI: Supplementary Information; SIMS: Secondary ion mass spectrometry; STIS: Short-term inhalation study; TEM: Transmission electron microscope; XPS: X-ray photoelectron spectroscopy; XRD: X-ray diffraction.

Based upon the outcome of the case studies, the following adaptations to the threshold values laid down in Arts et al. (2015) are made (*cf.* Section 4.1).

[a] While the threshold values for water solubility and dissolution are adequate for NMs that release ions with GHS classification for systemic effects, they may have to be reconsidered for substances that dissolve into non-toxic components.

[b] NMs may be assigned to MG2 on account of high aspect ratio, fibre diameter, and insolubility/low dissolution in water or biological media, even though their length does not meet the WHO criterion (>5 μm).

[c] This threshold value applies for cytotoxicity tests performed with lung epithelial cells. For *in vitro* assays performed with alveolar macrophages, a threshold value of 4000 μm^2 particle surface area/macrophage is laid down since it corresponds to *in vitro* non-overload conditions (Wiemann et al., 2015b).

[d] The threshold value for STIS NOAEC Range I is amended to '<0.5 mg/m^3 and no regression or progression of effects'.

- DF4nanoGrouping – qualifiers: Release (dustiness);
- DF4nanoGrouping – supplementary criteria: Surface area, surface chemistry, surface charge, and hydrophobicity.

Rigidity that Arts et al. (2015) had specified as criterion for NM assignment to MG2 (biopersistent HAR NMs) was not addressed in the case studies. To date, there are no established methods to determine this criterion. Generally, fibre diameter may be used as a proxy for rigidity, if the diameter of the material under investigation is comparable to the diameter of a MWCNT with known fibre toxicity, e.g. Mitsui-7 (Poulsen et al., 2015). Further, the supplementary criterion corona formation was not addressed in the case studies: The predictive value of this system-dependent property is sufficiently addressed by the intrinsic material properties hydrophobicity and surface charge, supplemented by the system-dependent property dispersibility. Finally, droplet size, which Arts et al. (2015) indicated as potential qualifier to evaluate NM release, was exempt from the case studies since it is only relevant for very specific use and release scenarios.

2.4. Nanomaterial assignment to the four main groups of the DF4nanoGrouping

Criterion-by-criterion and tier-by-tier the recorded data were evaluated against the respective criteria-specific threshold values laid down within the DF4nanoGrouping (*cf.* Table 1 for an overview of the criteria and threshold values). In brief, the three tiers and the grouping criteria of the DF4nanoGrouping serve to answer the following sequence of questions:

1. Is the NM soluble? Does it dissolve in biological media? Does it have low biopersistence? In Tier 1, NMs are assigned to MG1 on account of their solubility in water (>100 mg/L; BAuA (2013)), or 'non-MG1' if solubility is below this threshold value. Data from Tier 2 may be used to additionally assign those NMs to MG1 which are not water soluble, but that highly dissolve in biological media (>100 mg/L). Likewise, in Tier 3, NMs may be assigned to MG1 if their pulmonary half-life (t_{50}) is less than 40 days (the threshold value set for biopersistent fibres in BAuA (2014)). The further hazard assessment of NMs that have been assigned to MG1 is based upon read-across to the bulk counterpart and/or the dissolved ions as well as available data from the NM itself.
2. Does the size, aspect ratio, and biopersistence of the NM indicate that it is a biopersistent HAR NM? An indication for assignment to MG2 is based upon size and aspect ratio in Tier 1 (aspect ratio: >3:1, length: >5 μm; diameter: <3 μm; WHO (2005)). The final assignment is based upon low dissolution in biological media in Tier 2 (≤100 mg/L) or high *in vivo* biopersistence in Tier 3 (t_{50}: ≥40 days). The further hazard assessment of biopersistent HAR NMs may address their potential to exert asbestos-like 'fibre toxicity' (Poland et al., 2009).
3. Are 'non-MG1 – non-MG2' NMs passive or active (i.e. do they have an inherent potential for toxicity)? NM assignment into MG3 (passive NMs) is based upon fulfilment of ***all*** of the following Tier 1/Tier 2 criteria:
   a. Lack of (or no release of) components that have been assigned a GHS category (<0.1% of the respective elements or molecules);
   b. Low dissolution in biological media (≤100 mg/L);
   c. Low surface reactivity (<10% of the reactivity of the reference material Mn_2O_3 in the Ferric Reducing Ability of Serum (FRAS) assay);
   d. Low dispersibility (average agglomeration number (AAN) ≥3);
   e. Low cytotoxic potency (no effects up to 10 μg/cm^2; i.e. over the entire range of *in vitro* effective dosages that do not reflect *in vivo* pulmonary overload conditions; Kroll et al. (2011)).

By contrast, NMs are assigned to MG4 if any single decisive property (or combinations of properties) listed for MG1, MG2, or MG3 is (or are) not met. The Tier 2 assignment to MG3 or MG4 may be confirmed or revised by Tier 3 data from *in vivo* short-term inhalation studies (STIS; MG4 if the no observed adverse effect concentration (NOAEC) is <10 mg/m^3), for the inhalation route of exposure.

4. Do specific grouping criteria, qualifiers or supplementary criteria enable sub-grouping of active NMs? Sub-grouping is especially relevant for the MG4 active NMs because they may possess specific hazards. Since no rules for sub-grouping were established in Arts et al. (2015), the comprehensive data recorded for the case study materials were evaluated to determine whether specific grouping criteria, qualifiers or supplementary criteria may provide a more detailed indication of the toxic potential or potency of the NMs assigned to MG4.

In summary, for all NMs, the Tier 1 intrinsic material properties chemical composition, morphology (i.e. size and aspect ratio) and water solubility were evaluated as essential criteria, thereby providing an indication for NM assignment as MG1 soluble NMs or MG2 HAR NMs. In Tier 2, the water solubility-based NM assignment as MG1 or MG2 (or non-MG1 or non-MG2) was reassessed evaluating the system-dependent property dissolution in biological media. Further in Tier 2, for the 'non-MG1 – non-MG2' NMs, data on the system-dependent properties surface reactivity and dispersibility as well as on cellular effects were used as essential criteria to distinguish MG3 passive NMs from MG4 active NMs. Accordingly, based upon the outcomes of the non-animal testing Tiers 1 and 2, the test materials were eventually assigned to one of the four MGs.

Of note, materials or impurities were considered potentially active if they had been assigned a GHS category for human health or environmental hazards (United Nations (2011); *cf.* the ECHA's 'Classification and Labelling inventory'; www.echa.europa.eu) and, in the case of impurities, their proportion was ≥0.1%. Further, the potential to release substances (e.g. copper ions) which elicit cellular damage was recorded (either via assessment of water solubility or dissolution in biological media). Of note, there is no standardized *in vitro* assay having received regulatory acceptance to determine whether released ions (or substances in general) may damage cells. Similarly, prediction models for *in vitro* assay evaluation in regard to *in vivo* toxic effects in the respective target tissue are currently limited (Schrage et al., 2011; Sauer et al., 2014a). Therefore, for ions released from NMs, GHS categories of corresponding soluble salts may provide some indication of their potential cytotoxicity. Effects observed with a salt (e.g. CuCl2) after oral administration may, however, not accurately reflect the toxic potential of the released ions in the respiratory tract (e.g. copper ions would be harmful, but not toxic, as judged by the acute systemic toxicity of CuCl2). Taking into account these limitations, preferably data from the corresponding bulk material, which release the same ions, were taken into consideration – while noting that these bulk materials may exhibit different release kinetics.

The following materials, material components or impurities had been assigned the following GHS categories:

- **CuO** (CAS No. 1317-38-0): Aquatic acute toxicity category (cat.) 1 (H400): Very toxic to aquatic life; Aquatic chronic toxicity cat. 3 (H412): Harmful to aquatic life with long lasting effects.
- **ZnO** (CAS No. 1314-13-2): Aquatic acute toxicity cat. 1 (H400): Very toxic to aquatic life; Aquatic chronic toxicity cat. 1 (H410): Very toxic to aquatic life with long lasting effects.
- The MWCNT impurity **cobalt nitrate** (CAS No. 10141-05-6): Skin sensitization cat. 1 (H317): May cause an allergic skin reaction; Respiratory sensitization cat. 1 (H334): May cause allergy or asthma symptoms or breathing difficulties if inhaled; Mutagenicity cat. 2 (H341): Suspected of causing genetic defects; Carcinogenicity cat. 1B (H350): May cause cancer (inhalation route of exposure); Reproductive toxicity cat. 1B (H360): may damage fertility; Aquatic acute toxicity cat. 1 (H400): Very toxic to aquatic life; Aquatic chronic toxicity cat. 1 (H410): Very toxic to aquatic life with long lasting effects.

2.5. Evaluation of the Tier 1 and Tier 2 main group assignment using Tier 3 short-term toxicity data

In Tier 3, the assignment of NMs to MG1 (soluble NMs) or MG2 (biopersistent HAR NMs) based upon Tier 1 and Tier 2 criteria was evaluated focussing on the *in vivo* STIS pulmonary half-life (biopersistence). Similarly, Tier 3 evaluation of passive (MG3) or active NMs (MG4) was based upon the STIS NOAEC (indicating toxic potency *in vivo*). The STIS NOAEC was further used to sub-group the NMs assigned to MG4 by specific levels of toxic potency, and it was assessed whether further criteria could be discerned that would support sub-grouping of these materials.

In Arts et al. (2015), the STIS NOAEC threshold values were inadvertently misrepresented: NOAEC intervals are half-bound, right-open, i.e. they extend upwards from the respective NOAECs. The correct threshold values for the DF4nanoGrouping STIS NOAEC ranges are:

- Range I: <0.1 mg/m^3 (and not: ≤ 0.1)
- Range II: <1 mg/m^3 (and not: ≤ 1)
- Range III: <10 mg/m^3 (and not: ≤ 10)
- Range IV: ≥ 10 mg/m^3 (and not: >10 mg/m^3)

In the case studies, the correct NOAEC threshold values were applied (Table 1). STIS NOAEC Range IV confirms NM assignment as MG3 (passive), whereas STIS NOAEC Ranges I-III support the assignment of 'non-MG1 and non-MG2' NMs as active (MG4) and allow their sub-grouping by the given toxic potency range. Additionally, Tier 3 STIS data may be used to determine the nature of toxic effects and to sub-group MG4 active NMs by the progression/reversibility of effects or by their extent of systemic availability (no systemic availability/systemic availability only in the MPS/systemic availability also outside the MPS).

For Tier 3 data, published or unpublished STIS from members of the ECETOC Nano Task Force were available for all case study materials with the following exceptions: For SiO_2 NM-200 and NM-203, STIS data published by a member of the ECETOC Nano Task Force were available for SiO_2 NMs that had been assessed as 'equivalent' in the corresponding OECD dossiers based upon production process, minimum degree of material purity, and comparability of specific surface area and agglomerate size (Arts et al., 2007; OECD, 2015c,d). For 10 nm-CuO, STIS data conducted within the EU SUN project (Gosens et al., 2015) were used.

The STIS protocol (*cf.* OECD, 2015e, pages 122–127) was developed in the EU 6th research framework-funded project NANOSAFE2 (www.nanosafe.org) and the German Federal Ministry for Education and Research-funded project nanoCare (http://www.nanopartikel.info/projekte/abgeschlossene-projekte). It is essentially an adaptation of the OECD test guideline (TG) 412 *Subacute inhalation toxicity: 28-day study* (OECD, 2009) test protocol. This protocol was amended by appropriate aerosol generation and characterisation, bronchoalveolar lavage parameters and lung burden assessments. Instead of 28 days of exposure and an optional post-exposure observation period, the STIS protocol foresees five days of exposure and a 2- to 13-week post-exposure observation period (Arts et al., 2007; Ma-Hock et al., 2007, 2009a; Landsiedel et al., 2014a). NM acute inhalation toxicity, which is scarcely investigated in the first place, was not used for NM grouping (or evaluation of the DF4nanoGrouping) since the outcome of such studies is expected to provide little useful information on NM toxicity following repeated inhalation exposure (Landsiedel et al., 2014c).

2.6. Overall evaluation of the DF4nanoGrouping using long-term NOAEC

Data from long-term (as a rule, 90-day) studies were collected to assess the suitability of the DF4nanoGrouping in predicting the toxic potential and potency of NMs (and, more generally, of particles in aerosols), i.e. the appropriateness of NM (material) MG assignment.

2.7. Evaluation of in vitro and in vivo genotoxicity, the qualifier dustiness and the supplementary criteria

A definite assessment of the *in vitro* or *in vivo* genotoxic potential of NMs is still under discussion. Prevailing knowledge gaps had precluded the determination of threshold values or benchmark materials for *in vitro* or *in vivo* genotoxicity in DF4nanoGrouping (Arts et al., 2015). Therefore, available data for these endpoints were collected for the case studies, but these data were not used for NM assignment to the MGs. Similarly, dustiness data, indicating a material's emission potential, were recorded but not used for NM assignment to the MGs since the present assessment of the suitability of 'DF4nanoGrouping' is unrelated to exposure. Also data for the supplementary criteria surface area, surface charge, surface chemistry, and hydrophobicity were collected to further evaluate their potential relevance for NM grouping.

2.8. Reassessment of the DF4nanoGrouping criteria, threshold values and benchmark materials

Upon finalization of the DF4nanoGrouping MG assignment, adequacy of the DF4nanoGrouping criteria as well as their corresponding threshold values and benchmark materials as laid down by Arts et al. (2015) was reassessed. Further, the need for additional grouping criteria was reconsidered.

3. Outcome of the DF4nanoGrouping case studies

The following Section 3.1 presents the outcome of Tier 1 and Tier 2 of the DG4nanoGrouping in assigning the case study materials to one of the four MGs (MG1: soluble NMs; MG2: biopersistent HAR NMs; MG3: passive NMs; MG4: active NMs). Subsequently, Section 3.2 compares the outcome of the Tier 1 and Tier 2 MG assignment to the Tier 3 MG assignment by STIS data. Section 3.3 compares the overall outcome of the DF4nanoGrouping to available long-term NOAECs, and Section 3.4 summarizes the evaluation of the genotoxicity data, supplementary criteria, and qualifiers. Finally, a reassessment of all grouping criteria, threshold values and benchmark materials is provided in Section 4.1.

3.1. Nanomaterial MG assignment by Tiers 1 and 2 of the DF4nanoGrouping

3.1.1. Carbonaceous NMs

Table 2 presents the details of the application of the Tiers 1 and 2 of the DF4nanoGrouping to the case study 'carbonaceous NMs'. Two materials (MWCNT NM-400 and NM-402) are assigned to MG2 (biopersistent HAR NMs), one material (low surface carbon black) to MG3, and two materials (graphene and graphite nanoplatelets) to MG4 (active NMs).

The WHO definition of fibres (WHO, 2005) was not specifically conceived in regard to NMs, and, in fact, it may not be fully applicable to NMs. MWCNT NM-402 was assigned to MG2 on account of its high aspect ratio, fibre diameter, and insolubility in water/low dissolution in biological media, even though its length (1.1 μm) did not meet the WHO criterion (>5 μm). Further research is needed to determine the critical length of biopersistent, high-aspect ratio NMs. By contrast, graphene also has a high aspect ratio (in two dimensions), but research is still ongoing to determine if graphene and other two-dimensional platelet-like materials have the potential to elicit 'fibre toxicity'. Therefore, it was not assigned to MG2 in the current case studies.

3.1.1.1. MWCNT NM-400 and MWCNT NM-402

- In Tier 1, high aspect ratio and low water solubility indicate MG2.
- Additionally, for MWCNT NM-400, the Tier 1 criterion 'impurities' indicates the presence of toxic cobalt nitrate at a proportion ≥0.1% which would be indicative of an active NM (MG4) if it had not yet been assigned to MG2.
- In Tier 2, low dissolution confirms MG2 assignment.

3.1.1.2. Graphene and graphite nanoplatelets

- Tier 1 criteria indicate non-MG1 (non-soluble in water), non-MG2 (not fibres, see above for high aspect ratio of two-dimensional NMs). There are no (or only <0.1%) material components or impurities that have been assigned a GHS category.
- In Tier 2, non-MG1 cannot be refuted since data on their dissolving properties in biological media are not available. However, graphene is not expected to dissolve in different solvents.
- Further in Tier 2, the essential MG3 criterion surface reactivity could not be determined due to the hydrophobicity of graphene and graphite nanoplatelets. Therefore, the respective activity cannot be excluded, which by conservative default implies MG4.

3.1.1.3. Low surface carbon black

- Tier 1 criteria indicate non-MG1 and non-MG2. There are no (or only <0.1%) material components or impurities that have been assigned a GHS category.
- In Tier 2, low dissolution in different solvents confirms non-MG1.
- Further in Tier 2, lack of cellular effects up to 10 μg/cm^2 and agglomeration in Dulbecco's Modified Eagle's Medium supplemented with 10% foetal calf serum (in the following: DMEM + FCS) consistently indicate passivity resulting in assignment to MG3.

Table 2
Assignment of the case study 'carbonaceous nanomaterials' to one of the four main groups of the DF4nanoGrouping by application of its Tiers 1 and 2.

Tier	Grouping criterion	Threshold value for grouping	MWCNT NM-400	MWCNT NM-402	Graphene	Graphite nanoplatelets	Low surface carbon black
1	Water solubility	>100 mg/L = MG1	Insoluble	Insoluble	Insoluble	Insoluble	Insoluble
	Particle size and shape	Aspect ratio >3:1, length >5 μm, Ø <3 μm = MG2	**Ø 5-15 nm, 0.1-10 μm length [a,b]**	**15 nm outer diameter; 1.1 μm length [c,d] #**	Up to 10 μm; flakes [c]	Up to 30 μm Flakes [c]	50-100 nm; globular [b,c]
	Composition, shape	≥0.1% of toxic component = MG4	Carbon-based [a]	Carbon-based [c,d]	Carbon-based [c]	Carbon-based [c]	Carbon-based [c]
	Purity; impurities	≥0.1% of toxic component = MG4	>90%; Al: 42192; Co 1911; Fe 3455 (mg/kg) [e]	99%; no significant impurities [c]	<85%; sulphur impurity; 3D graphite impurities [c]	<85%; Na-, Ca-, Si-containing minerals; 3D graphite impurities [c]	98%; variable amounts of O, H, sulphur [f]
2	Dissolution in biological media	>100 mg/L = MG1	N/A	N/A	N/A	N/A	Insoluble in different solvents [f]
	Surface reactivity	≥10% of Mn_2O_3 reactivity = MG4	With the present FRAS protocol, determination not possible due to high substance hydrophobicity		**With the present FRAS protocol, determination not possible due to high substance hydrophobicity**		Low surface reactivity [g]
	Dispersibility	AAN <3 or diameter ≤100 nm = MG4	DMEM+FCS: 77000 nm [b]	DMEM+FCS: several μm [c]	Water: <0.01 wt% <100 nm [c]	Water: <0.01 wt% <100 nm [c]	DMEM+FCS: 67 μm [b]
	Cellular effects	Effect at ≤10 μg/cm^2 = MG4	No *in vitro* AMA data for technical reasons [h]	N/A	N/A	Passivity [h]	Cellular effects (A549 cells, MTT, LDH): only at 25 μg/cm^2 [i]
Tier 1 / Tier 2 MG assignment			**MG2**	**MG2**	**MG4**	**MG4**	**MG3**

Abbreviations: AAN: Average agglomeration number; AMA: (*in vitro*) Alveolar macrophage assay; DMEM+FCS: Dulbecco's modified Eagle's Medium supplemented with 10% fetal calf serum; FRAS: Ferric reducing ability of serum; LDH: Lactate dehydrogenase; MG: Main group; MTT: C,N-diphenyl-N′-4,5-dimethyl thiazol-2-yl tetrazolium bromide; N/A: Not available; TEM: Transmission electron microscopy; wt%: Weight percentage.
For applied test methods, *cf.* Table 1.
MWCNT NM-402 is assigned to MG2 in spite of its length < 5 μm (see text for further details).
Colour legend:
• Bold print with bold framing indicates nanomaterial assignment to MG2 (biopersistent HAR NMs); light grey: MG 3 (passive nanomaterials); black: MG4 (active nanomaterials).
• Dark grey shading indicates the presence of potentially active components (that however is only toxicologically relevant if these components may be released).
• Nanomaterials are assigned to MG2 based upon particle size and high aspect ratio. 'Activity' recorded for a single relevant grouping criteria results in nanomaterial assignment to MG4. For nanomaterial assignment to MG3, all grouping criteria have to indicate 'passivity'. This is highlighted by the continuous light grey shading.
The data for the case study 'carbonaceous nanomaterials' were retrieved from the following sources: Ma-Hock *et al.* (2009b); [b] Wohlleben *et al.* (2013); [c] Ma-Hock *et al.* (2013); [d] OECD (2015b); [e] NANOGENOTOX (2012); [f] SCCS (2014); [g] Hsieh *et al.* (2013); [h] Wiemann et al. (2015b); [i] Kuhlbusch *et al.* (2009).

3.1.2. Metal oxides and metal sulphates

Table 3 presents the details of the application of the Tiers 1 and 2 of the DF4nanoGrouping to the case study 'metal oxides and metal sulphates'. Three materials (10 nm-CuO, ZnO NM-110 and NM-111) are assigned to MG1 (soluble NMs), two materials ($BaSO_4$ NM-220 and 15 nm-Fe_2O_3 hematite) to MG3 (passive NMs). Three materials (CeO_2 NM-211 and NM-212, TiO_2 NM-105) are assigned to MG4 (active NMs) on account of their cellular effects.

3.1.2.1. $BaSO_4$ NM-220

- Tier 1 criteria indicate non-MG1 and non-MG2. There are no (or only <0.1%) material components or impurities that have been assigned a GHS category.
- In Tier 2, low dissolution in phagolysosomal simulant fluid (PSF) and phosphate buffered saline (PBS) confirms non-MG1.
- Further in Tier 2, surface reactivity <10% Mn_2O_3 reactivity, agglomeration, and lack of cellular effects consistently indicate passivity (assignment to MG3).

3.1.2.2. CeO_2 NM-211 and CeO_2 NM-212

- Tier 1 criteria indicate non-MG1 and non-MG2. There are no (or only <0.1%) material components or impurities that have been assigned a GHS category.
- In Tier 2, low dissolution in DMEM + FCS, PSF or PBS confirms non-MG1.
- Further in Tier 2, activity in the *in vitro* alveolar macrophage assay results in assignment to MG4.
- Additionally, CeO_2 NM-211 and NM-212 have positive surface charge (*cf.* Section 3.4), which may be used as supplementary indication for MG4.

3.1.2.3. 10 nm-CuO

- Tier 1 criteria indicate non-MG1 and non-MG2. Copper oxide has been assigned GHS categories.
- In Tier 2, high dissolution in PSF results in 10 nm-CuO assignment to MG1. This high dissolution also indicates that cytotoxic copper ions may be released into biological media and fluids.
- Available cytotoxicity data indicate activity of 10 nm-CuO. Presumably, the cellular effects are caused by released copper ions. This in return confirms MG1 assignment. Some authors discuss additional effects that may be related to the high surface reactivity of this material (Karlsson et al., 2008, 2014; Son et al., 2015), but the observed effects seem to be dominated by copper ions.

Table 3
Assignment of the case study 'metal oxides and metal sulphates' to one of the four main groups of the DF4nanoGrouping by application of its Tiers 1 and 2.

Tier	Grouping criterion	Threshold value for grouping	$BaSO_4$ NM-220	CeO_2 NM-211	CeO_2 NM-212	10 nm-CuO	15 nm-Fe_2O_3 (hematite)	TiO_2 NM-105	ZnO NM-110	ZnO NM-111
1	**Water solubility**	>100 mg/L = MG1	Ba: 6 mg/L [a]	<10 mg/L [b]	<20 mg/L [b]	0.4 % at pH 7.5 [c]	< 1mg/L [c]	Ti <0.1 mg/L [a]	Insoluble [d,e]	Insoluble [a,d]
	Particle size and shape	Aspect ratio >3:1, length >5 µm, Ø <3 µm = MG2	32 nm [a]	4-15 nm [b,f,g]	40 nm [b,f,g]	10 nm [h]	15-130 x 4-21 nm (d_{50}: 15 nm); rods [c]	21 nm [a,i]	70±46 nm; hexagonal [d,e]	82±45 nm; globular [a,d]
	Composition Shape	≥0.1% of toxic component = MG4	$BaSO_4$; crystalline, ortho-rhombic [a]	CeO_2 [b,f,g]	CeO_2 [b,f,g]	**CuO [h]**	Fe_2O_3 hematite [c]	TiO_2; rutile-anatase [a,i]	**ZnO [d,e]**	**ZnO; coated with triethoxy-caprylylsilane [a,d]**
	Purity; impurities	≥0.1% of toxic component = MG4	93.8%; water, org. additive [a]	>95%; few alkyls [b]	99.5%; 0.7% ester, alkyl [b]	Na: 505 ppm Pb: 36 ppm Ag: 13 ppm [h]	High purity, no relevant impurities [c]	>99% [i]	>99% [d,e]	ZnO content: 96-99%; ZnO core: ≥99% [a,d]
2	**Dissolution in biological media**	>100 mg/L = MG1	PSF (28-d): 7 mg/L, disappearance of smallest particles; PBS (28-d): 12 mg/L [j]	DMEM+FCS (24-h) / PSF (28-d): <10 mg/L, each, structural changes with increasing particle size [b]	DMEM+FCS (24-h) / PSF (28-d / PBS (28-d): <10 mg/L, each, structural changes with increasing particle size [b]	**PSF (pH 4.3; 28-d): 120 mg/L (6%), recrystallizes to $CuCO_3$; Gamble (pH 7.5; 28-d): 1 mg/L (0.05%) [c]**	PSF (pH 4.3; 28-d): <1 mg/L; Gamble (pH 7.5; 28-d): <1 mg/L, no significant structural changes [c]	PSF (28-d): 1.5 mg/L; PBS (28-d): 0; no significant structural changes [a]	**Gamble (pH 7.4; 72-h): <100mg/L (<0.05%); ALF (pH 4.5; 72-h): >1800 mg/L (>90%) [k]**	**PSF (pH 4.3; 28-d): 640 mg/L (32%), strong increase of polydispersity; Gamble (pH 7.5; 28-d): 2 mg/L (0.1%); recrystallizes in PSF & Gamble; ALF (pH 4.5; 72-h): >1800 mg/L (90%) [c,k]**
	Surface reactivity	≥10% of Mn_2O_3 reactivity = MG4	0.0503 µUFRAS/m^2*h; intermediate [c]	0.0073 µUFRAS/m^2*h non oxidative [c]	0.0324 µUFRAS/m^2*h intermediate [c]	2.205 µUFRAS/m^2*h active [c]	0.0372 µUFRAS/m^2*h intermediate [c]	0.0244 µUFRAS/m^2*h intermediate [c]	0.0978 µUFRAS/m^2*h intermediate [c]	0.0389 µUFRAS/m^2*h intermediate [c]
	Dispersibility	AAN <3 or diameter ≤100 nm = MG4	DMEM+FCS: AAN 9 [a]	DMEM+FCS: >1 µm [c]	DMEM+FCS: AAN 7 [c]	PSF: AAN 5 and 23; bimodal [c]	DMEM+FCS: >1 µm [c]	DMEM+FCS: >>2 µm [a]	DMEM+FCS: AAN 10 [e]	DMEM+FCS: AAN 7 [a]
	Cellular effects	Effect at ≤10 µg/cm^2 = MG4	Passivity [l]	**Activity [l]**	**Activity [l]**	Alamar blue, macrophages: Activity [c]	Passivity [l]	**Activity [l]**	A549 cells, LDH release at 25 µg/cm^2 [m]	Activity [l]
Tier 1 / Tier 2 MG assignment			**MG3**	**MG4**	**MG4**	**MG1**	**MG3**	**MG4**	**MG1**	**MG1**

Abbreviations: AAN: Average agglomeration number; ALF: Artificial lysosomal fluid; AMA: Alveolar macrophage assay; DMEM+FCS: Dulbecco's modified Eagle's Medium supplemented with 10% fetal calf serum; FRAS: Ferric reducing ability of serum; Gamble: Gamble's solution; MG: Main group; N/A: Not available; PSF: Phagolysosomal simulant fluid; wt%: Weight percentage.
For applied test methods, *cf.* Table 1.
Colour legend:
• Bold print with bold framing indicates assignment to MG1 (soluble nanomaterials); light grey: MG 3 (passive nanomaterials); black: MG4 (active nanomaterials).
• Dark grey shading indicates the presence of potentially active components (that however is only toxicologically relevant if these components may be released).
• Nanomaterials are assigned to MG1 that have a high dissolution in biological media, 'Activity' recorded for a single relevant grouping criteria results in nanomaterial assignment to MG4. For nanomaterial assignment to MG3, all grouping criteria have to indicate 'passivity'. This is highlighted by the continuous light grey shading.
The data for the case study 'metal oxides and metal sulphates' were retrieved from the following sources: [a] Wohlleben *et al.* (2013); [b] Keller *et al.* (2014); [c] unpublished study report; [d] Singh *et al.* (2011); [e] Izak-Nau and Voetz (2014); [f] Singh *et al.* (2014); [g] OECD (2015a); [h] Gosens *et al.* (2015); [i] Rasmussen *et al.* (2014); [j] Konduru *et al.* (2014); [k] Fraunhofer ITEM, 2015; [l] Wiemann et al. (2015b); [m] Kuhlbusch *et al.* (2009).

3.1.2.4. 15 nm-Fe_2O_3 (hematite)

- Tier 1 criteria indicate non-MG1 and non-MG2. There are no (or only <0.1%) material components or impurities that have been assigned a GHS category.
- In Tier 2, low dissolution in PSF and Gamble's solution confirms non-MG1.
- Further in Tier 2, surface reactivity <10% Mn_2O_3 reactivity, agglomeration, and lack of cellular effects consistently indicate passivity (assignment to MG3).

3.1.2.5. TiO_2 NM-105

- Tier 1 criteria indicate non-MG1 and non-MG2. There are no (or only <0.1%) material components or impurities that have been assigned a GHS category.
- In Tier 2, low dissolution in PSF and PBS confirms non-MG1.
- Further in Tier 2, cellular effects observed in different test methods result in assignment to MG4.

3.1.2.6. ZnO NM-110 and NM-111

- Tier 1 criteria indicate non-MG1 and non-MG2. Zinc oxide has been assigned GHS categories.
- In Tier 2, their high dissolution in different media results in ZnO NM-110 and NM-111 assignment to MG1. This high dissolution also indicates that cytotoxic zinc ions may be released into biological media and fluids.
- Available cytotoxicity data indicate activity of both ZnO NMs. ZnO NM cellular effects are caused by released zinc ions (Xia et al., 2008), which in return confirms MG1 assignment.

3.1.3. Amorphous silica NMs

Table 4 presents the details of the application of the Tiers 1 and 2 of the DF4nanoGrouping to the case study 'amorphous silica NMs'. Two materials (SiO_2.amino and SiO_2.PEG) are assigned to MG3 (passive NMs). Three materials are assigned to MG4 (active NMs): aSiO_2-susp on account of its cellular effects and SiO_2.acrylate and SiO_2.phosphate on account of their high dispersibility indicating the potential for mobility in the body, i.e. systemic availability. Finally, two materials (SiO_2 NM-200 and NM-203) are assigned as 'borderline' MG1 (soluble NMs) or MG4 (active NMs).

3.1.3.1. SiO_2 NM-200 and SiO_2 NM-203

- Tier 1 criteria indicate borderline MG1/non-MG1 (partial solubility in water, but below the DF4nanoGrouping threshold value) and non-MG2. There are no (or only <0.1%) material components or impurities that have been assigned a GHS category.
- In Tier 2, effects in the *in vitro* alveolar macrophage assay indicate activity.
- Partial dissolution in DMEM + FCS, but high dissolution in Gamble's solution results in 'borderline' assignment to MG1 or MG4.

Table 4
Assignment of the case study 'amorphous silica nanomaterials' to one of the four main groups of the DF4nanoGrouping by application of its Tiers 1 and 2.

Tier	Grouping criterion	Threshold value for grouping	SiO_2 NM-200	SiO_2 NM-203	aSiO_2-susp	SiO_2.acrylate	SiO_2.amino	SiO_2.PEG	SiO_2.phosphate
1	**Water solubility**	>100 mg/L = MG1	67 mg/L [a] 115 mg/L [b]	210 mg/L [c] Si: 56 mg/L [d]	Si: 3 mg/L [e]	#CP_ID	#CP_ID	#CP_ID	#CP_ID
	Particle size and shape	Aspect ratio >3:1, length >5 µm, Ø <3 µm = MG2	10-15 nm (spherical) [a,f]	20 nm (spherical, irregular) [c,f]	15 nm [e]	20 nm [g]	15 nm [g,h]	15 nm [g,h]	15 nm [g,h]
	Composition, shape	≥0.1% of toxic component = MG4	amorphous SiO_2 [a,f]	amorphous SiO_2 [c,f]	amorphous SiO_2 [e]	SF: Polyacrylate [g]	SF: LMM silane, pos. charged amino on C_3-linker [g,h]	SF: LMM silane, PEG (500 g/mol) [g,h]	SF: LMM silane, neg. charged phosphonate on C_3-linker [g,h]
	Purity, impurities	≥0.1% of toxic component = MG4	>96.5%; no relevant impurities [a,f]	≥99.8%; no relevant impurities [c,f]	>99% [i]	as for aSiO_2-susp	as for aSiO_2-susp	as for aSiO_2-susp	as for aSiO_2-susp
2	**Dissolution in biological media**	>100 mg/L = MG1	**Soluble in Gamble (24-h) [j] DMEM+FCS (24 h): 39 mg/L [k]**	**Soluble in Gamble (24-h) [j]**	PSF (28-d): 65 mg/L [e]	N/A	N/A	N/A	N/A
	Surface reactivity	≥10% of Mn_2O_3 reactivity = MG4	N/A	0.0059 µUFRAS/m^2*h non-oxidative [d]	0.015 µUFRAS/m^2*h non-oxidative [d]	N/A	0.0112 µUFRAS/m^2*h non-oxidative [d]	non-oxidative in ESR with CPH spin trap [h]	0.00715 µUFRAS/m^2*h non-oxidative [d]
	Dispersibility	AAN <3 or diameter ≤100 nm = MG4	DMEM+FCS: AAN 55 [d]	DMEM+FCS: AAN 23 [d]	DMEM+FCS: AAN 28 [e]	**DMEM+FCS: AAN 1 [f]**	DMEM+FCS: 90 [g,h]	DMEM+FCS: AAN 16 [g,h]	**DMEM+FCS: AAN 2 [g,h]**
	Cellular effects	Effect at ≤10 µg/cm^2 = MG4	Activity [l]	Activity [l]	**Activity [l]**	N/A	Passivity [l]	Passivity [l]	Passivity [l]
Tier 1 / Tier 2 MG assignment			**MG1**	**MG1**	**MG4**	**MG4**	**MG3**	**MG3**	**MG4**

Abbreviations: AAN: Average agglomeration number; CPH: Centrophenoxine; DMEM+FCS: Dulbecco's modified Eagle's Medium supplemented with 10% fetal calf serum; ESR: Electron spin resonance; FRAS: Ferric reducing ability of serum; Gamble: Gamble's solution; LMM: Low molar mass; MG: Main group; N/A: Not available; PEG: Polyethylene glycol; SF: Surface functionalization; TEM: Transmission electron microscopy; wt%: Weight percentage.
For applied test methods, *cf.* Table 1.
#CP_ID: The core particle is the identical batch as the aSiO_2.susp: Solubility cannot reasonably be higher than the solubility of the core particle.
Colour legend:
• Bold print with bold framing indicates assignment to MG1 (soluble nanomaterials); light grey: MG 3 (passive nanomaterials); black: MG4 (active nanomaterials). 'Activity' recorded for a single relevant grouping criteria results in nanomaterial assignment to MG4. For nanomaterial assignment to MG3, all grouping criteria have to indicate 'passivity'. This is highlighted by the continuous light grey shading.
The data for the case study 'silica nanomaterials' were retrieved from the following sources: [a] OECD (2015c); [b] Personal communication from 14 September 2015 from Dr. Jürgen Nolde, Director Product Stewardship CT & MT EMEA, Grace GmbH & Co. KG, Worms, Germany [c] OECD (2015d); [d] unpublished study report; [e] Wohlleben *et al.* (2013); [f] NANOGENOTOX (2012); [g] Landsiedel *et al.* (2014a); [h] Izak-Nau and Voetz (2014); [i] Schaefer *et al.* (2012); [j] Rasmussen *et al.* (2014); [k] Fraunhofer IKTS (2012); [l] Wiemann et al. (2015b).

3.1.3.2. $aSiO_2$-susp

- Tier 1 criteria indicate non-MG1 (very low solubility in water) and non-MG2. There are no (or only <0.1%) material components or impurities that have been assigned a GHS category.
- In Tier 2, low dissolution in PSF confirms non-MG1.
- Further in Tier 2, activity in the *in vitro* alveolar macrophage assay results in assignment to MG4.

Research on the water solubility and dissolution in biological media of amorphous SiO_2 NM is ongoing. At the time of writing the present article, most published data indicate partial solubility of SiO_2 NM-200 and SiO_2 NM-203 in water or DMEM + FCS (below the DF4nanoGrouping threshold value), but high dissolution in Gamble's solution. Therefore, SiO_2 NM-200 and NM-203 were assessed as 'borderline MG1 or MG4'. By contrast, for $aSiO_2$-susp, both water solubility and dissolution in biological media were well below the respective threshold values, and this NM was assigned to MG4 on account of its cellular effects.

Even though $aSiO_2$-susp was assessed in a different medium (PSF) than SiO_2 NM-200 or NM-203 (DMEM + FCS and Gamble's solution), the choice of medium was assessed as not affecting dissolution (and hence MG assignment). As reported by Luoto et al. (1994), for amorphous SiO_2-based man-made vitreous fibres, dissolution is more strongly affected by the materials' chemical composition (and impurities) than by the choice of medium.

In light of the ongoing research in respect to the solubility of amorphous SiO_2 (without surface functionalization), MG1 and/or MG4 assignment of these NMs should not be considered irrevocable. These materials may even be borderline cases, possibly indicating that the DF4nanoGrouping threshold values of 100 mg/L for water solubility and dissolution in biological media will eventually require reconsideration for NMs that dissolve into non-toxic components. Furthermore, test methods to assess NM dissolution have not yet been standardized to the same extent as dissolution methods and benchmark materials for man-made vitreous fibres (WHO, 1988). This lack of test method standardization for NMs seriously restricts inter-laboratory comparability of test results. For instance, NM dissolution in Gamble's solution was recorded as the absolute ionic concentration in mg/L in a static medium after different incubation times ranging from 24 h to 28 days (*cf.* Tables 3 and 4, specifically the corresponding values for the MG1 and borderline MG1 substances 10 nm-CuO, ZnO NM-110 and NM-111 and SiO_2 NM-200 and NM-203). In some studies, the percentage of ions per total solids was reported. While no cut-off value has been proposed for this metric, it may provide a basis to harmonize the assessment of NM dissolution with the one for vitreous fibres. Notwithstanding, if amorphous SiO_2 NMs without surface functionalization are not assigned to MG1 (soluble NMs), they are assigned to MG4 (active NMs) on account of consistent recordings of cellular effects in protein-free medium (*cf.* Section 4.1).

3.1.3.3. SiO_2.amino and SiO_2.PEG

- Tier 1 criteria indicate non-MG1 and non-MG2. There are no (or only <0.1%) material components or impurities that have been assigned a GHS category.
- In Tier 2, non-MG1 cannot be refuted since data on dissolution in biological media are unavailable. However, it is not expected that the surface coating would increase the solubility of $aSiO_2$-susp which is non-MG1.
- Further in Tier 2, low surface reactivity, agglomeration and lack of cellular effects consistently indicate likelihood of passivity (assignment to MG3).

Table 5
Assignment of the case study 'organic pigments' to one of the four main groups of the DF4nanoGrouping by application of its Tiers 1 and 2.

Tier	Grouping criterion	Threshold value for grouping	DPP orange (bulk)	DPP orange (nano)	Pigment red 254-2	Pigment blue 15:1
1	Water solubility	>100 mg/L = MG1	<<1 mg/L	<<1 mg/L	<<1 mg/L	<1 mg/L
	Particle size and shape	Aspect ratio >3:1, length >5 µm, Ø <3 µm = MG2	0.3-3 µm x 70-200 nm	30-400 nm x 10-50 nm	43 nm	17 nm
	Composition, shape	≥0.1% of toxic component = MG4	Pyrrolo[3,4-c]pyrrole-1,4-dione, 3,6-bis(3-chlorophenyl)-2,5-dihydro-	Pyrrolo[3,4-c]pyrrole-1,4-dione, 3,6-bis(3-chlorophenyl)-2,5-dihydro-	Bis-(p-chrolopheny)-1.4-diketopyrrolo(3.4-c)pyrrole	Cu-Phthalocyanin; XRD: Cu in alpha crystalline phase
	impurities	≥0.1% of toxic component = MG4	No impurities	No impurities	No impurities	≥99%; 0.1% MgO; heavy metals below detection limit
2	Dissolution in biological media	>100 mg/L = MG1	PSF & Gamble (28-d): <<1 mg/L; in Gamble increased agglomeration; in PSF significant reduction of particle sizes	PSF & Gamble (28-d): <<1 mg/L; but slight reduction of particle sizes	PSF & PBS (28-d): 0.1 mg/L; no significant structural changes	PSF & PBS (28-d): <1 mg/L; in PSF rearrangement to larger globules at loss of nanoscale structures
	Surface reactivity	≥10% of Mn_2O_3 reactivity = MG4	0.0196 µUFRAS/m^2*h intermediate	0.0256 µUFRAS/m^2*h intermediate	0.0006 µUFRAS/m^2*h non-oxidative	N/A
	Dispersibility	AAN <3 or diameter ≤100 nm = MG4	DMEM+FCS: >>1 µm	DMEM+FCS: AAN 103	DMEM+FCS: AAN 18	DMEM+FCS: AAN 19 PSF: 498 µm
	Cellular effects	Effect at ≤10 µg/cm^2 = MG4	Passivity	Passivity	Passivity	Activity
Tier 1 / Tier 2 MG assignment			MG3	MG3	MG3	MG4

Abbreviations: AAN: Average agglomeration number; DMEM+FCS: Dulbecco's modified Eagle's Medium supplemented with 10% fetal calf serum; FRAS: Ferric reducing ability of serum; Gamble: Gamble's solution; MG: Main group; N/A: Not available; TEM: Transmission electron microscopy; wt%: Weight percentage.
For applied test methods, *cf.* Table 1.
Colour legend:
• Light grey shading indicates nanomaterial assignment to MG 3 (passive nanomaterials); black: MG4 (active nanomaterials).
• 'Activity' recorded for a single relevant grouping criteria results in nanomaterial assignment to MG4. For nanomaterial assignment to MG3, all grouping criteria have to indicate 'passivity'. This is highlighted by the continuous light grey shading.
All data for the case study 'organic pigments' were retrieved from unpublished study reports. For the cellular effect data recorded for DPP orange (bulk or nano) and Pigment blue 15:1: Wiemann et al. (2015b).

3.1.3.4. SiO_2.acrylate and SiO_2.phosphate

- Tier 1 criteria indicate non-MG1 and non-MG2. There are no (or only <0.1%) material components or impurities that have been assigned a GHS category.
- In Tier 2, non-MG1 cannot be refuted since data on dissolution in biological media are unavailable. However, it is not expected that the surface coating would increase the solubility of aSiO_2-susp which is non-MG1.
- Further in Tier 2, dispersibility in DMEM + FCS indicating potential for mobility in the body, results in assignment to MG4.

3.1.4. Organic pigments

Table 5 presents the details of the application of the Tiers 1 and 2 of the DF4nanoGrouping to the case study 'organic pigments'. DPP orange (bulk and nano) as well as Pigment red 254-2 are assigned to MG3 (passive NMs). Pigment blue 15:1 is assigned to MG4 (active NMs) on account of cellular effects.

3.1.4.1. DPP orange 1 (bulk), DPP orange 2 (nano), and pigment red 254-2

- Tier 1 criteria indicate non-MG1 and non-MG2. There are no (or only <0.1%) material components or impurities that have been assigned a GHS category.
- In Tier 2, low dissolution in PSF, PBS or Gamble's solution confirms non-MG1.
- Further in Tier 2, low surface reactivity, agglomeration and lack of cellular effects consistently indicate passivity (assignment to MG3).

3.1.4.2. Pigment blue 15:1

- Tier 1 criteria indicate non-MG1 and non-MG2. Pigment blue 15:1 contains ≥0.1% copper (that is tightly bound into the molecule).
- In Tier 2, low dissolution in PSF and PBS confirms non-MG1. Further, these data indicate that copper ions are not released into biological media. Hence, this is not an indication for MG4 (as would have been concluded from potential for ion release).
- Further in Tier 2, activity in the *in vitro* alveolar macrophage assay results in assignment to MG4.

3.1.5. Additional materials

The Tier 1 and Tier 2 data recorded for the additional materials quartz dust DQ12 and C_{60} fullerene are presented in Table 6.

3.1.5.1. Non-nanosized crystalline quartz dust DQ12

- Tier 1 criteria indicate non-MG1 and non-MG2. There are no (or only <0.1%) material components or impurities that have been assigned a GHS category.
- In Tier 2, low dissolution in PSF confirms non-MG1.
- Further in Tier 2, high surface reactivity and observation of cellular effects result in assignment to MG4.

3.1.5.2. C_{60} fullerene

- Data on Tier 1 criteria obtained in the literature for different preparations of C_{60} fullerene consistently indicate non-MG1 and non-MG2. There are no (or only <0.1%) material components or impurities that have been assigned a GHS category.

Table 6
Assignment of the additional materials to one of the four main groups of the DF4nanoGrouping by application of its Tiers 1 and 2.

Tier	Grouping criterion	Threshold value for grouping	Quartz dust DQ12)	C_{60} fullerene
1	Water solubility	>100 mg/L = MG1	Si: 41 mg/L [a]	0.4 mg/L [b]
	Particle size and shape	Aspect ratio >3:1, length >5 µm, Ø <3 µm = MG2	3000 nm; platelets [a]	Particle size: Fine fraction 6±3 µm; produced under argon, suspended in 'culture medium' [c] PPS: 0.7 nm, sonicated in water [d] Mean particle size in distilled water supplemented with 0.1% Tween 80: 33-59 nm [e] C_{60} fullerene aerosol: count median diameter 55 nm (aerosolized without the use of water or solvents) – no primary particle characterization [f] 30-100 nm aggregates in water [g] 10.5 nm – 13 µm (peaks at 235 and 857 nm) suspended in saline containing 0.05% Tween 80 [h]
	Composition, shape	≥0.1% of toxic component = MG4	crystalline SiO_2 [i]	C_{60} fullerene
	impurities	≥0.1% of toxic component = MG4	87% crystalline SiO_2; rest: amorphous SiO_2, 0.2% Al_2O_3; 0.03% Fe_2O_3 [i]	99.5% pure [b]
2	Dissolution in biological media	>100 mg/L = MG1	PSF (pH 4.3; 28-d): 38 mg/L; [in Gamble (pH 7.5; 28-d): 100 mg/L]; recrystallization towards smaller structural sizes [a]	Insoluble in water and other solvents [b]
	Surface reactivity	≥10% of Mn_2O_3 reactivity = MG4	**0.316 µUFRAS/m²*h = active [j]**	No data found in the published literature
	Dispersibility	AAN <3 or diameter ≤100 nm = MG4	Above cut-off value due to large primary size	Peaks at 150 and 700 nm in MiliQ water, and 311 nm in DMEM-F12 [d]
	Cellular effects	Effect at ≤10 µg/cm² = MG4	**Activity [k]**	Different macrophages, no cellular effects [c] Mouse lung epithelial cells, 3-h exposure: Slightly >ROS at 100 µg/mL [d]
Tier 1 / Tier 2 MG assignment			**MG4**	**Non-MG1, non-MG2 MG3 / MG4? - no surface reactivity data**

Colour legend: Black shading indicates nanomaterial assignment to MG4 (active nanomaterials).
Data for quartz dust DQ12 and C_{60} fullerene were retrieved from the following sources: [a] Wohlleben *et al.* (2013); [b] Marcus *et al.* (2001); [c] Baierl *et al.* (1996); [d] Jacobsen *et al.*, (2008); [e] Morimoto *et al.* (2010); [f] Baker *et al.* (2008); [g] Oberdörster (2004); [h] Totsuka *et al.* (2009); [i] supplier's product information (Dörentrup Quarz GmbH & Co. KG, Germany, www.doerentrup.de); [j] unpublished study reports; [k] Wiemann et al. (2015b).

- In Tier 2, available information on the overall low dissolution of C_{60} fullerene in different solvents confirms non-MG1.

- Further in Tier 2, the available information points to C_{60} fullerene agglomeration, and none of the retrieved *in vitro* studies reports pronounced cellular effects. However, since data on C_{60} fullerene surface reactivity were not found in the published literature, a decisive Tier 2 distinction between MG3 or MG4 was not possible.

3.2. Evaluation of Tier 1 and Tier 2 nanomaterial MG assignment using Tier 3 short-term toxicity data

Table 7 provides an overview of the evaluation of the DF4nanoGrouping Tier 1 and Tier 2 nanomaterial MG assignment using Tier 3 short-term toxicity data. Further, Figs. 1–4 illustrate application of the DF4nanoGrouping for carbonaceous NMs, soluble ZnO NMs, amorphous SiO_2 NMs, and organic pigments, respectively.

3.2.1. Case study materials assigned to main group 1 (soluble NMs)

For ZnO NM-110 and NM-111, rapid elimination during the 2–3-week post-exposure period was reported in the corresponding STISs (Bellmann, 2011; Landsiedel et al., 2014a). These findings reflect high solubility also in the lung, thereby confirming MG1 assignment of the ZnO NMs.

For all three metal oxide NMs that were assigned to MG1, low STIS NOAEC values (Range II) were recorded. For ZnO NM-111, a NOAEC of 0.5 mg/m^3 was assigned (Landsiedel et al., 2014a) and for 10 nm-CuO a NOAEC of 0.6 mg/m^3 (Gosens et al., 2015). In a rat 14-day inhalation study, ZnO NM-110 and NM-111 and a micron-scale ZnO showed comparable effects: The NOAEC values were <8 mg/m^3 for ZnO NM-110 and the micron-scale ZnO (only one concentration tested) and 2 mg/m^3 (LOAEC 8 mg/m^3) for ZnO NM-111 (Bellmann, 2011). Concordantly, a NOAEC of 2.0 mg/m^3 was recorded for ZnO NM-111 in the 5-day range finding study to the 14-day inhalation

Table 7
Evaluation of Tier 1/Tier 2 nanomaterial MG assignment using Tier 3 STIS biopersistence or toxic potency (NOAEC).

Tier 1 and Tier 2 MG assignment	Test material	Tier 3 STIS NOAEC (mg/m^3)	Tier 3 STIS biopersistence	Tier 3 MG assignment	Progression of effects?	MG4 sub-grouping by NOAEC range	Reference *in vivo* data
MG1 'soluble NMs'	ZnO NM-110	<8 [1]	Rapid clearance	Solubility confirmed	Full reversibility	[Different NOAEC ranges; grouping by 'solubility' does not *per se* relate to toxic potency]	a
	ZnO NM-111	0.5	Rapid clearance		Partial reversibility		b
	10 nm-CuO	0.6	N/A	N/A	Full reversibility		c
	SiO_2 NM-200	1 [2]	1 and 5 mg/^{3}: always < detection limit; 25 mg/m^3: Full clearance	Solubility supported	Partial reversibility		d
	SiO_2 NM-203	1 [2]			Partial reversibility		d
MG2 'biopersistent HAR NMs'	MWCNT NM-400	<0.5	d.n.p.	Biopersistence	Progression	[Range I or II]	e
	MWCNT NM-402	<0.5	d.n.p.				f
MG 3 'passive NMs'	LS carbon black	≥10	d.n.p.	MG3 'passive NMs'	No adverse effects	[Range IV = MG3]	f
	$BaSO_4$ NM-220	≥50	t_{50} <40 days				b [3]
	15 nm-Fe_2O_3	≥30	t_{50} <40 days				g
	SiO_2.amino	≥50	t_{50} <40 days				b
	SiO_2.PEG	≥50	t_{50} <40 days				b
	DPP orange (bulk)	≥10	Not decisive for grouping				g
	DPP orange (nano)	≥30					g
	Pigment red 254-2 (nano)	≥30					g
MG 4 'active NMs'	SiO_2.phosphate	≥50	t_{50} <40 days		No adverse effects		b
	Pigment blue 15:1	≥30	N/A				g
	Graphite nanoplatelets	≥10	d.n.p.				f
	aSiO_2-susp	2.5	N/A	MG4 'active NMs'	Progression	Range III	b
	Graphene	<2.5	d.n.p.		Persistence of effects		f
	TiO_2 NM-105	<2	t_{50} >40 days		Partial reversibility		h
	SiO_2.acrylate	0.5 (splenic effects; pulmonary effects: ≥10)	t_{50} >40 days		Full reversibility of splenic effects; no pulmonary effects at any time point	Range II	b
	CeO_2 NM-211	<0.5	t_{50} >40 days		Progression		i
	CeO_2 NM-212	<0.5	t_{50} >40 days		Progression		i
	Quartz dust DQ12	0.1 [4]	t_{50} >40 days		Progression	Range I or II	j [4]

Abbreviations: d.n.p.: Determination not possible for technical reasons (the (non-radio-labelled) main component C is indistinguishable from the biological environment), N/A: Not available.
The STIS NOAEC ranges correspond to: Range I: <0.1 mg/m^3; Range II: <1 mg/m^3; Range III: <10 mg/m^3; Range IV: ≥10 mg/m^3.
Colour legend: Grey shading: In Tier 2, SiO_2 phosphate was assigned to MG 4 on account of its high dispersibility, Pigment blue 15:1 was assigned to MG4 on account of its activity in the in vitro alveolar macrophage assay, and graphite nanoplatelets were assigned to MG4 since determination of surface reactivity was not possible for technical reasons. In Tier 3, high STIS NOAEC (Range IV) are recorded for all 3 substances indicating MG3 'passivity'. Further, the pulmonary half-life of SiO_2. phosphate is <40 days.
STIS data were retrieved from the following sources: [a] Bellmann (2011); [b] Landsiedel et al. (2014a); [c] Gosens et al. (2015); [d] Arts et al. (2007); [e] Ma-Hock et al. (2009b); [f] Ma-Hock et al. (2013); [g] Unpublished study report; [h] Ma-Hock et al. (2009a); [i] Keller et al. (2014); [j] Henderson et al. (1995).
[a] 14-day exposure, only 1 test substance concentration (i.e. 8 mg/m^3).
[b] For equivalent substance.
[c] Furthermore, there are strong indications that $BaSO_4$ is at least partially soluble *in vivo* after inhalation (Konduru et al., 2014).
[d] The findings from Henderson et al. (1995) were recorded for alpha-quartz (median particle size 1.7 μm) in a 28-day subacute inhalation study. Additionally, quartz dust DQ12 was used as positive control in two rat STIS, in which it was only tested at one (high) concentration, each, i.e. at 25 mg/m^3 at which concentration it produced progressively severe effects over the 3-month post-exposure period (Arts et al., 2007) and at 100 mg/m^3, where additionally biopersistence (t_{50} > 40 days) was reported (Van Ravenzwaay et al., 2009).

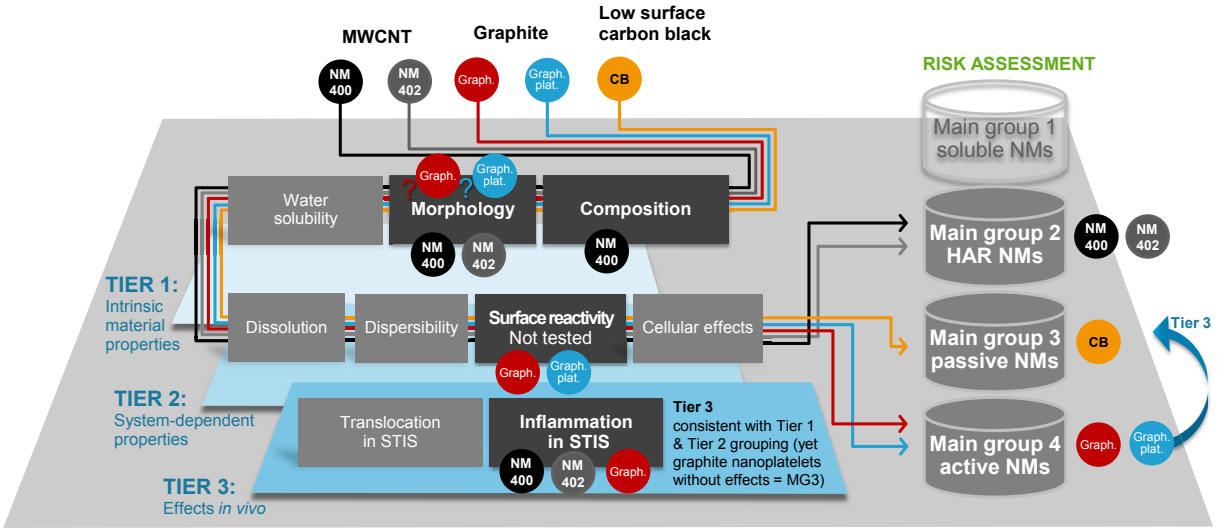

Fig. 1. Application of the DF4nanoGrouping to the case study 'carbonaceous nanomaterials'.

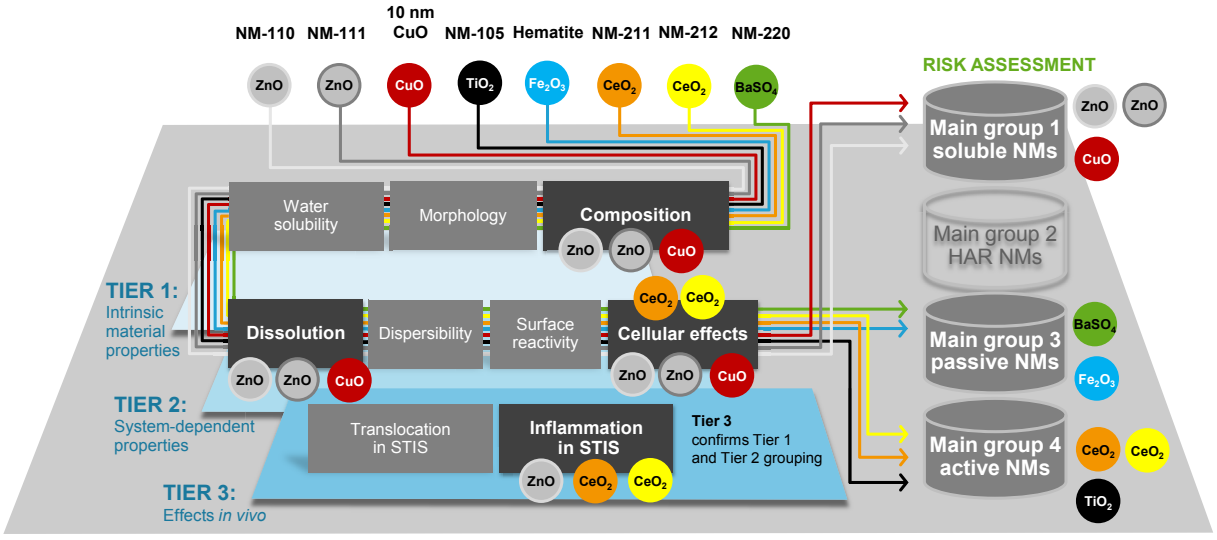

Fig. 2. Application of the DF4nanoGrouping to the case study 'metal oxides and metal sulphates'.

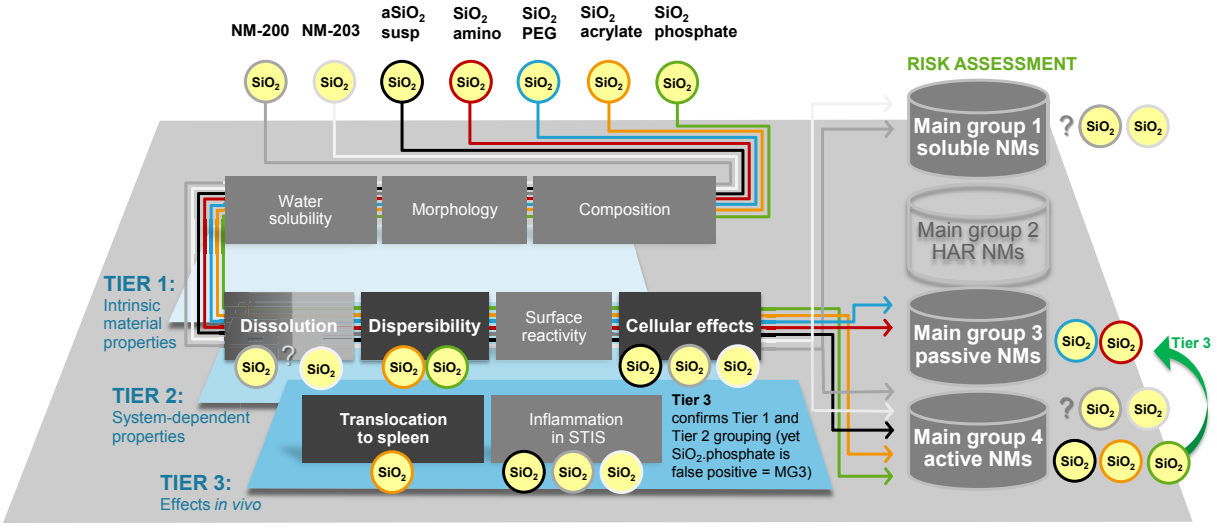

Fig. 3. Application of the DF4nanoGrouping to the case study 'amorphous silica nanomaterials'.

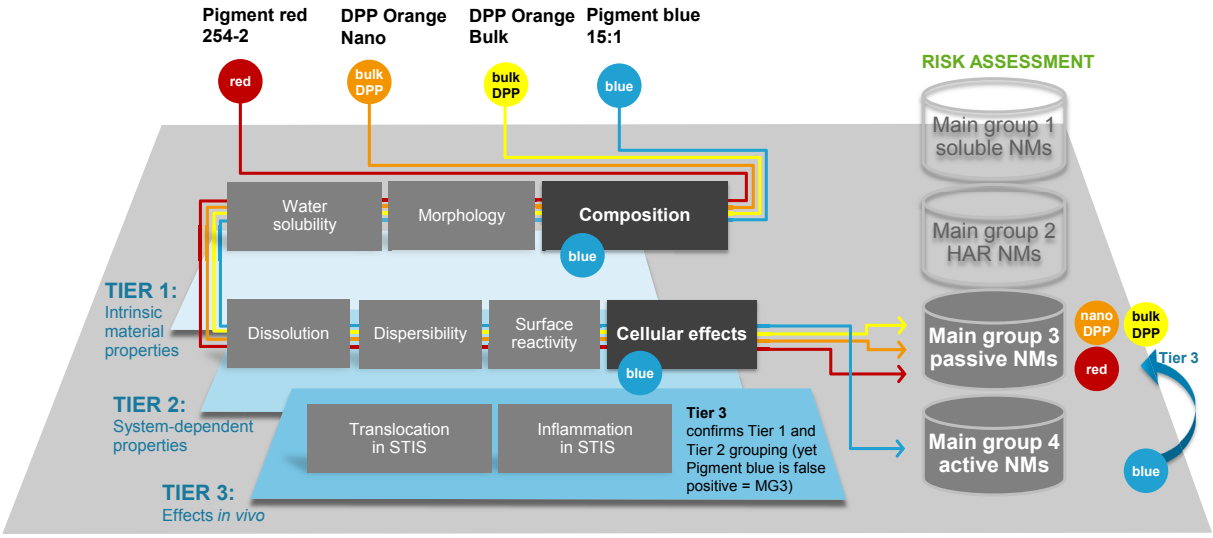

Fig. 4. Application of the DF4nanoGrouping to the case study 'organic pigments'.

study (Creutzenberg, 2009). These low STIS NOAECs are consistent with the dissolution of hazardous ions.

3.2.2. Case study materials assigned borderline main group 1 (soluble NMs) or main group 4 (active NMs)

For SiO_2 NM-200 and NM-203 (respectively, the equivalent materials that were tested in the STIS), full clearance within the 1-month post-exposure period was recorded for the high concentration test group (25 mg/m^3). For the lower concentration test groups (1 and 5 mg/m^3), pulmonary Si-content was below the detection limit at all time points (i.e. on the day after the final exposure and 1 and 3 months thereafter; Arts et al., 2007).

Further, STIS NOAECs of 1 mg/m^3 (Range III) were recorded for the two equivalent materials to SiO_2 NM-200 and NM-203. The differing NOAEC ranges (as compared to the MG1 metal oxide NMs) underline that NM assignment as soluble (or partially soluble) is not 'effect-based'. The intrinsic and system-dependent properties water solubility and dissolution in biological media are not directly related to *in vivo* toxic potential or potency. Depending on their chemical composition, the dissolved materials may be toxic, or not, at the given lung burden and dissolution. For SiO_2 NM-200 and NM-203, the rapid elimination following 5-day inhalation exposure reflects MG1, while the pulmonary effects that are not caused by released toxic ions indicates MG4. Hence, the STIS data confirm the assignment of these two materials as 'borderline MG1 or MG4'.

3.2.3. Case study materials assigned to main group 2 (biopersistent HAR NMs)

Also for NM assignment to MG2, the essential grouping criterion HAR does not refer to a biological effect, but to a material property that is critical to develop and exhibit a biological effect. Tier 3 organ burden or pulmonary half-life of the MWCNTs could not be assessed in order to confirm low solubility in water or biological media, since the (non-radio-labelled) main component of the material, carbon, can hardly be quantified in lung tissue. However, the outcomes of the STISs indicate a progression of effects for both MWCNT NM-400 and NM-402. This supports their assignment as *biopersistent* HAR NMs. Additionally, for both MWCNT NM-400 and NM-402, Tier 3 STIS NOAEC values of <0.5 mg/m^3 (NOAEC Ranges I or II) indicate toxic potency of these materials which is consistent with fibre toxicity. The available STISs were, however, not designed to identify a mode-of-action according to the fibre paradigm. In fact, some of the NMs assigned to MG2 in Tier 1 and 2 may turn out to act more like granular particles due to the agglomeration and tangling of the fibres (Poland et al., 2008).

3.2.4. Case study materials assigned to main group 3 (passive NMs)

For all 8 NMs assigned to MG3 in Tiers 1 and 2 of the DF4nanoGrouping, STIS NOAEC ≥10 mg/m^3 were recorded in Tier 3 confirming assignment of these materials as passive NMs. Accordingly, NMs assigned to MG3 are expected to induce adverse effects in the lung only if aerosol concentrations, lung deposition and impaired clearance result in lung overload conditions (ECETOC, 2013).

3.2.5. Case study materials assigned to main group 4 (active NMs)

For 7 of 10 materials assigned to MG4 in Tiers 1 and 2 of the DF4nanoGrouping (i.e. aSiO_2-susp, graphene, TiO_2 NM-105, SiO_2.acrylate, CeO_2 NM-211 and NM-212 and quartz dust DQ12), STIS NOAEC <10 mg/m^3 were recorded in Tier 3 confirming assignment of these materials as MG4 active NMs. For graphite nanoplatelets, SiO_2.acrylate and Pigment blue 15:1, high STIS NOAEC values (≥10 mg/m^3) indicated MG3 passivity, thereby refuting the Tier 2 assignment as MG4 active NMs.

Graphite nanoplatelets were assigned to MG4 in Tier 2, since data on surface reactivity were unavailable for technical reasons, and hence, the respective activity of this material could not be excluded. In Tier 3, however, the STIS NOAEC (≥10 mg/m^3) indicates MG3 passivity of graphite nanoplatelets.

For SiO_2.acrylate, the NOAEC for lung effects was ≥10 mg/m^3. At aerosol concentrations of 10 mg/m^3, however, the material was detected in the spleen (albeit below 1% of the total mass in the body) where it caused organ changes. This indicates extra-pulmonary translocation (inside the MPS) of the inhaled NM which qualifies SiO_2.acrylate as an active NM. Indeed, this NM had been assigned to MG4 in Tier 2 on account of its high dispersibility, which is indicative of mobility in the body. For SiO_2.phosphate that had been assigned to MG4 in Tier 2 on account of its high dispersibility, a high STIS NOAEC (>50 mg/m^3) exceeding the threshold value for MG4 'active nanomaterials' was recorded, and no extra-pulmonary translocation or systemic alterations were observed (either clinically or during histopathological evaluation). This lack of findings and mobility in the STIS provides evidence that SiO_2.phosphate is unlikely to be an active NM. Hence, Tiers 1 and 2 over-predicted the outcome of the *in vivo* study.

Pigment blue was assigned to MG4 in Tier 2 on account if its activity in the *in vitro* alveolar macrophage assay. In Tier 3 its STIS

NOAEC of ≥30 mg/m^3 (Range IV) indicated MG3 'passivity'. Hence, again, Tiers 1 and 2 over-predicted the outcome of the *in vivo* study.

The NMs assigned to MG4 in Tier 3 encompass a broad spectrum of different materials, and their NOAEC values cover all three STIS NOAEC ranges allocated to MG4 (Range I: <0.1 mg/m^3; Range II: <1 mg/m^3; Range III: <10 mg/m^3; *cf.* Section 2.5 and Table 7). Hence hazard and risk assessment for different MG4 NMs will not be alike and may require further considerations to specify the toxic potential and potency. In this respect, sub-grouping of the MG4 NMs by STIS NOAEC range, reversibility/progression of effects and bioavailability may provide important indications to determine (and specify, if applicable) the need for additional information.

Sub-grouping of the MG4 NMs by STIS NOAEC range results in the following assignments:

- Range I or II: Non-nanosized quartz dust DQ12
- Range II: Both CeO_2 NMs and SiO_2.acrylate (the latter on account of translocation and splenic alterations; respiratory tract effects were not recorded at any point of time)
- Range III: aSiO_2-susp, graphene, graphite nanoplatelets and TiO_2 NM-105

Evaluation of the reversibility or progression of effects elicited by the MG4 NMs results in the following sub-groups:

- Full reversibility of splenic alterations: SiO_2.acrylate
- Partial reversibility of pulmonary effects: TiO_2 NM-105
- Persistence of pulmonary effects: Graphene and graphite nanoplatelets
- Progression of pulmonary effects: Quartz dust DQ12 (within the STIS NOAEC Ranges I or II); both CeO_2 NMs (within Range II); and aSiO_2-susp (within Range III)

Additionally, NMs may be sub-grouped by their pattern of biodistribution. Arts et al. (2015) foresee distinguishing between NMs that only become available in the primary organ (i.e. the respiratory tract for the inhalation route of exposure), NMs that are additionally found in the MPS, and, finally, NMs that become systemically available outside the MPS (at >1 mass% of the total dose, each).

- SiO_2.acrylate is assigned to the sub-group of NMs that may become available in the MPS.
- None of the case study materials is assigned to the sub-group of test materials with systemic availability outside the MPS.

Also in Tier 3, C_{60} fullerene could not be assigned to either MG3 or MG4 with certainty. However, the available data point to the low toxic potential of this material upon short-term exposure and no translocation to extra-pulmonary organs, which indicates MG3 passivity. This estimation is confirmed in an extensive literature review that was conceived to reflect *a "classical regulatory risk assessment"* (Aschberger et al., 2010). Also Aschberger et al. caution that their estimation should not be considered definite due to limitations in the dataset. In a 28-day rat inhalation study, 0.12 mg/m^3 C_{60} fullerene (*cf.* Table 6 [e] for material properties) did not elicit adverse effects either during treatment or throughout the 3-month post-exposure period (Morimoto et al. (2010). In a further rat short-term inhalation study, 2 mg/m^3 C_{60} fullerene (*cf.* Table 6 [f] for material properties) only affected a few parameters in the blood and bronchoalveolar fluid (Baker et al., 2008). However, in this study the rats were only exposed to the test material for 3 h per day (over a 10-day period), and Baker et al. (2008) assessed the outcome of their study as inconclusive. Of note, neither study (i.e. Baker et al., 2008; Morimoto et al., 2010) was performed as concentration-response study. In juvenile fish (largemouth bass), olfactory translocation of C_{60} fullerene (0.5 ppm; *cf.* Table 6 [g] for material properties) was observed after 48-h exposure resulting in oxidative stress reactions in the brain (Oberdörster, 2004). Clearly, it is not possible to relate these fish data to mammal NOAEC extra-pulmonary translocation upon inhalation exposure. Also Oberdörster et al. (2009) and Aschberger et al. (2010) caution that the human health implications of central nervous system findings for NMs, as such, remain to be determined.

3.3. Overall evaluation of the DF4nanoGrouping using long-term NOAEC

As Table 8 reveals, the two NMs assigned to MG3 for which data from long-term studies were available (i.e. low surface carbon black and $BaSO_4$ NM-220) had high 90-day NOAECs (50 mg/m^3, each), whereas all other NMs (assigned to MG1, MG2, or MG4) had considerably lower long-term NOAEC values ranging from 0.3 to 1.5 mg/m^3. Hence, even though long-term data were only available for 10 of the 25 materials (including quartz dust DQ12), these findings confirm the passivity of NMs that are assigned to MG3.

In regard to C_{60} fullerene, Shinohara et al. (2011) calculated the 90-day NOAEC to be 3.1 mg/m^3 basing this figure on a combined evaluation of published rat instillation and 28-day inhalation studies that assessed different forms of C_{60} fullerene. Since this long-term NOAEC was not derived from a 90-day inhalation study

Table 8
Evaluation of DF4nanoGrouping main group assignment using long-term NOAEC (as available).

DF4nanoGrouping MG assignment	Test substance	Published 90-day NOAEC (mg/m^3)	References
MG1 'soluble NMs'	ZnO NM-111	1.5	a
	SiO_2 NM-200	1	b
	SiO_2 NM-203	1 (for equivalent substance)	c
MG2 'biopersistent HAR NMs'	MWCNT NM-400	<0.1	d
	MWCNT NM-402	0.25	e [a]
MG 3 'passive NMs'	Low surface carbon black	50 (for equivalent substance)	f
	$BaSO_4$ NM-220	50	g
MG 4 'active NMs'	CeO_2 NM-212	0.3	g
	TiO_2 NM-105	0.5	h
	Quartz dust DQ12	2-yr: 1 (only one dose group)	i

Long-term *in vivo* data were retrieved from the following references: [a] Creutzenberg (2011); [b] Creutzenberg et al. (2014); [c] Reuzel et al. (1991); [d] Ma-Hock et al. (2009b); [e] Pothmann et al. (2015); [f] Elder et al. (2005); further discussed in UBA (2014); [g] Keller (2015); [h] Bermudez et al. (2004); further discussed in UBA (2014) and Sellers et al. (2015); [i] Muhle et al. (1995); further discussed in SCGOS (2014).

[a] Concordantly, for a variety of different MWCNTs, very low long-term NOAECs of 0.1 mg/m^3 or lower were recorded by Pauluhn (2010); DeLorme et al. (2012, 2015); Schuler et al. (2013).

Table 9
Supplementary grouping criteria for the case study 'carbonaceous nanomaterials'.

Supplementary criterion	MWCNT NM-400	MWCNT NM-402	Graphene	Graphite nanoplatelets	Low surface carbon black
Dustiness (mg/kg)	No respirable dust [a]	N/A	N/A	N/A	N/A
Specific surface area (m^2/g)	230 [a,b]	161 [c]	131 [c]	74 [c]	32 [c]
Surface chemistry	C: 99; O: 1; Al <1; Si <<1 [b]	C: 99; O: 1 [c]	C: 84.1; O: 8.8; S: 5.4; Na: 0.6; Si: 0.4; Cl: 0.6 [c]	C: 84.3; O: 9.0; S: 1.7; Na: 3.0; Ca: 1.5; Si: 0.6 [c]	C: 98; O: 1; S: 1; Cl: <<1 [c]
Surface charge	Determination not possible: Poor dispersibility				
Hydrophobicity (water contact angle)	Hydrophobic [d]	Hydrophobic [d]	Hydrophobic [d]	Hydrophobic [d]	Hydrophobic [d]

Abbreviations: N/A: Not available.
For applied test methods, *cf.* Table 1.
The data for the case study 'carbonaceous nanomaterials' were retrieved from the following sources: [a] Ma-Hock et al. (2009b); [b] Wohlleben et al. (2013); [c] Ma-Hock et al. (2013); [d] unpublished study report.

and Shinohara et al. further did not provide any estimations for a LOAEC (so that the true NOAEC may well be much higher than 3.1 mg/m^3), this study was not used to reassess the tentative assignment of C_{60} fullerene to MG3 (passive NMs) in Tier 3 of the DF4nanoGrouping.

3.4. Evaluation of in vitro and in vivo genotoxicity, the qualifier dustiness and the supplementary criteria

The Supplementary Information Table SI-1 presents the *in vitro* and *in vivo* genotoxicity data collected for the case study materials. Of note, only data from *in vivo* genotoxicity tests that were part of inhalation or instillation studies were used. Concordantly, *in vitro* genotoxicity studies were included in the evaluation if pulmonary cells or tissues had been used as test systems.

***In vitro* genotoxicity** data were available for all materials except for graphene, graphite nanoplatelets, and SiO_2 NM-203. For CuO, *in vitro* genotoxicity data were available for NMs with larger PPS than 10 nm-CuO, i.e. 42 nm- and 55 nm-CuO. Predominantly, the *in vitro* genotoxicity data had been obtained in the alkaline Comet assay or micronucleus test using standard cell lines (mostly A549 cells) or a three-dimensional reconstruct of the human airway epithelium (EpiAirway™). *In vitro* genotoxic effects that encompass a broad variety of different DNA damaging and mutagenic effects were recorded for case study materials from all four MGs, i.e.

MG1: ZnO NM-110 and NM-111 and 42 nm- and 55 nm-CuO.

- MG2: different types of MWCNTs
- MG3: SiO_2.phosphate (and C_{60} fullerene)
- MG4: Both CeO_2 NMs and $aSiO_2$-susp

Only for the MG1 materials ZnO NM-110 and NM-111 and 55 nm-CuO as well as for the MG2 material MWCNT NM-400, were these *in vitro* genotoxic effects recorded at $\leq$10 $\mu g/cm^2$, i.e. at test material concentrations lying within the DF4nanoGrouping range for relevant *in vitro* effects that do not correspond to *in vivo* overload conditions. By contrast, none of the MG3 or MG4 materials were reported to elicit *in vitro* genotoxic effects at $\leq$10 $\mu g/cm^2$. It remains to be determined whether the general threshold value of $\leq$10 $\mu g/cm^2$ set by Arts et al. (2015) for *in vitro* effects is specifically relevant for *in vitro* genotoxicity.

***In vivo* genotoxicity** data were available for ZnO NM-110 and NM-111, SiO_2 NM-200 and NM-203, MWCNT NM-400, $BaSO_4$ NM-220, both CeO_2 NMs, TiO_2 NM-105, $aSiO_2$-susp, and SiO_2.amino. Mostly, the *in vivo* tests were part of intratracheal instillation or short-term inhalation studies using rats and the data were obtained in alkaline Comet assays using lung cells or in micronucleus tests using bone marrow cells. The exceptions are MWCNT NM-400, for which data from rat and mouse intratracheal instillation studies were available, and ZnO NM-111, for which additionally genotoxicity data were obtained in the course of a mouse intraperitoneal

Table 10
Supplementary grouping criteria for the case study 'metal oxides and metal sulphates'.

Supplementary criterion	$BaSO_4$ NM-220	CeO_2 NM-211	CeO_2 NM-212	10 nm-CuO	15 nm-Fe_2O_3 (hematite)	TiO_2 NM-105	ZnO NM-110	ZnO NM-111
Dustiness (mg/kg)	Inhalable: 450 Respirable: 80 [a]	Inhalable: 2222 Respirable: 86 [b]	Inhalable: 2845 Respirable: 66 [b]	N/A	N/A	Inhalable: 1020 Respirable: 28 [c]	Inhalable: 2905 Respirable: 27 [d]	Inhalable: 5880 Respirable: 138 [d]
Specific surface area (m^2/g)	41 [e]	66 [e]	27 [e]	47 [f]	85 [g]	51 [h]	12 [i]	15 [h]
Surface chemistry	O: 52; Ba: 13; C: 17; S: 11; Cl: 3; P: 3; N: 1 [h]	Ce: 28.7; O: 57.2; C: 14.1 [e]	C: 79.9; O: 17.7; Ce: 2.4 [e]	Cu: 46; O: 47, C: 7 [g]	O: 54.2; Fe: 27.2; C: 15.7; P: 1.6; Ca: 0.9; S: 0.2; F: 0.1; Mg: 0.1 [g]	Ti: 16; O: 63; C: 9; Al: 7; Si: 5; Na: <1 [h]	O: 38; Zn: 35; C: 30; Cl: 3; Na: 3 [i]	O: 38; Zn: 35; C: 20; Cl: 3; Na: 3 [h]
Surface charge (mV)	-39 [h]	16 [e]	42 [e]	28 [f]	-27 [g]	-17 [h]	20 [i]	N/A
Hydrophobicity (water contact angle)	Hydrophilic (<10°) [g]	Hydrophilic (<10°) [g]	Hydrophilic (60°) [g]	Hydrophilic (<10°) [g]	Hydrophilic (<10°) [g]	Hydrophilic (60°) [g]	Hydrophilic (<10°) [g]	Hydrophobic (137°) [g]

Abbreviations: N/A: Not available.
For applied test methods, *cf.* Table 1.
Colour legend: Black shading indicates that positive surface charge may be used as supplementary criterion for nanomaterial assignment to MG4 (active nanomaterials).
The data for the case study 'metal oxides and metal sulphates' were retrieved from the following sources: [a] nanoGEM final report (2014); available at: http://nanopartikel.info/projekte/abgeschlossene-projekte/nanogem; [b] OECD (2015a); [c] NANOGENOTOX (2013b); [d] Tantra *et al.* (2012); [e] Keller *et al.* (2014); [f] Gosens et al. (2015); [g] unpublished study report; [h] Landsiedel *et al.* (2014a); [i] Izak-Nau and Voetz (2014).

Table 11
Supplementary grouping criteria for the case study 'amorphous silica nanomaterials'.

Supplementary criterion	SiO_2 NM-200	SiO_2 NM-203	aSiO_2-susp	SiO_2.acrylate	SiO_2.amino	SiO_2.PEG	SiO_2.phosphate
Dustiness (mg/kg)	Inhalable: 6459 Respirable: 293 [a,b]	Inhalable: 5800 Respirable: 218 [a,c]	N/A	N/A	N/A	N/A	N/A
Specific surface area (m^2/g)	190-220 [a,b]	200-226 [a,c]	200 [d,e]	200 [f]	200	200	200
Surface chemistry	O: 71.43; Si: 20.30; C: 5.96 *; Na: 1.83 [a,b]	NA	O: 66; Si: 29; C: 4 (C-C, C-H, C-O, C=O); Na: 1 [d,e]	Si: 21; O: 54; C: 24; Na: 1; SIMS: polymethacrylic acid/3-methacryl-oxypropyl [f]	SIMS: Aminosilane [f,g]	SIMS: PEG500 (CH_2CH_2O) [f,g]	O: 66; Si: 29; C: 4.5; Na: 0.5; SIMS: PO_2, PO_3 fragments [f,g]
Surface charge (mV)	-45 [a,b]	-35 [a,c]	-38 [d,e]	- 47 [f]	0 [f,g]	-26 [f,g]	-43 [f,g]
Hydrophobicity (water contact angle)	Hydrophilic (<10°) [h]	Hydrophilic (44°) [h]	Hydrophilic [h]	N/A	Hydrophilic [h]	Hydrophilic [h]	Hydrophilic [h]

Abbreviations: N/A: Not available; SIMS: Secondary ion mass spectrometry.
For applied test methods, *cf.* Table 1.
The data for the case study 'amorphous silica nanomaterials' were retrieved from the following sources: [a] NANOGENOTOX (2013b); [b] OECD (2015c); [c] OECD (2015d); [d] Wohlleben et al. (2013); [e] Schaefer et al. (2012); [f] Landsiedel et al. (2014a); [g] Izak-Nau and Voetz (2014); [h] unpublished study report.
[a] Presence of C considered to result from surface contamination.

administration study.

In vivo genotoxic effects were not recorded for any of these materials that cover all four MGs of the DF4nanoGrouping. Hence, the *in vitro* genotoxic effects (recorded at test material concentrations lying within ranges reflecting *in vivo* non-overload conditions) recorded for the MG1 and MG2 materials did not correlate with the outcomes of the available *in vivo* genotoxicity studies. This observation confirms the mentioned problems in correlating *in vitro* genotoxicity data for NMs with *in vivo* genotoxicity data (Landsiedel et al., 2010; Maser et al., 2015). Most likely, they further point to the overall low *in vivo* genotoxic potential of NMs.

The additional material C_{60} fullerene was the only material for which *in vivo* genotoxicity was recorded. These findings were obtained in an *in vivo* alkaline Comet assay performed as a part of an intratracheal instillation study with C57BL/6J mice (Totsuka et al., 2009).

In summary, the outcome of the case studies does not reveal principles allowing a grouping of NMs by *in vitro* or *in vivo* genotoxic effects, and a threshold value or benchmark material for *in vitro* or *in vivo* genotoxicity that had not been laid down in Arts et al. (2015) is again not suggested.

Tables 9–13 present the data on the DF4nanoGrouping qualifier dustiness and the supplementary criteria specific surface area, surface chemistry, surface charge and hydrophobicity recorded for the 24 case study materials, quartz dust DQ12 and C_{60} fullerene.

In regard to the qualifier dustiness, it should be noted that the case study materials do not cover the entire range of relevant degrees of dustiness. All of the case study materials are mono-constituent materials, and since most of them are delivered as powders, as such, they are more or less dusty (as compared to, e.g. resinated materials). Nevertheless, the qualifiers 'dustiness' or 'droplet size' may be used to prioritize the need for inhalation toxicity studies. They may further be used for the refinement of testing requirements, e.g. to select relevant biological media to assess dissolution and dispersibility.

No relevant principles for NM grouping (or relevant threshold values) could be recognized from the data collected for the supplementary criteria surface area, surface chemistry, and hydrophobicity. However, as the example of CeO_2 NM-211 and NM-212 reveals, the supplementary criterion surface charge may provide added value for NM grouping and hazard assessment (Marucco et al., 2015), especially when the DF4nanoGrouping is expanded to additionally include ecotoxicological assessment. Ruenraroengsak and Tetley (2015) recorded that positive surface charge of polystyrene nanoparticles may enhance *in vitro* toxicity in primary human alveolar macrophages and lung epithelial cells. Similarly, neutral or positively charged TiO_2 NMs, but not negatively charged ones, were taken up by *in vitro* airway epithelial cells (Boland et al., 2014).

4. Discussion

The four case studies that were performed to evaluate the

Table 12
Supplementary grouping criteria for the case study 'organic pigments'.

Supplementary criterion	DPP orange (bulk)	DPP orange (nano)	Pigment red 254-2	Pigment blue 15:1
Dustiness (mg/kg)	N/A	N/A	N/A	N/A
Specific surface area (m^2/g)	42	64	94	53
Surface chemistry	C: 73.1; Cl: 9; N: 9.5; O: 8.4	C: 73.6; Cl: 8.8; N: 8.7; O: 8.8	C: 77.1; O: 10.9; N: 5.9; Cl: 6.1	C: 80.5; O: 9.0; N: 8.5; Cl: 0.7; Cu: 1.2; Na: 0.2
Surface charge	-11.4	-12.3	-16	-11
Hydrophobicity	Hydrophilic (78°)	Hydrophobic (132°)	Hydrophobic (136°)	Hydrophobic (138°)

Abbreviations: N/A: Not available.
For applied test methods, *cf.* Table 1.
All data for the case study 'organic pigments' were retrieved from unpublished study reports.

Table 13
Supplementary grouping criteria for the 'additional materials'.

Supplementary criterion	Quartz dust DQ12	C_{60} fullerene
Dustiness (mg/kg)	N/A	N/A
Specific surface area (m^2/g)	5.9 [a]	<20 (sonicated in water) [b]
Surface chemistry	N/A	>99.9% C [b]
Surface charge	Strongly ionic (IEP <3 pH)	approx. -70 mV (stirred in water) [c]
Hydrophobicity (water contact angle)	N/A [a]	Hydrophobic [d]

Abbreviations: IEP: Iso-electric point; N/A: Not available.
The data for quartz dust DQ12 and C_{60} fullerene were retrieved from the following sources: [a] Wohlleben et al. (2013); [b] Jacobsen et al. (2008); [c] Chen and Elimelech (2009); Aschberger et al. (2010).

appropriateness of the DF4nanoGrouping were set up to encompass three main types of inorganic NMs, i.e. carbonaceous NMs, metal oxides and metal sulphates, silica NMs, as well as nanosized and non-nanosized organic pigments. Further, for each of the case studies, materials were selected that are economically relevant and cover a broad spectrum of intrinsic material properties.

4.1. Reassessment of the DF4nanoGrouping criteria, threshold values and benchmark materials

4.1.1. Reassessment of Tier 1 and Tier 2 criteria

Arts et al. (2015) identified a limited number of Tier 1 (intrinsic material properties) and Tier 2 (system-dependent properties and cellular effects) criteria as essential for NM assignment to one of the four MGs. For Tier 1, these were water solubility, morphology and composition (including impurities). For Tier 2, these were dissolution in biological media, surface reactivity, dispersibility and cellular effects.

The outcome of the case studies confirms the adequacy of these DF4nanoGrouping criteria (Table 14). All Tier 1 and Tier 2 criteria proved relevant in assigning NMs to one of the four MGs. Further, the high concordance between Tier 1 and Tier 2 MG assignment and Tier 3 MG assignment confirms the relevance and adequacy of all grouping criteria and threshold values for NM hazard assessment. The threshold values set for the Tier 1 and Tier 2 criteria are conservative in that hazard may be over-predicted (as the examples of SiO_2.phosphate and Pigment blue 15:1 reveal), but it was never under-predicted. Finally, also the available long-term NOAECs that were used as 'gold standard' for the evaluation of the case studies are consistent with the overall assignment of the case study materials to the four MGs.

Hence, the overall outcome of the case studies confirms the usefulness of the DF4nanoGrouping as a relevant tool to support NM hazard assessment. At the same time, the outcome of the case studies highlights prevailing knowledge gaps which stand in the way to the final assignment of individual NMs to one of the MGs.

The metal oxide NMs assigned to MG1 (soluble NMs) were assigned to this MG based upon their high dissolution in biological media. By contrast, these materials did not meet the threshold value for the Tier 1 criterion water solubility that was only altered in the borderline findings for SiO_2 NM-200 and NM-203. As compared to the soluble metal oxides, amorphous SiO_2 NMs have a complex dissolution and reaction behaviour. In aqueous media, amorphous SiO_2 NMs gradually transform into polymeric silicic acids, which in water and under certain conditions may again condense to colloidal structures. Hence, for amorphous SiO_2 NMs, it is not appropriate to strictly differentiate between *solubility* (Tier 1) and *dissolution* (Tier 2). Instead, both criteria should be assessed jointly. Of note, in a modified OECD TG 105 solubility test, using molybdate to precipitate orthosilicic acid released from SiO_2 NMs, a borderline value of 115 mg/L was recorded for precipitated SiO_2 NM-200 and 210 mg/L for pyrogenic SiO_2 NM-203 (unpublished study reports). Depending on the methodologies applied to remove and detect the released species, the recorded results may differ between studies.

Generally, the available data on water solubility and dissolution were found to be very heterogeneous in terms of methodologies and dose metrics applied. Especially, the testing methods to assess dissolution in biological media have obviously not yet been clearly defined or standardized. Applied test material concentrations ranged between 0.1 and 10 g/L. Further, for a given medium, incubation times ranged between 24 h and 28 days, and results were either expressed in dissolved mass per volume or relative to the total concentration. In Arts et al. (2015), the grouping criterion 'dissolution rate in biological media' (i.e. dissolved amount after a pre-determined incubation time) had been set. However, due to the mentioned inconsistencies in assessing and expressing the rate of dissolution, for the present case studies, 'dissolution in biological media' was used instead (still recording the applied incubation times).

Further research should aim at determining cut-off values for the incubation times that are relevant for the respective media. The evaluation of test results should be standardized to express the amount of dissolved material both as absolute mass per volume as well as relative to the total applied material. The latter option is recommended by the WHO for the *in vitro* assessment of man-made vitreous fibres, for which the key role of benchmark materials and dissolution rates (in units of $ng/cm^2/h$) is well-established (WHO, 1988). For NMs, a cut-off value in rate units does not exist. In this respect, an OECD draft technical guidance on the *Dissolution rate of nanomaterials in aquatic media* is currently being finalized (OECD, 2015f), and the DF4nanoGrouping criterion dissolution in biological media should be amended in accordance with its provisions, as relevant.

Finally, it has been recommended to assess particle dissolution (for the inhalation route of exposure) both at acidic (4.5) and neutral (7.5) pH values to reflect both the acidic environment of the lung lining fluid and the neutral environment of the alveolar macrophages (Guldberg et al., 1995). In the present case studies, dissolution in PSF or artificial lysosomal fluid (ALF) was assessed at acidic pH values and dissolution in Gamble's solution at neutral pH values.

For soluble metal oxides (e.g. ZnO and CuO NMs), effects are expected to be dominated by the released toxic ions. Nevertheless,

Table 14
Reassessment of the DF4nanoGrouping benchmark materials as laid down in Arts et al. (2015).

Benchmark material	DF4nanoGrouping criterion or qualifier	DF4nanoGrouping benchmark for:	Consistency with outcome of case studies/necessary adaptations
MWCNT NM-400	Toxic potency (STIS)	STIS NOAEC Range I; recovery: no	With the current threshold, for MWCNT NM-400, a STIS NOAEC Range of I or II was recorded. Additionally, the original Range I threshold does not address the reversibility or progression of effects which is a further relevant toxicological parameter for hazard assessment. Therefore, the threshold value for STIS NOAEC Range I is amended to '<0.5 mg/m^3 and no regression or progression of effects'.
$BaSO_4$ NM-220	1 Surface reactivity 2 Cellular effects 3 Toxic potency (STIS) 4 Clearance [a]	1 Not oxidative 2 Passive 3 STIS NOAEC Range IV 4 Accelerated	1 yes 2 yes 3 yes 4 yes
CeO_2 NM-211 and NM-212	1 Cellular effects 2 Toxic potency 3 Clearance [a] 4 Bioavailability 5 Surface charge	1 Activity 2 STIS NOAEC Range II 3 Decelerated 4 Local 5 Positive	1 yes 2 yes 3 yes 4 yes; available data indicate a lack of systemic translocation or effects 5 Supplementary criterion for 'activity'
TiO_2 NM-105	1 Dustiness powders 2 Water solubility 3 Toxic potency 4 Regression of effects 5 Clearance [a]	1 High 2 Low 3 STIS NOAEC Range III 4 Recovery: yes 5 Physiological	1 No, but all case study substances have some degree of dustiness 2 yes 3 yes 4 Effects partially reversible 5 yes, at substance concentrations below pulmonary overload in rats
ZnO NM-110 and NM-111	1 Water solubility 2 Cellular effects	1 Limited 2 Activity (shedding of toxic ions)	1 yes 2 *In vitro* activity was recorded that is most likely attributable to dissolved ions. Therefore, this criterion appears appropriate for the benchmark material ZnO, even though it is not essential for nanomaterial assignment to MG1.
SiO_2 NM-200 and NM-203	1 Toxic potency (STIS) 2 Dispersibility	1 SiO_2 NM-200 (large agglomerates); SiO_2 NM-203 (small agglomerates) 2 STIS NOAEC range III	1 and 2. While for both SiO_2 NM-200 and NM-203 agglomeration and the STIS NOAEC Range III were recorded, these criteria are not essential for nanomaterial assignment to MG1. Instead, the following benchmark materials are suggested: SiO_2.acrylate for dispersible substances (AAN <3), and aSiO_2-susp for agglomeration and STIS NOAEC Range III.
10 nm-CuO	1 Water solubility 2 Dissolution	1 Limited 2 High (active)	10 nm-CuO had not yet been assigned as benchmark material in Arts et al. (2015). Based upon the outcome of the case studies, it appears as appropriate benchmark material for the essential MG1 criteria water solubility (limited) and dissolution (high).
Quartz dust DQ12	1 Surface reactivity	1 High	Non-nanosized crystalline quartz dust DQ12 had not yet been assigned as benchmark material in Arts et al. (2015). Based upon the outcome of the case studies, it appears as appropriate benchmark material for the essential MG4 criterion high surface reactivity.

Abbreviations: MG: Main group; NOAEC: No observed adverse effect concentration; STIS: Short-term inhalation study.
The STIS NOAEC ranges correspond to: Range I: <0.1 mg/m^3; Range II: <1 mg/m^3; Range III: <10 mg/m^3; Range IV: ≥10 mg/m^3.
[a] Of note, physiological alveolar macrophage-derived pulmonary clearance of inhaled particles in rats corresponds to a pulmonary half-time of approximately 60 days (ECETOC, 2013), i.e. longer than the threshold value of >40 days set for biopersistent fibres in BAuA (2014).

particle effects may additionally contribute to or modify these effects. The proposed solubility threshold of 100 mg/L is low. However, based upon the outcome of the case studies, it appears adequate to indicate that a sufficient amount of ions is released that cause adverse effects. By contrast, if materials release substances that do not cause adverse effects, such as in the case of SiO_2 NM-200 and NM-203, only effects of the particles will be seen. This is reflected in the relatively low STIS NOAECs recorded for these two materials. As discussed above taking the example of SiO_2 NM-200 and NM-203, the threshold values for water solubility and dissolution in biological media may have to be reconsidered for materials that dissolve into components that do not cause adverse effects. For the time being, these two SiO_2 NMs are assessed as partly dissolving 'borderline MG1 or MG4'.

Within the DF4nanoGrouping, particle size and shape are assessed to distinguish HAR from globular NMs. Currently, the essential criteria for NM assignment to MG2 (biopersistent HAR NMs) are based upon the WHO definition of fibres (WHO, 2005) and the biopersistence threshold value set by BAuA (2014). However, the WHO definition was not specifically conceived in regard to NMs. Further research is needed to determine the critical length of biopersistent, high-aspect ratio NMs that have the potential to elicit 'fibre toxicity' and to determine if two-dimensional platelet-like materials, such as graphene, may also elicit such *in vivo* effects. Based upon the outcome of such research, the threshold values for NM assignment to MG2 (biopersistent HAR NMs) may have to be adapted (and for the time being the name 'biopersistent fibres' may be more appropriate for MG2 than 'biopersistent HAR NMs').

The Tier 2 grouping criterion dispersibility, assessed in media that are relevant for the route of exposure under investigation, allows predicting a NM's mobility in the organism. Therefore, it is an important criterion to distinguish between passive (MG3) and active NMs (MG4). The example of SiO_2.acrylate that was assigned to MG4 in Tier 2 on account of its high dispersibility and for which splenic effects were observed in the Tier 3 STIS underlines the relevance of the criterion dispersibility for NM grouping. The influence of NM agglomeration (or dispersibility) on NM hazard may be manifold. Outside the body, NM agglomeration greatly reduces human exposure. After uptake, agglomeration reduces NM translocation across the pulmonary barrier (or the skin and

gastrointestinal tract), preventing exposure of secondary organs (Bruinink et al., 2015). Further, Bruinik et al. caution that agglomeration may represent a risk factor if it occurs after translocation across the primary barriers, which may result in reduced clearance efficiency.

Nevertheless, high dispersibility alone does not provide conclusive evidence of a NM's potential for extra-pulmonary translocation, let alone its potential to elicit systemic effects: SiO_2.phosphate was one of the three 'false positive' case study materials: It was assigned to MG4 in Tier 2 on account of its high dispersibility in DMEM + FCS, but this assignment was corrected to MG3 in Tier 3. In the rat STIS, SiO_2.phosphate neither elicited pulmonary (or systemic) effects, nor was it detected in extra-pulmonary organs. By comparison, there were no 'false negative' case study materials, i.e. none of the Tier 1 and Tier 2 assignments to MG3 (passive NMs) had to be corrected in Tier 3.

Surface reactivity was the only Tier 2 criterion that was not pivotal – on its own – for NM assignment to MG3 or MG4: High surface reactivity was only observed for one MG4 material, i.e. non-nanosized quartz dust DQ12, and additionally for the MG1 material 10 nm-CuO. Quartz dust DQ12 further tested positive in the *in vitro* alveolar macrophage assay. While 10 nm-CuO was grouped as a soluble NM and its effects may largely be attributed to released Cu-ions, other mechanisms, such as surface reactivity, may have a minor contribution to the biological effects. While surface reactivity alone was not decisive for the grouping of the case study materials, nevertheless, low surface reactivity confirms NM assignment to MG3. The relevance of surface reactivity for NM hazard assessment has also been confirmed by other research groups (Karlsson et al., 2008, 2014).

Apart from the data recorded for SiO_2.PEG (that were obtained with electron spin resonance (ESR)), all surface reactivity data collected for the present case studies were obtained in the FRAS assay. This assay has proven robust, and it is well adaptive to routine testing. Even though the application of the FRAS assay for NMs is relatively new (Hsieh et al., 2013), its results are promising: The ranking of results from the FRAS assay is concordant with those from the cytochrome c assay, that was not used for the case studies (in-house data and Zhang et al. (2012)), or ESR with centrophenoxine (CPH) spin trap (Izak-Nau and Voetz, 2014). In contrast to the ESR or cytochrome c assay, the FRAS signal of the reference material Mn_2O_3 is at least a factor 100 above noise. Hence, the differentiation between active and passive NMs is more reliable, and the FRAS assay was used for the present case studies.

Research to further optimize the FRAS assay is ongoing. In addition to the Mn_2O_3–related FRAS assay threshold value of ≥10% of Mn_2O_3 surface reactivity indicating high surface reactivity (laid down in Arts et al. (2015)), an absolute threshold value of >0.1 μUFRAS/m^2*h may be suggested. In the present case studies, a second Mn_2O_3–related threshold value was introduced to relate values >1% and <10% of the reference material Mn_2O_3 to 'intermediate surface reactivity'. Such intermediate surface reactivity was recorded for all metal oxides and metal sulphates for which the respective data are available as well as for the nanosized and bulk forms of pigment orange. Further investigations should aim at determining the hazard implications of different levels of surface reactivity in order to hone the discriminatory power of this grouping criterion.

For five of the seven NMs that were correctly assigned to MG4 in Tier 2, cellular effects were the decisive grouping criterion. Hence, the outcome of the case studies underlines the relevance of *in vitro* investigations for NM hazard assessment. However, the case studies also highlight a number of requirements that have to be met to ensure that *in vitro* data are relevant for hazard assessment.

Taking into account recent research on the *in vitro* alveolar macrophage assay, prevalence was given to data from this assay since it has been shown to be predictive of *in vivo* respiratory tract effects (Wiemann et al., 2015a, 2015b). The *in vitro* alveolar macrophage assay uses the NR8383 rat alveolar macrophage cell line which is similar to alveolar macrophages in the rat lung that further sequester the vast majority of particles inhaled during the STIS. The NR8383 assay jointly assesses cellular release of lactate dehydrogenase (LDH), glucuronidase, tumour necrosis factor alpha, and reactive oxygen species. Significant effects observed at 'non *in vitro* cellular overload' conditions are interpreted as indicating activity. Particles usually agglomerate in the *in vitro* alveolar macrophage assay since it uses a protein-free culture medium. Hence, they sediment to the bottom of the wells, and NM effects elicited at 'non *in vitro* cellular overload' conditions are assessed as specific biological effects that are not merely caused by the burden of the particle volume on the cells. While the cellular effect range of ≤10 μg/cm^2 laid down in Arts et al. (2015) applies for cytotoxicity tests performed with lung epithelial cells, for *in vitro* assays performed with alveolar macrophages, a threshold value of 4000 μm^2 particle surface area per macrophage is laid down. This value corresponds to non-overload conditions in the phagocytically-active cells (Wiemann et al., 2015b).

In the current case studies, the example of SiO_2 NM-200 and NM-203 highlights how FCS-supplementation of the culture medium may affect the outcome of cytotoxicity assays. These two MG1 materials tested positive in the *in vitro* alveolar macrophage assay that uses a protein-free culture medium (Minimum Essential Medium; MEM). This outcome correctly reflects their low STIS NOAEC in Tier 3 (1 mg/m^3, each, for equivalent materials) that, however, is not essential for NM assignment to MG1. By contrast, these same materials did not induce cellular effects in the MTT or colony forming efficiency assays using BALB/3T3 mouse fibroblasts cultured in FCS-supplemented MEM at test material concentrations from 1 to 100 μg/mL and up to 72-h incubation (Uboldi et al., 2012). Concordantly, also other studies report that the cytotoxicity of SiO_2 NMs is mitigated in the presence of FCS (Landsiedel et al., 2014c).

However, in the *in vitro* alveolar macrophage assay MWCNT NM-400 and carbon black could not be assessed because these NMs could not be dispersed in the protein-free culture media. If data from the *in vitro* alveolar macrophage assay were unavailable, data from test methods addressing standard cell viability endpoints, such as LDH release, reduction in metabolic activity (reduction of the tetrazolium salt MTT), or cell proliferation (resazurin reduction in the alamarBlue® assay) in standard cell lines with respiratory tract origin were recorded (Kuhlbusch et al., 2009; Landsiedel et al., 2014b). Accordingly, low surface carbon black and ZnO NM-110 did not elicit cellular effects in A549 cells using the LDH and MTT tests up to test material concentration of 25 μg/cm^2, whereas 10 nm-CuO was assessed as causing cellular effects, based upon the positive outcome of an alamarBlue® assay using macrophages.

4.1.2. Reassessment of Tier 3 grouping and sub-grouping criteria

In Tier 3, pulmonary biopersistence in the STIS serves to confirm or refute NM assignment to MG1 or MG2. NMs that are assessed as soluble in water or quickly dissolving in biological media in Tier 1 and Tier 2 are expected to have short pulmonary half-lives. By contrast, NMs that are assigned to MG2 (biopersistent HAR NMs) in Tier 1 and Tier 2 are expected to have prolonged pulmonary half-lives. Nevertheless, NM grouping based on water solubility and dissolution in biological media does not always reflect the *in vivo* situation. This is highlighted by the example of $BaSO_4$ NM-220 that is not soluble in water or biological media. Nevertheless, this material was observed to possess only very short *in vivo* biopersistence (Keller et al., 2014; Konduru et al., 2014). The mechanisms by which $BaSO_4$ NM-220 is rapidly eliminated from the lung and Ba is transported to extra-pulmonary

locations are currently under investigation (Cefic LRI, 2015). The DF4nanoGrouping tiered assessment of biopersistence is conservative: Lower tiers may under- but not over-estimate. Besides aerosol concentration and deposition efficiency, *in vivo* biopersistence affects lung burden over a given period of time. Low lung clearance may result in high lung burdens, which may result in pulmonary overload conditions. Moreover, slow clearance may prolong or abolish the regression of lung effects after the inhalation exposure period has ended (*cf.* sub-grouping by STIS NOAEC, below). Accordingly, *in vivo* biopersistence has to be taken into account when assessing a material's hazard at actual exposure concentrations and exposure durations. Of note, however, rats are especially sensitive to pulmonary overload and resulting tumour formation, whereas the human health relevance of these effects is at least questionable (ECETOC, 2013).

For the assessment of NMs as active (MG4) based on systemic bioavailability (extra-pulmonary translocation for the inhalation route), Arts et al. (2015) laid down a threshold value for organ burden of 1 mass% of the total dose further distinguishing whether this level was recorded in the MPS or outside the MPS. In addition to this original threshold value, organ burden <1% that is accompanied by extra-pulmonary effects (as was observed for SiO_2.acrylate) may be used as a further grouping parameter.

As laid down in Arts et al. (2015), the STIS NOAEC may be used for the sub-grouping of MG4 NMs by potency. Using a threshold value for STIS NOAEC Range I (<0.1 mg/m^3), no single case study material was clearly assigned to this potency range. For technical reasons, the lowest aerosol concentration applied in inhalation studies oftentimes does not undercut 0.5 mg/m^3. Additionally, the original Range I as described by Arts et al. (2015) does not address the reversibility or progression of effects which is a further relevant toxicological parameter for hazard assessment. Therefore, the threshold value for STIS NOAEC Range I should be amended to '<0.5 mg/m^3 and no regression or progression of effects'. Further, evaluation of STIS data should take into account that the progression of effects is not only determined by material pathogenicity, but also by its respective organ clearance.

Generally, grouping and sub-grouping of NMs assigned to MG4 (active NMs) provides information to identify and specify the need for further testing.

- If NMs are detected outside the respiratory tract and/or induce effects in extra-pulmonary tissues, specific testing of systemic effects is warranted.
- Likewise, if pulmonary effects other than inflammation or upper airway necrosis are observed, further testing may be necessary for an in-depth assessment of these effects.
- If a NM's pulmonary clearance in the STIS is prolonged, it may accumulate in the lung, and studies covering an appropriate exposure and/or post-exposure period may be warranted.
- For NMs that exhibit inflammatory responses in the lung, but do not elicit any other effects, sub-grouping based on STIS data (STIS NOAEC range, the progression or reversibility of effects, systemic bioavailability) may be useful. Whether this information is sufficient for hazard assessment largely depends on the availability of data for read-across-source NMs within the same sub-group that may serve the specific read-across for the NM under investigation.

Of note, for those NMs evaluated in the present case studies for which data from sub-chronic studies were available, these data did not reveal a new quality of effects or a toxic potency in a different order of magnitude. However, *in vivo* chronic studies with NMs are largely unavailable, and little is known about the progression of short-term effects during life-time exposure. A rat life-time study assessing two NMs ($BaSO_4$ NM-220 and CeO_2 NM-212) is currently ongoing (Gebel and Landsiedel, 2013), and it remains to be seen to which extent its results will be applicable to other NMs. Given the limited number of potential modes-of-action of NMs in the lung, most of the testing needs beyond Tier 3 of the DF4nanoGrouping are expected to arise from indications of extra-pulmonary effects or the need to consider for distinct biokinetics due to increased lung deposition or prolonged clearance.

4.1.3. Reassessment of benchmark materials

As presented in Table 14, the outcome of the case studies generally confirms adequacy of the benchmark materials suggested in Arts et al. (2015), with the following adaptations:

Using the revised threshold value for NM assignment to the STIS NOAEC Range I (i.e. '≤0.5 mg/m^3 and no regression or progression of effects'), MWCNT NM-400 (STIS NOAEC <0.5 mg/m^3) is maintained as benchmark material for this STIS NOAEC range. Nevertheless, its toxic potency in the STIS does not *per se* provide a definite indication that this MWCNT – or other MWCNTs – will act according to the fibre paradigm (Pauluhn, 2010; DeLorme et al., 2012, 2015; Ma-Hock et al., 2013; Schuler et al., 2013).

Taking into account that the case study materials do not cover the entire range of relevant degrees of dustiness (*cf.* Section 3.4), TiO_2 NM-105 is not a suitable benchmark material for 'high dustiness'. However, the case study confirms the usefulness of TiO_2 NM-105 as benchmark material for low water solubility, STIS physiological pulmonary clearance, STIS NOAEC Range III as well as recovery of *in vivo* effects (that were only partially reversible within the 21-day post-exposure observation period which stands in accordance with the lung clearance rate). Of note, physiological alveolar macrophage-derived pulmonary clearance of inhaled particles in rats corresponds to a pulmonary half-life of approximately 60 days (ECETOC, 2013), i.e. longer than the threshold value of >40 days set for biopersistent fibres in BAuA (2014).

SiO_2 NM-200 and NM-203, which have been assigned as 'borderline MG1 or MG4' on account of research accomplished since the publication of Arts et al. (2015), should not be benchmark materials for 'dispersibility', since this is an essential criterion for MG3 and MG4 grouping. Instead, SiO_2.acrylate is put forward as benchmark materials for dispersible materials (AAN <3) and aSiO_2-susp as benchmark material for agglomeration and NOAEC Range III.

Additionally, 10-nm CuO is suggested as new benchmark material for the essential MG1 criteria water solubility and dissolution in biological media supplementing the benchmark materials ZnO NM-110 and NM-111. Furthermore, non-nanosized crystalline quartz dust DQ12 is suggested as benchmark material for the MG4 criterion high surface reactivity.

4.2. Usefulness of the DF4nanoGrouping for nanomaterial hazard assessment

The DF4nanoGrouping *Decision-making framework for the grouping and testing of nanomaterials* is the first decision-tree-based tool for the grouping of NMs that comprehensively addresses all aspects of a NM's life cycle and biological pathways that are relevant for hazard and risk assessment with a focus in the inhalation route of exposure. The number of criteria that are essential for NM assignment to one of the four MGs is limited and clearly defined, and the present case studies confirm the usefulness of these criteria for NM grouping. Criteria that are relevant for the further sub-grouping of the heterogeneous group of MG4 active NMs are likely to be more complex and therefore have not been defined, but some suggestions were made. In the case studies, the NMs assigned to MG4 (active NMs) were sub-grouped by *in vivo* parameters recorded in the STIS, i.e. toxic potency range, the reversibility of effects, and systemic bioavailability. Such sub-grouping may

contribute to identifying the need for further testing, e.g. if effects are progressive or if materials have the potential for extra-pulmonary translocation.

Generally, the need for decision-tree based tools and methods enabling a rapid categorization of NM hazard potential for regulatory purposes is unanimously acknowledged. It has been requested that such tools and methods should enable to target materials of high concern for additional scrutiny, while material categories that pose the least hazard should receive expedited review (Godwin et al., 2015). In this respect, the four MGs defined in DF4nanoGrouping (i.e. soluble, biopersistent HAR, passive and active NMs) stand in line with the requirements and stipulations from different organisations, authorities and research groups (ISO, 2014; UBA, 2014; EPA, 2015; Sellers et al., 2015).

The DF4nanoGrouping allows such an expedited review for soluble NMs (MG1), biopersistent HAR NMs (MG2) and passive NMs (MG3). For NMs assigned to MG1 because they rapidly release hazardous constituents, these dissolved constituents or the bulk material may serve as a basis for the hazard assessment. Actual dose considerations may, however, be more complex since deposition and dissolution in biological media have to be figured in. Future research should aim at specifying test methods to measure dissolution in relevant media, and it should address whether the MG1 100 mg/L threshold value needs to be revised. Moreover, it should be discussed whether NMs that dissolve into non-toxic components may merit a different threshold value than the MG1 threshold value established for NMs that release cytotoxic ions. For NMs assigned to MG2 (biopersistent HAR NMs), the general fibre OEL will apply. Here, further research should aim at clarifying whether the general fibre paradigm is equally applicable to NMs (and further, specifically, to NMs that have a HAR in two dimensions) and if so, whether the same OEL is suitable for all MG2 NMs.

Just as NMs assigned to MG1 or MG2, NMs assigned to MG3 (passive NMs) may not require further testing for human hazard assessment. In respect to long-term human exposure to NMs, the DF4nanoGrouping assignment to MG3 versus MG4 may serve to determine whether a general dust OEL is applicable or not. Passive NMs are those for which a general dust OEL is sufficient, whereas active NMs (MG4) are those requiring specific, lower OELs (and, accordingly, specific further investigations, as relevant). Passive NMs may nevertheless have the potential to induce inflammation and potentially lung tumours in rats, if the aerosol concentration is high and pulmonary clearance is sufficiently prolonged, even though the human health relevance for such overload conditions is questionable (ECETOC, 2013). Of note, even though the term passive, on its own, might not be self-explanatory, the MG3 for passive NMs is precisely defined. Generally, this MG addresses a similar type of particles as are subsumed by the terms ‘dusts’, ‘poorly soluble low toxicity (PSLT) particles’, or ‘granular biodurable particles (GBP)s, but many of these terms lack a detailed definition.

Just as the MGs defined in the DF4nanoGrouping reflect groups of NMs recognized by other research groups, authorities or organisations, the essential criteria for NM assignment to the four MGs of the DF4nanoGrouping stand in accordance with the groups and criteria put forward by different organisations and authorities. Also the need to distinguish between ‘intrinsic’ material properties (Tier 1) and ‘extrinsic’, environment-related properties or ‘functionality’ (Tier 2) for proper NM hazard assessment is widely recognized (Lynch et al., 2014; Hendren et al., 2015; Nel et al., 2015). The present case studies confirm the usefulness of these criteria and distinctions for NM grouping and for the prediction of the likelihood of NM *in vivo* activity.

The DF4nanoGrouping MG assignments and grouping criteria also provide preliminary information on the mode-of-action of NMs. Generally, the four MGs relate to four specific modes-of-actions of NMs (Landsiedel et al., 2014c, Landsiedel, 2015). MG1 relates to toxic ion release (Nel et al., 2013), MG2 to the fibre paradigm (Poland et al., 2009), MG3 to lung overload as the only cause for pulmonary effects (Moreno-Horn and Gebel, 2014), and MG4 to specific surface properties that result in cellular effects and/or mobility in the organism (Nel et al., 2013, 2015; Wiemann et al., 2015b). These principles may lay a foundation for the future development of adverse AOPs (Ankley et al., 2010) for NMs and the subsequent development of AOP-based integrated approaches for the testing and assessment (IATAs) of NMs (Arts et al., 2014; Oomen et al., 2014). In fact, the DF4nanoGrouping may provide key elements for such IATAs (Arts et al., 2015). The DF4nanoGrouping provides a mechanistic and biokinetic rationale to steer the assessment. Thereby, the DF4nanoGrouping provides a sound scientific basis for read-across and weight-of-evidence approaches to derive information on specific endpoints (*cf.* ‘hypothesis development’ in Oomen et al. (2015)). Generally, the DF4nanoGrouping will recognize common hazard potentials of materials belonging to different or same nanoforms (*cf.* Information box for definition). Depending on the regulatory context, this may be applied widely (occupational regulations on fibre OELs and general dust OELs) or restrictively (only within a nanoform).

A number of grouping criteria laid down in the DF4nanoGrouping that were assessed as essential for grouping, such as water solubility, dissolution in biological media, surface reactivity, dispersibility or *in vitro* cellular effects are not yet regularly addressed in NM hazard assessment. In recognition of the relevance of these parameters, collection of data for these criteria should be encouraged. Evidently, this may imply the need to introduce new test methods into a company's or laboratory's test method portfolio. Nevertheless, application of the DF4nanoGrouping may make NM hazard assessment more efficient, and it may serve to reduce the need for animal testing. Evidently, none of the Tier 1 or Tier 2 criteria require animal studies. The STIS requires 40 animals per test material, whereas the 90-day inhalation study performed in accordance with OECD TG 413 requires 80 animals (and 120 animals if the optional post-exposure observation period is included) for each single material.

The DF4nanoGrouping truly uses animal studies only as a last resort as is mandatory by Article 25(1) of the EU REACH regulation (EP and Council of the EU, 2006): It exclusively relies on non-animal studies in Tier 1 and 2 in order to group those NMs into MG1, MG2 and MG3 that do not require animal testing to perform a hazard assessment. MG4 NMs may have specific hazardous properties upon inhalation exposure. These can be identified and possibly subgrouped by refined and reduced animal testing using the STIS protocol. After testing in the STIS, only a fraction of the NMs may require further animal testing. This will be tailored to the specific information needs, thereby reducing the overall testing programme. In summary, even if animal testing is required for the DF4nanoGrouping, significantly less animals will be needed than when a standard testing battery is applied.

In spite of the possible need to introduce the determination of properties that are not yet routinely addressed, the present case studies show that, provided that data for the essential grouping criteria have been collected, the DF4nanoGrouping can be easily applied. Similarly, existing information can be used, if the test materials and applied test methods are unequivocally described. Thereby, redundant testing may be avoided. The example of C_{60} fullerene supports this presupposition. Data for this material were exclusively taken from the peer-reviewed literature. Albeit not having a consistent data base to serve all DF4nanoGrouping criteria, it was possible to tentatively assign C_{60} fullerene to MG3 based on the available literature data only. The DF4nanoGrouping was also

applied to non-nanosized quartz dust DQ12, which resulted in its assignment to MG4 in Tier 2 based upon cellular activity and high surface reactivity. In Tier 3, this assignment was confirmed by *in vivo* activity. Likewise, for the non-nanosized DPP orange (bulk) the Tier 1 and Tier 2 assignment to MG3 was confirmed by a high STIS NOAEC. These observations may indicate that the DF4nanoGrouping is potentially applicable to inhaled particles irrespective of their size.

Even though intended use and release-related exposure scenarios were addressed as important qualifiers for NM testing and grouping in Arts et al. (2015), the present case studies were conceived and performed without taking into account specific NM uses or release scenarios. Thereby, the case studies highlight that the DF4nanoGrouping may be applied irrespective of specific exposure scenarios. Overall, knowledge on how different life cycle changes may affect the release potential and different properties and effects of NMs is only beginning to evolve (Froggett et al., 2014). A few examples for no or low release scenarios have been published, such as use of the concrete hardening accelerator X-Seed® (Bräu et al., 2012) or TiO_2, Ag and SiO_2 NMs that are firmly embedded in complex paint matrices (Smulders et al., 2015). If NM release from a given product may be ruled out with certainty, exposure-based waiving should be justifiable. Observations that particles that are embedded in products during use and/or storage may be significantly changed compared to the pristine NMs underline the need for a holistic view on the impact of NMs through the entire value chain from production, through use and finally to disposal (Mitrano et al., 2015).

5. Conclusion

The present case studies assessing a broad spectrum of economically relevant inorganic NMs covering carbonaceous NMs, metal oxide and metal sulphate NMs, amorphous silica NMs and non-nanosized and nanosized organic pigments confirm the usefulness of the DF4nanoGrouping *Decision-making framework for the grouping and testing of nanomaterials* as a relevant tool for NM hazard assessment (with very minor modifications as compared to the framework published in Arts et al., 2015; *cf.* footnotes to Table 1). In two tiers that rely exclusively on non-animal test methods followed by a third tier, if necessary, in which data from the STIS are evaluated, NMs are assigned to one of four MGs. In the case studies, the DF4nanoGrouping has proven highly efficient in sorting out NMs that may undergo hazard assessment without further testing. These are the MG1 soluble NMs, whose further hazard assessment should rely on read-across to the dissolved materials, MG2, HAR NMs, which may be assessed as asbestos-like fibres, and the MG3 passive NMs for which a general dust threshold may be applicable. Thereby, the DF4nanoGrouping allows identifying MG4 active NMs that merit further in-depth investigations. It provides a solid approach to sub-group active NMs which in return provides a scientific rationale to determine specific additional information needs.

NM assignment to one of the four MGs of the DF4nanoGrouping further provides preliminary information on the mode-of-action of NMs. This may also lay a foundation for the future development of AOPs. Finally, the present case studies may form the scientific basis for the justification of read-across applications, e.g. by using the DF4nanoGrouping benchmark materials as source NMs for read-across. Since the DF4nanoGrouping is a hazard assessment strategy that strictly uses animal studies as a last resort as required by the REACH regulation (EP and Council of the EU, 2006), its general application for NM hazard assessment not only serves scientific, but also animal welfare needs.

Conflicts of interest

This manuscript was prepared by members of the ECETOC Task Force on nanomaterials. TP represents and JA, MAI, RK, MM, NN, DW, KW, WW, RL are employees of companies producing and/or marketing nanomaterials. UGS was hired by ECETOC to support this Task Force. The authors alone are responsible for the content and writing of the paper.

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Appendix A. Supplementary data

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Transparency document

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