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# Prioritising nano- and microparticles: identification of physicochemical properties relevant for toxicity to *Raphidocelis subcapitata* and *Daphnia magna*

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## Abstract

**Background:** Advanced/innovative materials are an undefined group of nano- and micro-particles encompassing diverse material compositions, structures and combinations. Due to their unique properties that enable specific functions during applications, there are concerns about unexpected hazards to humans and the environment.

In this study, we provide ecotoxicity data for 36 nano- and microparticles of various inorganic species (single constituents and complex compositions; materials releasing toxic ions and others), morphologies (spheroidal, cubic, flaky, elongated/fibrous) and sizes (10 nm–38 µm). By applying *Raphidocelis subcapitata* algae growth inhibition and *Daphnia magna* immobilisation tests according to OECD test guidelines 201 and 202, and extensive material characterisation, we aimed to identify indicators of concern. This would allow better predictions of the hazardous properties of these materials in the future.

**Results:** The chemical identity (toxic ion-releasing materials vs. other materials) and agglomeration behaviour, which is affected by size (nm vs. µm) and morphology (fibres vs. others), were obvious drivers of ecotoxicity on *R. subcapitata*. Differences in morphology had an impact on agglomeration behaviour. Fibres formed agglomerates of varying sizes with entrapped and attached algae. Small compact (e.g. spheroidal) particles attached to algae. A high coverage resulted in high ecotoxicity, while less toxic materials attached to a much lesser extent. No agglomeration of algae and particles was observed for particles with a µm size. Small toxic components of large hybrid materials did not affect ecotoxicity. For *D. magna*, despite uptake of all materials studied into the gut, the sole indication of toxicity was the release of toxic ions. This is in line with previous observations on nanomaterials. Based on the identified criteria, charts were developed to indicate the expected toxicity of advanced/innovative materials toward algae and daphnia.

**Conclusion:** Indicators for the toxicities of advanced materials differ for algae and daphnia. Thus, different materials give rise to concerns for the two aquatic organisms. For *D. magna,* only the toxic ion-releasing materials are relevant, but for *R. subcapitata,* more complex interactions between particular materials and cells must be considered.

**Keywords:** Advanced materials, Innovative materials, Nanomaterials, Micromaterials, Ecotoxicity, *Daphnia magna*, *Raphidocelis subcapitata*

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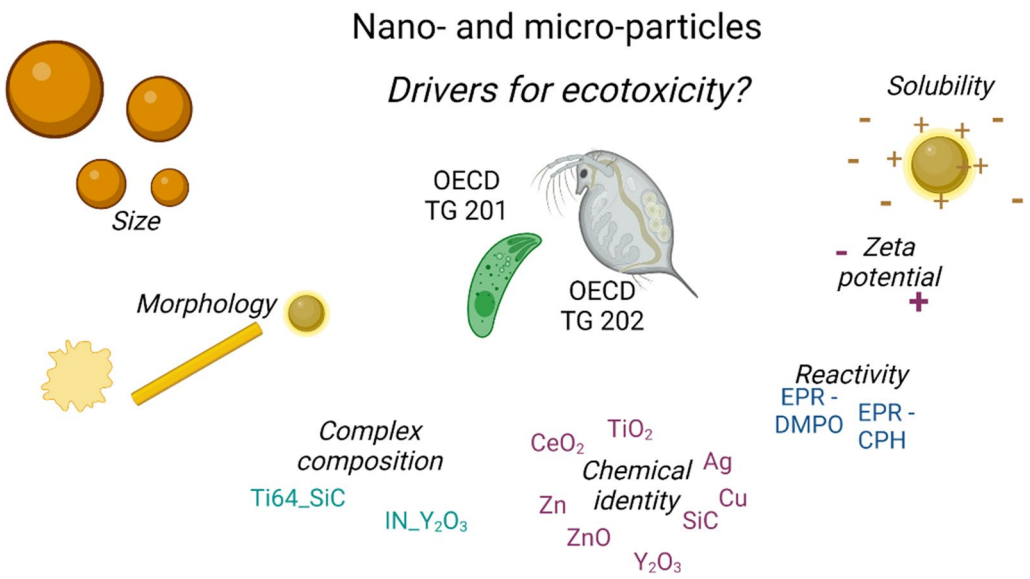
**Graphical Abstract**




## Introduction

Engineered nanomaterials (ENMs) have been comprehensively studied during the last 15 years by, among others, the OECD working party on nanomaterials, who have used their testing programme of manufactured nanomaterials (OECD). Over the years, it became evident that several issues discussed for nanomaterials can also be of importance for materials exceeding the 100 nm size threshold. The term "Advanced Materials" (AdMa) was created and describes a diverse group of materials (European [16]. There is no official definition, but as a working description not intended as a basis for regulation, AdMa are described as materials that are rationally designed to meet new functional requirements [55]. Schwirn et al. [55] proposed implementing the term "materials of concerns" along with the underlying criteria and pointed out that concern could differ from the "very high concern" of the REACH Regulation. Eight clusters of advanced materials have been proposed [19], and these are Advanced Alloys, Advanced Polymers, Biopolymers, Porous Materials, Particulate Systems, Advanced Fibres, Composites and Metamaterials. Testing of all materials might not be feasible, and the development of strategies to prioritise them for hazard assessment has been proposed. An early warning system for AdMa, which addresses ENMs as well as larger materials, could enable prioritisation [48]. The most advanced grouping framework, GRACIOUS, was developed specifically for monoconstituent nanomaterials [30, 59]. With a screening strategy named Early4AdMa, an early warning, prioritisation and action system was developed [43]. It consisted of several steps designed to identify, describe, prioritise and respond to warnings for AdMa. To provide a safety assessment for the environment physicochemical properties, hazard, fate and exposure/environmental release are addressed. For hazard, among others, it has to be decided whether there is an indication of short- or long-term toxicity. Criteria for these decisions were not included.

The freshwater alga growth inhibition test [42] and the immobilisation test with daphnids [41] are key tests used for environmental hazard testing of chemical substances as well as for ENMs in the framework of regulations [14], ECHA [15]). To provide information on the relationship between material properties and ENM ecotoxicity, systematic studies were performed using both tests [24, 25, 35]. Nanoscale monoconstituent metals and metal oxides, mainly comprising spheroidal particles with narrow size ranges, were investigated. For the green algae *R. subcapitata*, no relationship between physicochemical properties such as particle size, surface area, agglomeration size, reactivity, crystalline structure and growth inhibition according to OECD Test Guideline (TG) 201 was obvious. However, their attachment to algae was found to be related to their ecotoxicity [27]. The toxicity of particles with high attachment efficiency exceeded the toxicity of particles with a lower tendency for attachment. For example, the $CeO_2$ particles (NM-211, NM-212) that densely covered the algae cells were more toxic by a

factor of approximately 10 than the material (NM-213), which resulted in nearly no attachment ($EC_{50}$—NM-211: 8.5 mg/L; NM-212: 11 mg/L vs. NM-213: 99 mg/L). The underlying properties causing agglomeration remained unknown. For materials releasing toxic ions, the chemical identity was of relevance. For example, $EC_{50}$ values of 0.02 to 0.08 mg/L were determined for the spherical Ag material which exceeded the $EC_{50}$ of ZnO by a factor of approximately 10 ($EC_{50}$: 0.1 to 0.6 mg/L). An influence of the fibre morphology for the Ag material could not be excluded, but the data situation with two fibres was very limited. The ecotoxicity of the investigated fibres differed by a factor of approximately 100 ($EC_{50}$ of 0.02 vs. 2.4 mg/L). The thinner and more toxic fibre, in contrast to the thicker fibre, showed attachment to the algae [24, 25]. For *D. magna*, the most toxic materials were those releasing toxic ions (Ag-, Cu-, and Zn-containing materials), while materials releasing nontoxic ions (e.g. some titanium nanomaterials) or not releasing ions at all (such as silica nanomaterials) did not have an impact on *D. magna* mobility in previous experiments. ENMs composed of silver, copper or zinc exhibited dose-dependent toxicity [35], for example, $EC_{50}$ values between 0.0016 and 0.043 mg/l were determined for Ag nanomaterials, between 3.43 and 8.25 mg/l for Zn nanomaterials, and between 0.0132 and 0.2481 mg/l for Cu nanomaterials. In addition, there were some indications that fibre morphology played a role in determining toxicity. Silver fibres ($EC_{50}$ 0.0016 mg/l) were approximately 30 times more toxic than spheroidal silver particles ($EC_{50}$ 0.043 mg/l) without releasing substantially more ions [35]. For any of the other nanomaterial properties, no relationship to toxicity for daphnids could be established.

Building on these experiences, we extended the spectrum of particles by investigating additional advanced nano- and micromaterials due to their unusual and rationally designed compositions and/or morphologies. We wanted to know whether observations of ENM toxicity to *R. subcapitata* and *D. magna* could be generalised. Selection of a wide variety of materials should allow data generation to assess the influence of morphology, hybrid composition (particles composed of materials with different chemical identities), size, zeta-potential and reactivity. A variety of inorganic materials were selected, including materials that release toxic ions as well as others (materials that release no ions or only nontoxic ions). By considering results from 20 additional inorganic materials together with the results for the 16 previously studied ENMs, we aimed to identify indicators for ecotoxicity to key aquatic organisms and support the identification of "materials of concern".

## Materials and methods

### Test materials

A wide range of test materials was investigated. The underlying objectives of material selection are presented Table 1 and in Additional file 1: Table S1. The influence of morphology (spheroidal, cubic, flaky, elongated), size (nm and µm) and hybrid composition (monoconstituent substances vs. complex compositions) should be addressed. One selection criterion for Ag fibres was "different lengths, but similar diameters". No material was specifically produced for the study. Therefore, the materials that were compared differed in more than one property. When selecting the materials, preference was given to those that are commercially available to increase the practical relevance of the conclusions. Materials such as alloys, modified alloys, or silver materials are used in industrial processes and are therefore also of economic relevance. The characterisations showed that the reactivities and zeta-potentials of the materials also differed. Even though these parameters were not the focus of the study, they were also taken into account as much as possible. A compilation of characterisation data (morphology, state, primary particle sizes; test medium; zeta-potential, solubility, reactivity) and the methods used are presented in the Supplementary Information (Additional file 1: Table S2 to Table S5).

**Table 1** Assignment of the test materials to various objectives based on the effects of selected material properties on ecotoxicity

| Objective | Material | |
|---|---|---|
| Influence of morphology | Toxic ion-releasing materials:<br>• Ag: fibre, flake, sphere | • Cu: fibre, sphere |
| | Other materials (release of no ions or only nontoxic ions): | |
| | • $TiO_2$: fibre, cube, sphere | • SiC: fibre, sphere, whisker |
| Influence of size and hybrid composition | Hybrids:<br>• IN_$Y_2O_3$ (µm-range)<br>• Ti64_SiC (µm-range) | Single components:<br>• IN718 (µm-range) + $Y_2O_3$ (nm-range)<br>• Ti64 (µm-range) + sSiC (nm-range) |
| Influence of size, reactivity, zeta-potential—monoconstituent materials | All materials including information from previous projects[a]: $TiO_2$, SiC, $Y_2O_3$, $CeO_2$, Ag, Cu, CuO, ZnO | |

[a] Data for $TiO_2$, $CeO_2$, Ag, ZnO were taken from previous investigations; Hund-Rinke et al. [25], Kühnel et al. [35]

## Ecotoxicological assays

### *Growth tests with the green algae R. subcapitata*

*Preparation of suspensions* The preparation method depended on the physicochemical properties (e.g. particle shape and availability as a dispersion or as a powder) of the test material. The test concentrations of the newly investigated materials were prepared as a tenfold dilution series. The number of test concentrations was selected according to the expected ecotoxicity, with 100 mg/L as the maximum. The $EC_{50}$ values of previous investigations [25, 35] were based on a geometric series with a factor not exceeding 3.2.

Except for fibres and the whisker, we used the dispersion method developed in the EU-nanOxiMet project [39] and adapted in the nanoGRAVUR project [38] for ecotoxicological testing [25, 35]. Briefly, a stock suspension of the particles was prepared by mixing $40 \pm 4$ mg of the powder with 40 mL of ultrahigh-quality water to reach a concentration of 1 g/L. The sample vial was placed 1 cm above the ultrasonic sensor in the middle of a cup horn (Bandelin, Germany) for ultrasonic treatment, and 230 mL of deionised water (4 °C) was added to the cup horn. The suspension was sonicated for 10 min using a pulse of two (0.2 s/0.8 s). To achieve a sufficient volume of the test dispersion, two 40-mL dispersions were prepared and combined. For the highest test concentration (100 mg/L), 50 mL of the test dispersion was added to 450 mL of the OECD test medium and manually shaken for 1 min to ensure that the test dispersion was sufficiently homogenous. Concentrations of 10, 1.0 and 0.1 mg/L were prepared by serial dilution.

The fibres were not processed with the cup horn device to avoid significant reductions in their lengths. The Cu, $TiO_2$ and SiC fibres were available as nondispersed materials and could not be dispersed by shaking or stirring. Therefore, we added 50 mg of each material to 500 mL of OECD test medium in a glass bottle (concentration 100 mg/L) and applied short pulses in a Bandelin Sonorex Type RK 510 ultrasonic bath (HF frequency 35 kHz, pulse 10 s, interval 10 s) (Bandelin, Germany). Between the short pulses, the dispersion was gently shaken to distribute the particles, and sufficient homogeneity was achieved after six pulses. No obvious shortening of the fibres by this procedure was observed. The stock dispersion, which corresponded to the highest test concentration, was serially diluted with the OECD test medium to 10, 1.0 and 0.1 mg/L. The Ag fibres were available as suspensions. The test concentrations were selected according to the expected toxicity. The required amount was added to the test medium and homogenised with an overhead shaker for 15 min.

*Test performance and assessment of attachment* The unicellular green alga *R. subcapitata* (Chlorophyceae, Chlorophyta, syn. *Pseudokirchneriella subcapitata*) is described in OECD test guideline 201 as the standard test organism representing primary producers in freshwater. The strain was purchased from SAG (Culture Collection of Algae; Pflanzenphysiologisches Institut of the University at Göttingen, Albrecht von Haller Institut, Untere Klarspüle 2, 37073 Göttingen, strain number 61.81 SAG). For cultivation, the stock cultures were maintained in the culture medium recommended by Bringmann and Kühn [8]. Prior to testing, a preculture was established in the OECD growth medium to obtain exponential algae growth for the tests. The duration of the precultures was 3 days. The cell density of the inoculum culture (preculture) was determined with a CASY Model TT-cell counter (Roche Innovatis, Germany), and aliquots of the inoculum culture (423–1105 µL) were added to the test vessels to obtain a density of 10,000 cells/mL. The inhibition of algal growth (*R. subcapitata*) was determined as described in OECD TG 201 [42] (four replicates per test concentration, control: six replicates. During the tests, all vessels (250-mL conical glass flasks containing 100 mL test dispersion were maintained at 21–24 °C. The tests were performed with a light intensity (OSRAM Standard cool white bulbs of ~ 100 µE $m^{-2}$ $s^{-1}$ (4440–8880 lx). During the tests, all vessels were placed on an Incubation Shaker Multitron (INFORS-HT, Switzerland) and shaken continuously at 150 rpm. Algae biomass was determined via chlorophyll fluorescence [26] followed by calculation of the growth rate. In vitro and in vivo data from valid fluorescence measurements were used. For the in vitro measurement, we applied a method that separated particles from chlorophyll by using locust bean gum. Both applied methods resulted in comparable effect data [26]. Every material was tested at several concentrations (see "Preparation of suspensions" Section). The presented data refer to nominal concentrations. The validity criteria listed in the test guideline were applied.

Agglomeration of the particles to the algal cells was determined via light microscopy in a short-term assay (3-h incubation period) at a particle concentration of 100 mg/L and an algae concentration of approximately 2 million cells/mL, which corresponded to the cell concentration in the growth test at the end. To verify the results, the agglomeration behaviour was investigated in the test vessels at the end of the growth tests (72 h). Both approaches are described in Hund-Rinke et al. [27].

*Statistics* ToxRat (ToxRat Solutions, Germany) was used to evaluate the effect concentrations and confirm fulfilment of the validity criteria. We calculated the percent inhibition of growth rate [r] compared to controls for the

exposure period, as this is the relevant regulatory parameter. Biological data were analysed to determine $EC_{50}$ values together with 95% confidence intervals when possible. For the calculations, the following settings were used. Pretesting: normal distribution—Shapiro–Wilk´s; significance level—0.01; variance homogeneity—Levene; significance level—0.01; final testing ($EC_{50}$): test procedure—Williams; significance level—0.05; test direction—one-sided smaller; ECx computation: selected method—nonlinear regression; optimisation—Levenberg–Marquardt (IRLS); dose/response function metric—3-parametric normal; calculation of confidence limits—Monte Carlo simulation.

*Principal component analysis (PCA)* Principal component analysis was performed with the studied materials by considering the values for size, solubility, zeta-potential and $EC_{50}$ in the algebra test using the function 'prcomp' of the package stats in R [5, 37, 60]. Materials for which numerical values for at least one of these parameters were not available were excluded from the analysis. Prior to PCA, the values for all parameters were standardised by centring them around the mean for all materials included in the analysis. Solubility was not considered in the separate analysis of the materials not releasing ions.

#### *Immobilisation test with D. magna*

*Preparation of particle suspensions* Particles supplied as dispersions were first agitated on an overhead shaker for 24 h and then vortexed at 220 rpm for 2 min. Particles supplied as a powder were added to the medium and vortexed at 220 rpm for 10 min. Stock solutions were always freshly prepared on the day of the experiment at a nominal concentration of 100 mg/L in ADaM (Aachener Daphnien Medium, according to Klüttgen et al. [32]. The stock solutions were then further diluted with ADaM to achieve the respective test concentrations.

Suspensions were first tested at concentrations of 1 µg/L, 10 µg/L, 100 µg/L, 1 mg/L, 10 mg/L and 100 mg/L. After the first results indicated a concentration range for $EC_{50}$, suspensions were tested with 6 concentrations based on the suspected $EC_{50}$, with sample concentrations ranging from no effect to maximum effect.

*Miniaturised D. magna acute immobilisation assay* *D. magna* were cultured in mass culture, with each flask holding 30 specimens of the same age range in 1.2 L of ADaM. The culture was kept at 20 ± 2 °C under a natural day–night cycle. The feeding regime was derived from [33] and involved feeding thrice weekly with the green algae *Scenedesmus vacuolatus* ($1*10^9$–$4.1*10^9$ algae cells per neonate, depending on age). On Fridays a volume of 1 mL of a 1 g/L yeast solution was added to each culture jar as described by Knops [33]. Adult survival, number of offspring and number of ephippia (if present) were tracked to ensure healthy culture conditions. Medium replacement and extraction of neonates not older than 24 h for testing was performed through staggered sieving.

The 48-h acute toxicity test with *D. magna* (Crustacea, Branchiopoda, Cladocera) was based on OECD TG 202 [41]. Deviating from the OECD guideline, daphnids were exposed in miniaturised assays in 24-well microplates (TPP®, Switzerland) to enable use of lower volumes of the particle dispersions. The miniaturised approach based on previous studies [22] suggesting the use of lower medium volumes. The studies concurringly demonstrate no impact on organism's sensitivity and equal toxicities for a number of test substances in miniaturised test setups compared to the standard test. Two of the studies [4, 50] dealt with nanomaterials, specifically supporting the applicability of the miniaturised approach for particulate test materials.

Each treatment and control consisted of 4 replicates with 5 daphnids per well (20 animals per treatment in total), and they were exposed in a total volume of 1.5 mL of ADaM per well. Each test was repeated at least three times, starting the experiments on different days.

During the 48-h exposure period, no food was provided. Immobility was evaluated after 24 and 48 h of exposure. Potassium dichromate served as the reference chemical for the positive control. Attachment and internalisation of particles by daphnids were monitored by light microscopy after the end of the 48-h exposure period, and pictures were taken.

All tests met the test validity criteria suggested by OECD TG 202, with an immobilisation < 10% in the negative control (observed: 0%), pH of the medium between 6 and 9 (observed: 7 to 7.8), dissolved oxygen concentration in the medium ≥ 3 mg/L (observed: > 8 mg/L), and toxicity of the reference substance potassium dichromate ($K_2Cr_2O_7$) within the $EC_{50/24\,h}$ range of 0.6 mg/L to 2.1 mg/L (observed: 1.1 mg/L to 1.9 mg/L).

*Calculation of* $EC_{50}$ The results of the experiments for each particulate were collected, and a sigmoidal curve was fitted for all valid tests. The $EC_{50}$s as well as the respective 95% confidence intervals were determined through probit analysis (own Python code).

## Results

### Growth tests with the green algae *R. subcapitata* and agglomeration of algae with particles

The ecotoxicities of industrially relevant materials were investigated, and the influence of selected properties was determined. When selecting the materials, preference was given to those that are commercially available

to increase the practical relevance of the conclusions. No material was specifically produced for the study (see "Test materials" Section Table 1, Additional file 1: Table S1). Therefore, it was unavoidable that the materials differed in more than one property, which had to be considered during interpretation of the results. PCA of size, solubility, zeta-potential, and $EC_{50}$ values in algae explained 54.75% of the total variance in the dataset (PC1 29.90%, PC2 22.85%) with all materials for which all values were available (Additional file 1: Figure S1). The analysis showed that in PC1, the materials releasing toxic ions differed from the other materials (Additional file 1: Figure S1A). Separation of materials not releasing toxic ions (Additional file 1: Figure S1B) and those releasing toxic ions (Additional file 1: Figure S1C) for PCA resulted in increases in the explained data variance to 78.67% and 71.06%, respectively, supporting the decision to evaluate these materials separately. Therefore, for presentation of the results, the materials were separated into toxic ion-releasing materials and others. Within these two groups, the materials were ordered according to their $EC_{50}$ values. Based on this compilation, the various objectives regarding the influence of selected material properties, as shown in Table 1, were addressed. Other properties were also taken into account if an influence on the results could not be ruled out. For some materials, this presentation mode avoided the need to list the results more than once.

Different types of agglomeration behaviour were observed. Algae can be covered by small particles (sizes smaller than the size of the algae), which is visible as a shell around the cell. Algae can attach to individual particles with sizes exceeding the size of the algae. Furthermore, materials can form agglomerates with each other. Algae can attach to large agglomerates or be entrapped within them.

#### *Materials not releasing toxic ions*

Table 2 provides data on the ecotoxicological effects on algae. Furthermore, selected physicochemical properties (morphology, size, reactivity) and agglomeration behaviour are also listed. The zeta-potentials are listed in Additional file 1: Table S3. The agglomeration behaviour determined with the short-term assay was also reflected at the end of the growth test at a concentration of 100 mg/L. At lower test concentrations, the agglomeration behaviour in the growth test was less pronounced.

Toxicity is presented as $EC_{50}$ values. For the materials tested with a dilution factor of 10, the inhibitory values for the individual test concentrations are presented in the Supplementary information (Additional file 1: Table S6). Based on the ecotoxicity, three groups were identified with high, low and no observable toxic effects. We defined $EC_{50}$ values up to approximately 10 mg/L as high toxic effects, whereas toxic effects in the range 10 to 100 mg/L (mainly around the highest test concentration of 100 mg/L) were considered low.

#### Morphology focus

Independent of the chemical identity, with spheriodal and elongated materials various morphologies are present in the group of materials with high and low toxicity. This indicates that not only morphology affects ecotoxicity.

Different shapes with the same chemical identity were available for $TiO_2$ and SiC. For $TiO_2$, five spheroidal, one cubic and two elongated materials were investigated. Despite the morphological differences, the $EC_{50}$ values were within the range 0.4 to 5 mg/L. Only the spheroidal $TiO_2$ NM-104 with a hydrophobic coating was less toxic, with an $EC_{50}$ of 60 mg/L. However, the differences in morphology were expressed in the agglomeration behaviour. The fibres formed agglomerates of varying sizes with entrapped and attached algae. The small spheroidal materials attached to the algal cells showed high coverage for the more toxic materials, while the less toxic spheroidal material attached to a much lower extent. These differences were also reflected in the PCA (Additional file 1: Figure S1B).

The two elongated and the spheroidal SiC materials differed in their toxicities and agglomeration behaviours. With an $EC_{50}$ of 7.8 mg/L for the spheroidal material, the toxicity exceeded the toxicity of the elongated materials. At the highest test concentration, the elongated particles showed a 50% effect (SiC_thin) and a 30% effect (SiC whisker). All materials formed agglomerates with attached and entrapped algae. Additionally, the more toxic spheroidal material covered the algal cells and formed a shell.

The results for the three $CeO_2$ particles confirmed the results for spheroidal $TiO_2$ particles. They showed that the coverage density has to be considered. While NM-211 and NM-212 showed high coverage and $EC_{50}$ values of 8.5 and 5.6 mg/L, respectively, NM-213 exhibited weaker coverage and lower ecotoxicity ($EC_{50}$ 43.8 mg/L).

#### Hybrid composition focus

To address the influence of complexity, the two complex materials IN_$Y_2O_3$ and Ti64_SiC consisted of large (INT718; Ti64) and nanoscale ($Y_2O_3$; sSiC) components. While the nanoscale components were toxic with $EC_{50}$ values of 2.6 and 7.8 mg/L, respectively, the large components and the large complex material showed no toxicity at all.

**Table 2** Ecotoxicity to algae and selected physicochemical properties of nontoxic ion-releasing nano- and microparticles available in powder form (for material details, see Additional file 1: Table S3 and Table S5)

| Material | Morphology | Size Ø [nm] | Size length [µm] | Reactivity | Description of agglomeration | $EC_{50}$ [mg/L] |
|---|---|---|---|---|---|---|
| Materials with toxic effects to *R. subcapitata* | | | | | | |
| Non-doped $TiO_2$ | Spheres | 19 | – | No | Algae densely covered by particles | 0.38 [0.33–0.43] |
| Eu-doped $TiO_2$ | Spheres | 10 | – | No | Algae densely covered by particles | 0.91 [0.75–1.10] |
| $TiO_2$ cubes | Cubes | 16 | – | Not determined | Loose agglomerates of large particles with attached and entrapped algae | 0.92 [0.55–1.51] |
| $TiO_2$_fibre IG | Fibre | 61 | 1.4 | No | Small and large, mainly loose agglomerates with entrapped and attached algae | 1.3 [n.d.] |
| $Y_2O_3$ | Spheres | 32 | | No | Particles attached to algae; no dense coverage; additionally, large agglomerates of algae and particles | 2.6 [2.2–3.1] |
| Fe-doped $TiO_2$ | Spheres | 23 | | No | Algae densely covered by particles | 3.6 [2.6–4.8] |
| $TiO_2$_fibre RG | Fibre | 65 | 1.3 | No | Small and large, mainly loose agglomerates with entrapped and attached algae | 4.2 [0.033–668] |
| $TiO_2$ NM-105 | Spheres | 21 | | No | Algae densely covered by particles | 4.7 [3.5–5.5] |
| $CeO_2$ NM-212 | Spheres | 30 | – | No | Algae densely covered by particles | 5.6 [3.0–10.4] |
| sSiC | Spheres | 75 | – | Yes | Small particles attached to some algae (half of the cell covered); algae attached to agglomerates exceeding size of algae; potentially entrapment of algae | 7.8 [4.4–13.8] |
| $CeO_2$ NM-211 | Spheres | 10 | – | No | Algae densely covered by particles | 8.5 [7.7–9.3] |
| Materials with low toxicity to *R. subcapitata* | | | | | | |
| $CeO_2$ NM-213 | Spheres | 33 | – | No | Minor attachment of particles to algae; no dense coverage | 43.8 [n.d.] |
| $TiO_2$ NM-104 | Spheres | 30 | – | No | Minor attachment of particles to algae; no dense coverage | 60.1 [13.9–234.2] |
| SiC_thin | Fibres | 190 | 11.7 | Not determined | Individual fibres; small, loose agglomerates of the fibres with entrapped and attached algae | 99.9 [29.1–315.6] |
| SiC whisker | Whisker | 314 | 3.7 | Not determined | Individual whiskers and loose agglomerates with some attached and entrapped algae | Small toxicity (–30% effect at 100 mg/L) |
| Materials without toxicity to *R. subcapitata* | | | | | | |
| INT718 | Spheres | $10.8 * 10^3$ | – | No | Individual particles or agglomerates comprising a few particles; only some algae are attached on the surface of the particles or agglomerates | No toxicity |
| IN_$Y_2O_3$ | Platelet | $26.1 * 10^3$ | Thickness: 3.6 | No | | No toxicity |
| Ti64 | Platelet | $17.7 * 10^3$ | – | No | | No toxicity |
| Ti64_SiC | Spheres | $37.9 * 10^3$ | Thickness: 11.0 | No | | No toxicity |

*n.d.* not determinable due to mathematical reasons or inappropriate data

### Size focus

The three toxicity groups differ with regard to the sizes and attachment/agglomeration behaviours of the materials (* MERGEFORMAT Table 2). The materials exhibiting toxic effects (high and low) had sizes in the nm range, while the nontoxic particles were in the µm range. The nanomaterials with high ecotoxicity were highly likely to agglomerate.

### Reactivity focus

While the ecotoxicity values ranged from none to high, most materials were nonreactive, except for sSiC, which had a low reactivity value of 2.5. For $TiO_2$, with anatase (NM105, $TiO_2$_cubes; non-doped $TiO_2$, $TiO_2$_fibre IG and RG) and rutile (NM-104; Eu-doped $TiO_2$; Fe-doped $TiO_2$), two forms with different reactivities in the presence of UV light, were included. A relationship between the crystalline forms and ecotoxicity was not obvious. For example, similar $EC_{50}$ values were obtained for Eu-doped $TiO_2$ (rutile) and undoped $TiO_2$ (anatase) as well as for Fe-doped $TiO_2$ (rutile) and NM-105 (anatase).

### Zeta-potential focus

Independent of the ecotoxicity values, the zeta-potentials of most materials were negative. Only for $TiO_2$ NM-104

a positive value was determined (13.3 mV) that can be explained by the $Al_2O_3$ coating.

#### *Toxic ion-releasing materials*

Information on the toxic ion-releasing particles is summarised in Table 3 for Cu and Zn; silver is addressed in Table 4. The toxicities are presented as $EC_{50}$ values. The materials are sorted by their chemical compositions and ecotoxicity values. For materials that were tested based on geometric series with a factor of 10, the inhibitory values for the individual test concentrations are presented in the Supplementary Information (Additional file 1: Table S6).

### Morphology focus

Cu was tested as spheroidal material and as fibre. Furthermore, spheroidal CuO particles were considered. The toxicities of the three copper materials differed by a factor of 70. Spheroidal nCu was the most toxic, followed by Cu fibres, and CuO was the least toxic. The agglomeration behaviours of the three materials also differed. The fibres formed agglomerates of varying sizes and densities. nCu and CuO formed dense covers on the algae. Additionally, CuO formed large agglomerates with entrapped algae.

Three Zn materials comprising spheroidal particles with sizes ranging from 34 to 42 nm were investigated and exhibited $EC_{50}$ values of 0.09 to 0.55, respectively. Taking the confidence intervals into account, NM-111 was slightly less toxic than the other two Zn materials by a factor of approximately 5. The ecotoxicity is not reflected by the agglomeration behaviour. While NM-110 densely covered the algal cells, NM-111 and NM-113 showed only minor attachment.

For Ag, one spheroidal, two flaky and eight fibrous materials were investigated. Three groups of ecotoxicity values were observed, with $EC_{50}$ values in the µg range, between 1 and 10 mg/L and between 10 and 100 mg/L. There was no obvious relationship between ecotoxicity and morphology. The ecotoxicity of the spheroidal ENM (NM-300 K; EC50 0.062 mg/L) and of one of the platelets (Ag B190; EC50 1.6 mg/L) were in the toxicity range of the fibres.

The materials differed in their agglomeration behaviours. Two fibrous materials (Ag-1340, SRM 110525), the spheroidal ENM (NM-300 K) and the two platelet materials (Ag B190; Ag ES-4) did not agglomerate, while the other fibrous materials remained as individual fibres or formed agglomerates with varying sizes and densities. A relationship between agglomeration behaviour and ecotoxicity was not obvious, but an influence of the producer/production process, including the stabilising agent, cannot be excluded. The materials forming no agglomerates were produced by RAS and DUDOKO, while the other materials were purchased from nanoGAP and ACS. The latter two were dispersed in water, while the DUDOKO material was available as a powder and dispersed in the test medium. The RAS materials were available as aqueous dispersions (Ag 1340, SRM 110525) and in an aqueous mixture of polyoxyethylene glycerol trioleate and polyoxyethylene sorbitan monolaurate (NM-300 K).

### Size, reactivity, zeta-potential, and solubility focus

The solubilities and reactivities of the Cu materials differed. There was no relationship between solubility and ecotoxicity. The ecotoxicities of the most toxic material (nCu) and the least toxic material (CuO) differed by a factor of 70, while the solubility values were comparable (0.10–0.14 mg/L, respectively). The fibres showed medium ecotoxicity and the highest solubility (1.1 mg/L). The orders for CPH reactivity and ecotoxicity were comparable, with nCu showing the highest toxicity and reactivity (307). However, for DMPO reactivity, the order was

**Table 3** Cu and Zn nano- and microparticles available in powder form—ecotoxicity on algae and selected physicochemical properties form (for material details, see Additional file 1: Table S4 and Table S5)

| Material | Morphology | Size ∅ [nm] | Size length [µm] | Solubility [mg/L] | Reactivity | Agglomeration/attachment [2] | $EC_{50}$ [mg/L] |
|---|---|---|---|---|---|---|---|
| nCu | Sphere | 76 | – | 0.14 ± 0.093 | Yes | Algae densely covered by particles | 0.020 [0.019–0.021] |
| Cu_fibre | Fibre | 228 | 6.1 | 1.2 ± 0.23 | Yes | Small and large, loose and dense agglomerates of fibres with attached algae; obvious entrapment of algae not visible | 0.16 [0.07–0.078} |
| CuO | Sphere | 24 | – | 0.1 | Yes | Algae densely covered by particles; agglomerates of algae and particles | 1.4 [1.2–1.6] |
| ZnO-NM-110 | Cube | 41 | – | 2.7 ± 1.5 | No | Algae densely covered by particles | 0.09 [0.08–0.10] |
| ZnO-NM-113 | Cube | 42 | – | 2.8 ± 0.7 | No | Minor attachment of particles to algae | 0.11 [0.05–0.21] |
| ZnO-NM-111 | Cube | 34 | – | 1.7 ± 0.5 | No | Minor attachment of particles to algae | 0.55 [0.38–0.80] |

**Table 4** Ag nano- and microparticles—ecotoxicity on algae and selected physicochemical properties sorted by ecotoxicity form (for material details, see Supporting Information Additional file 1: Tables S4 and S5)

| Material/producer | Morphology | State of material | Size Ø [nm] [2] | Size length [µm] | Solubility [mg/L] | Reactivity | Agglomeration/attachment [2] | $EC_{50}$ [mg/L] |
|---|---|---|---|---|---|---|---|---|
| Ag–1340/RAS | Fibre | Dispersion | 44 | 3.8 | 0.8 | Yes | Individual fibres; no obvious attachment | 0.022 [n.d.] |
| Ag_Rod_3140/nanoGAP | Fibre | Dispersion | 53 | 14.0 | 0.03 ± 0.00 | Yes | Mainly individual fibres; loose agglomerates; no obvious attachment | 0.53 [0.23–1.35] |
| NM-300 K/RAS | Sphere | Dispersion | 15 | –- | n.d. [2] | No | Individual particles; no obvious attachment | 0.062 [n.d.] |
| Ag_long/ACS | Fibre | Dispersion | 52 | 4.4 | 0.1 ± 0.0 | Yes | Mainly individual fibres; loose agglomerates; no obvious attachment | 1.0 [n.d.] |
| Ag_Rod_DS_0471/nanoGAP | Fibre | Dispersion | 44 | 1.6 | 2.8 ± 4.3 | Yes | Individual fibres and dense agglomerates; no obvious attachment | 1.4 [n.d.] |
| Ag B190/DODUKO | Platelet | Powder | $3.9 * 10^3$ | Thickness: 0.2 | 0.1 ± 0.0 | Yes | No obvious agglomerates, no obvious attachment | 1.6 [0—n.d.] |
| Ag_Rod_3170/nanoGAP | Fibre | Dispersion | 63 | 6.5 | 0.1 ± 0.1 | Yes | Individual fibres and dense agglomerates; no obvious attachment | 1.7 [1.6–1.9] |
| SRM 110525/RAS | Fibre | Dispersion | 241 | 2.4 | 0.004 | No | Individual fibres; no obvious attachment | 2.4 [2.1–2.7] |
| Ag_Rod_3143/nanoGAP | Fibre | Dispersion | 41 | 1.6 | 0.71 ± 1.11 | Yes | individual fibres and dense agglomerates; no obvious attachment of fibres to algae | > 1 (extrapolated: 1–10) |
| Ag_short | Fibre | Dispersion | 52 | 1.3 | < 0.0009 (detection limit) | not determined | Individual fibres and loose agglomerates; no obvious attachment | 18.2 [14.8–22.1] |
| Ag ES-4/DODUKO | Platelet | Powder | $1.7 * 10^3$ | Thickness: 0.5 | < 0.0009 (detection limit) | No | Agglomerates of varying size, no obvious attachment | 10–100 ($EC_{50}$ n.d) |

*n.d.* not determinable due to mathematical reasons or inappropriate data

reversed. The zeta-potentials of the Cu fibres and the CuO were around the isoelectric point, whereas the ecotoxicity values differed by a factor of 10.

The three cubic Zn particles showed comparable ecotoxicities, solubilities, zeta-potentials and reactivities, and influencing factors could not be identified.

For the 11 Ag materials, there were differences in the states (powder, dispersion), reactivities, solubilities and zeta-potentials. We were not able to identify relationships involving the state of the material (powder, dispersion), morphology (spheroidal, fibrous, flaky) or ecotoxicity, as there was no combination that showed a difference

in only one parameter. For example, both platelets were available in powder form and all fibres as dispersions with different dispersants. Reactivity and zeta-potential were excluded as the main drivers of ecotoxicity. Two materials (NM-300 K; SRM 110525) showed no reactivity, but one of them revealed high ecotoxicity (NM-300 K). The ecotoxicity of the nonreactive NM-300 K was comparable or even exceeded the ecotoxicity values of all reactive materials. However, an effect of morphology (spheroidal vs. fibrous, flaky) cannot be excluded. The reactivities of Ag_Rod_3140, Ag_Rod_3143 and Ag_Rod_DS_0471 were high compared to those of the other fibrous materials, which was not reflected by the ecotoxicity values. All Ag materials had negative zeta-potentials, independent of their ecotoxicity values. With regard to the solubility, two approaches must be distinguished. If all materials were considered together, no relationship was observed between solubility and ecotoxicity (Table 4). The ecotoxicity values of the materials with comparable solubilities, such as Ag 1340 and Ag_3143 (0.8 and 0.7 mg/L), differed by a factor of 100. However, a relationship between solubility and ecotoxicity was indicated if the materials were considered separately according to the producers (Additional file 1: Table S7).

### Immobilisation test with *D. magna* and particle uptake

#### *Materials not releasing toxic ions*

#### Morphology, size, reactivity and hybrid composition focus

All materials not releasing toxic ions, including the hybrid materials (see Additional file 1: Table S3), were compared with regard to their effects on immobilisation of *D. magna* based on the $EC_{50}$ values determined in the 48-h immobilisation assay. The toxicities of fibrous and nonfibrous materials with the same composition but differing morphologies (spheres, platelets, cubes) were compared. For hybrid materials, the individual materials (IN718, $Y_2O_3$, Ti64, sSiC) as well as the combined materials (IN718 + $Y_2O_3$, Ti64 + sSiC) were compared. In addition, information on material uptake into the gut of the daphnid was collected, and the results are summarised in Additional file 1: Tables S8 and S9.

For all materials not releasing toxic ions, no toxicity was observed, and the calculated $EC_{50}$ values were over 1000 mg/L.

For all of the materials, uptake into the gut of *D. magna* was observed (examples in Fig. 1).

#### *Toxic ion-releasing materials*

#### Morphology, size, reactivity and solubility focus

Different observations were made for all materials releasing toxic ions. Zinc, copper and silver materials exhibited dose-dependent toxicities towards *D. magna*. The $EC_{50}$ values for the individual materials are listed in Table 5. Differences in morphology were found to modulate the toxicities of ion-releasing materials. However, the trend differed depending on material type. The copper materials with spheroidal shapes showed higher toxicities ($EC_{50}$ 0.0132 and 0.2481 mg/L) than the fibre material ($EC_{50}$ 1.947 mg/L). There was no relationship with size or reactivity. Zinc materials were comparable in morphology (cubic) and solubility (1.2–2.1 mg/L) but differed slightly in their zeta-potentials (− 15.6–5.9 mV) and mean sizes (34–42 nm). Nevertheless, their ecotoxicities were comparable ($EC_{50}$ 3.43–8.25).

The $EC_{50}$ values of silver fibres with different diameters and lengths were between 0.0016 and 0.614 mg/L. However, despite these variations in $EC_{50}$ values across two orders of magnitude, a clear relationship between fibre dimensions and toxicity could not be established. All silver fibres, except SRM 110525, were reactive, but the material was the second most toxic among the silver materials tested ($EC_{50}$ 0.0085 mg/L). Additionally, there was no relationship to ion solubility (see Additional file 1: Table S4).

It was not possible to subject the silver flake materials to testing in the daphnia immobilisation assay. The flake dispersions were extremely unstable, and the particles settled quickly and adhered to the walls and surfaces of the test vessels, leading to irreproducible test conditions. Introduction of the surface agent polyvinylpyrrolidone (40 kDa) did not sufficiently improve dispersion stability.

All materials were highly toxic according to the classification given above for algae. Uptake into the gut was evident for all materials irrespective of morphology and dimension.

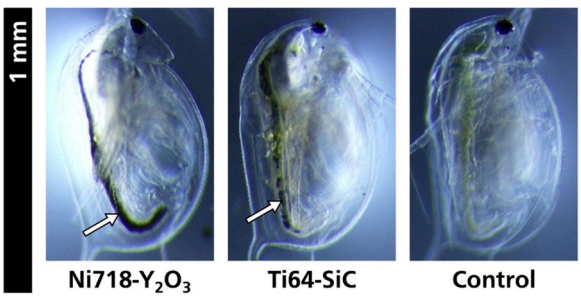



**Fig. 1** Uptake of hybrid materials (Ni718–$Y_2O_3$, Ti64–SiC) into the gut of *D. magna*. In the light microscopy images, a black colouration of the gut (arrows) is evident for the hybrid materials. In control animals, a slightly green colouration of the gut is visible due to the algae feed

**Table 5** Ecotoxicity for *D. magna* and uptake of toxic ion-releasing materials with various morphologies. Selected physicochemical properties of the materials are shown (for material details see Additional file 1: Tables S4 and S5)

| Materials | Selected PC parameters | | | $EC_{50}$ (mg/L) [CI] | | | Uptake into gut |
|---|---|---|---|---|---|---|---|
| | Length [µm] | Size Ø [nm] | Reactivity | Fibre | Sphere | Platelet | |
| Copper | | | | | | | |
| Cu_fibre | 5 | 100 | Yes | 1.947 [1.191–2.703] | | | Yes |
| nCu | – | 76 | Yes | | 0.0132 [0.0007–18.1792] | | n.a |
| CuO | – | 24 | n.d | | 0.2481 [-0.0307–0.5268] | | n.a |
| Zinc | | | | | | | |
| ZnO-NM-110 | – | 41 | No | | 3.43 [2.85–4.02] | | n.a |
| ZnO-NM-111 | – | 34 | No | | 8.25 [4.70–11.80] | | n.a |
| ZnO-NM-113 | – | 42 | No | | 5.63 [3.26–8.00] | | n.a |
| Silver | | | | | | | |
| Ag—1340/RAS | 3.8 | 44 | Yes | 0.0016 [— 0.014–0.018] | | | Yes |
| SRM 110525/RAS | 2.4 | 241 | No | 0.0085 [0.002–0.015 | | | Yes |
| Ag_short/ACS | 1.3 | 52 | n.d | 0.614 [0.549–0.669] | | | Yes |
| Ag_long/ACS | 4.4 | 52 | Yes | 0.221 [0.198–0.243] | | | Yes |
| Ag_Rod_3140/nanoGAP | 14 | 53 | Yes | 0.265 [0.251–0.279] | | | Yes |
| Ag_Rod_3143/nanoGAP | 1.6 | 41 | Yes | 0.220 [0.212–0.229] | | | Yes |
| Ag_Rod_3170/nanoGAP | 6.5 | 63 | Yes | 0.213 [0.191–0.235] | | | Yes |
| Ag_Rod_DS_ 0471/nanoGAP | 1.6 | 44 | Yes | 0.122 [0.110–0.134] | | | Yes |
| NM-300 K/RAS | – | 15 | No | | 0.043 [0.038–0.047] | | Yes |
| Ag B190/DODUKO | 0.2 (thickness) | $3.9 * 10^3$ | Yes | | | n.t | n.t |
| Ag ES-4/DODUKO | 0.5 (thickness) | $1.7 * 10^3$ | Yes | | | n.t | n.t |

Grey shaded fields—not relevant

*CI* confidence interval, *n.t.* not testable in *D. magna* assay, *n.a.* not assessed

## Discussion

Advanced/innovative materials are not a strictly defined group of materials, but encompass diverse material compositions, structures and combinations, and it is considered impossible to unambiguously structure AdMa without any overlaps. A total of 25 fact sheets on materials in several clusters were developed in the research project "Advanced materials—Overview of the field and screening criteria for relevance assessment". They provide structured information on AdMa [12, 19]. Among others, advanced alloys and inorganic fibres are addressed, which were also considered in our investigations. Although commercially available for the last several years, Cu fibres and Ag fibres still fulfil the AdMa criteria of relative novelty and rational design of the specific shape for unique functionality in transparent, flexible, conductive electrodes. The sheets demonstrate that information on environmental concerns is limited. As it will be impossible to test all materials, identification of the general principles for ecotoxicity of AdMa exemplified with *R. subcapitata* and *D. magna* is a step forward and could support the application of Early4AdMa. We focused on the assessment of initial particles according to the REACH Regulation. Modifications such as ageing or interactions with other substances relevant to environmental behaviour are not considered.

### Material parameters related to toxicity in the green algae *R. subcapitata*

With size, shape, solubility and biological reactivity in the form of agglomeration, some of the criteria listed by ECHA for grouping and read-across of nanomaterials [13] were identified as important drivers of ecotoxicity for *R. subcapitata*. Light microscopy verification of agglomeration behaviour is a simple approach, but is limited by resolution. Electron microscopy is considered less suitable for this issue due to the sample preparation required. However, quantitative approaches have been successfully applied, such as flow cytometry for spheroidal metallic oxide nanoparticles [49] or cell inductively coupled plasma–mass spectrometry techniques for gold particles of different shapes [1].

According to our results, zeta-potentials in the range − 39 to + 13 are of lower relevance as drivers for ecotoxicity and as indicators for agglomeration behaviour. Most of the investigated particulate materials had negative surface charges, while the ecotoxicities and agglomeration efficiencies obviously differed. Microalgae also have

negative surface charges [29, 36], and repulsion would be expected. Nevertheless, agglomeration and ecotoxicity were observed. The only material with a zeta-potential in the clearly positive range ($TiO_2$ NM-104) showed hardly any attachment to the algae. The low relevance of the zeta-potential, which had been shown for nanomaterials [25], was thus also confirmed for other and larger materials. In addition, reactivity measurements using CPH (1-hydroxy-3-carboxy-2,2,5,5-tetramethylpyrrolidine hydrochloride) [46] and DMPO (5,5-dimethyl-1-pyrroline-N-oxide) [56] proved to be of little significance.

Based on the observed relationships between material properties and ecotoxicity, the materials can be clustered to support identification of materials of concern (Fig. 2). First, due to the different toxicity mechanisms, differentiation according to the extent of toxic ion release is recommended. With this, the materials releasing toxic ions are separated from the other materials. Chemical composition as a main toxicity driver was demonstrated by testing $BaSO_4$, ZnO and Cu ENMs [58], which supports the proposed separation approach. Solubility is frequently discussed as a driving factor [3, 6, 17, 23, 28]. For example, Franklin et al. [17] demonstrated that nanoparticulate ZnO, bulk ZnO, and $ZnCl_2$ have comparable ecotoxicities on *Pseudokirchneriella subcapitata* (renamed *R. subcapitata*) if the $EC_{50}$ is related to Zn ions. However, according to our results for toxic ion-releasing particles (Fig. 2B), a dependency on the producer/production process must be considered as well; this is not reflected solely by solubility measurements in test media carried out independently of ecotoxicological assays. It is assumed that the production processes and applied substances are additional driving factors. This conclusion could not be verified based on these results, as no detailed information was available on production processes or other substances used to produce the silver particle suspensions. However, an argument in favour of considering the production process is that different synthesis approaches for Ag ENMs result in different particles [20]. Furthermore, the compilation of Kędziora et al. [31] shows that various physicochemical properties and biological activities of silver nanoparticles result from different synthesis methods. No conclusion is possible as to whether predictions of ecotoxicity can be improved with additional consideration of the state of the material or the particle shapes, since the materials with different shapes and states (powder or dispersion) were derived from different producers.

For materials not releasing toxic ions, several levels can be differentiated. We propose separating particles with considerably elongated shapes (e.g. fibre, rod, whisker) from other materials with compact shapes (spheres, platelets, cubes) due to their different agglomeration behaviours. Agglomeration of elongated materials can result in netlike agglomerates with varying densities in which algae can obviously be entrapped. A relationship between ecotoxicity and agglomerate density seems to be conclusive, as illumination levels for algae entrapped in denser agglomerates are lower, which affects growth. However, the underlying parameters leading to formation of the agglomerates could not be identified. Diameter and length do not seem to be the only causes. A relationship between size and ecotoxicity for our particles with diameters between 60 and 300 nm and lengths between 1 and 12 µm was not obvious. However, it can be concluded that high aspect ratio nanoparticles (HARNs) can be toxic to algae if they show a tendency to agglomerate.

We propose to consider various size ranges for materials with compact structures (spheres, platelets, cubes). For the algal species *R. subcapitata* used, lengths of 8–14 µm and diameters of 2–3 µm are described [54]. It was observed that materials with much smaller sizes than *R. subcapitata* behaved differently than materials whose sizes vastly exceeded the size of the algal cells. Small materials or their small agglomerates can form shell-like structures around the algae (Additional file 1: Table S10, Eu-doped $TiO_2$). It was already shown that the density of this coverage can be related to ecotoxicity [27]. In contrast, algae attach to materials that greatly exceed the size of the algae. These agglomerates can agglomerate further, resulting in entrapment of the algae. The extent of entrapment and shading can affect the level of ecotoxicity, with obvious entrapment resulting in high ecotoxicity. Based on our results and these considerations, we pragmatically recommend the following three size categories: 1–1000 nm, > 1–10 µm and > 10 µm. The use of median values is proposed as a basis for taking into account the size distribution of the materials. The probability of impact on algal growth differs depending on the agglomeration behaviour. Examples were available for elongated materials as well as for small and large particles. No medium-sized particles were available, so this category is only assumed. Furthermore, no large materials with obvious agglomeration behaviour resulting in toxic effects were available. Since we cannot exclude the existence of large particles with obvious tendencies to agglomerate, we have taken this factor into account. We used a mass-based test design, which resulted in lower particle numbers for larger materials compared to smaller ones. This can affect the agglomeration behaviour. Although particle number is proposed as an alternative and more suitable dose metric, mass is still usually applied for ecotoxicological hazard assessments [1]. As we used mass for calculations of the $EC_{50}$ values and as a basis for the agglomeration studies, the observed relationship is considered to be justified. For further studies, the particle number should also be considered. Abdolahpur Monikh

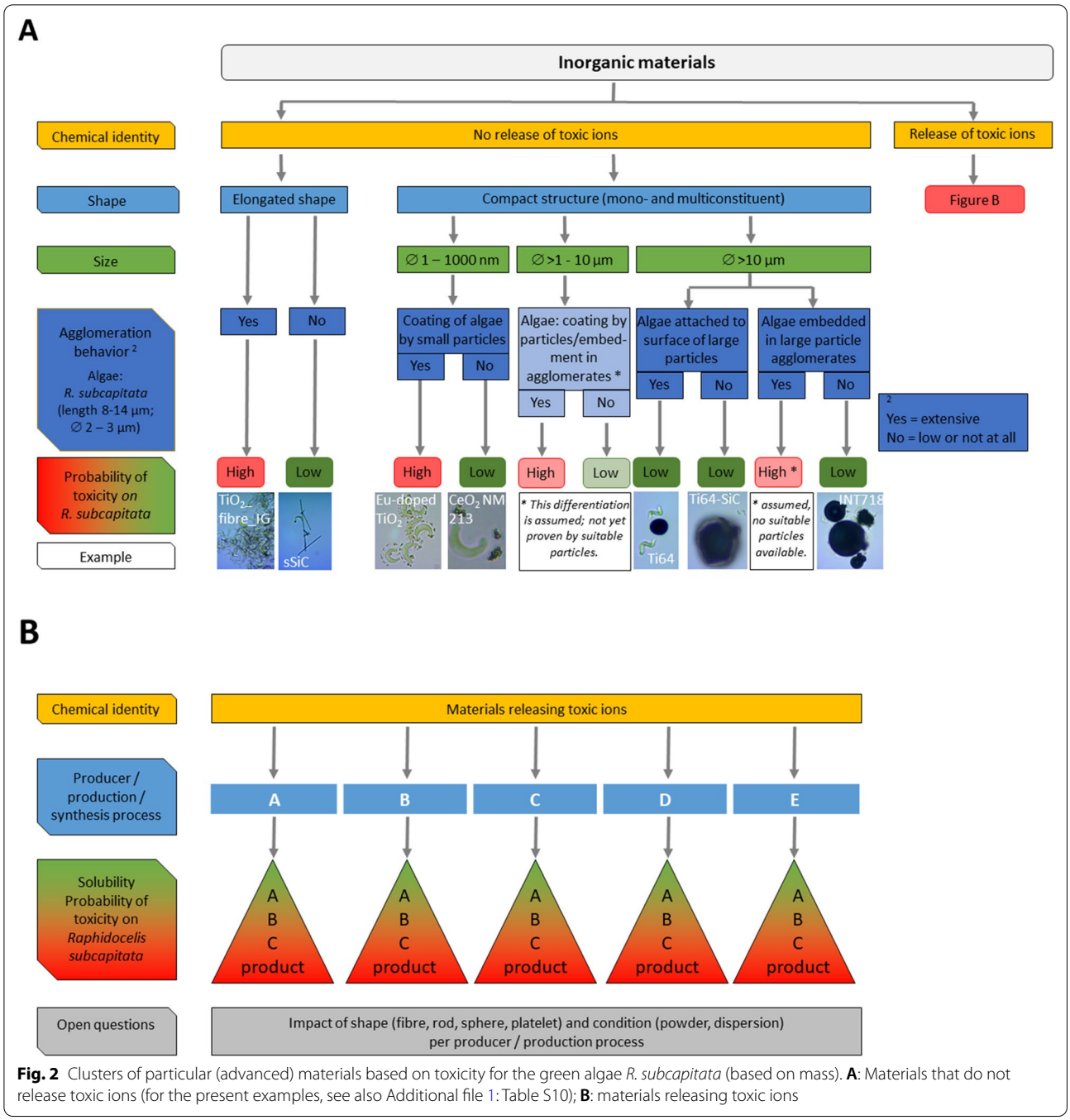



**Fig. 2** Clusters of particular (advanced) materials based on toxicity for the green algae *R. subcapitata* (based on mass). **A**: Materials that do not release toxic ions (for the present examples, see also Additional file 1: Table S10); **B**: materials releasing toxic ions

et al. [1] demonstrated that particle shapes and sizes did not influence cellular association if particle number was considered as a dose metric. In this study, a relationship to ecotoxicity was not investigated.

Parameters such as size, shape and reactivity have been discussed in the literature as driving factors for nanomaterial ecotoxicity [2, 10], George S. 2012; [31, 45]. For example, Chithrani et al. [10] demonstrated with an investigation of gold nanoparticles with different sizes (14, 50, 74 nm) and shapes that kinetics and saturation concentrations in cells depend on size. Auffan et al. [2] demonstrated the stronger effect of silver nanoplates compared to spheroidal Ag on *Gammarus fossarum* (Crustacea, Amphipoda) due to the high level of crystal defects affecting reactivity. Such small differences in size as well as the observed differences in morphology related to reactivity were not obvious in our study. We assume that their impact would become more

obvious if materials differing by only one property were investigated.

#### Material parameters related to toxicity in *D. magna*

For the materials not releasing toxic ions, morphology, size, reactivity and composition did not impact toxicity, and all inert materials were considered nontoxic to *D. magna*; no $EC_{50}$ values were determined up to a testing concentration of 1 g/L (Additional file 1: Table S8). For the hybrid materials, despite their novel functionalities, the combination of individual, nontoxic materials into materials with complex compositions did not lead to increased toxicity for *D. magna* after 48 h. The hybrid materials tested in this study are likewise considered nontoxic (Additional file 1: Table S9).

Toxic ion-releasing materials are the only ones exerting toxicities over several orders of magnitude in the acute 48 h assay with *D. magna*. The observed ecotoxicities varied across four orders of magnitude. However, specific parameters indicating toxicity, and hence usability for grouping or classification of the materials of interest, were not identified for any of the three materials (Ag-based, Cu-based, Zn-based) considered. Clearly, differences in morphology and dimensions, as well as reactivity, modified the toxicity, but no clear relationship between any of the parameters and ecotoxicity was established. For the silver fibres, there was some indication of an unknown effect for the silver suspension, since the two most toxic silver materials were from the same producer. However, we were not able to determine which component of the suspension caused this high toxicity. Testing the supernatants alone without particles indicated no toxicity (data not shown). Literature data indeed indicate a stronger effect for silver material manufacturing and/or dispersant used compared to the effect of particle dimensions for *D. magna* toxicity [11, 57]. Interestingly, one study likewise demonstrated that released Ag ions and capping agents from the filtrates of Ag ENM solutions had no significant toxic effects on the survival and mobility of Daphnia [11]. In general, our ecotoxicity data for the three toxic ion-releasing materials are in line with literature data [7, 51, 52].

Internalisation of particles with broad ranges of sizes and shapes was observed, consistent with the nonselective feeding mechanism of *D. magna* [21]. There is no definite size limit for particle internalisation reported in the scientific literature, one paper, for example, showed internalisation of prey with a diameter of 50 µm [44]. No difference was found for internalisation of ZnO particles with sizes between 20 and 300 nm [51]. There are some indications that prey shape influences uptake, e.g. spheroidal or oval shaped. Uptake of 63–75 µm fluorescent PE particles by *D. magna* was demonstrated, without acute toxic effects [9]. No uptake by *D. magna* was demonstrated for 90 µm plastic particles [53] or for 100 µm PE particles [47]. However, it was stated that uptake was influenced by both particle size and particle availability (e.g. if floating) as well as the life stage of the animal (internalised particle size increases with animal size). For our assays, only neonates less than 24 h old were used, but even for these animals, differences in body size were already evident. Further growth took place during the 48-h test duration. Roughly, there seems to be a cut-off at approximately 100 µm in diameter for filtration [34], but one must keep in mind that the sizes of the particles studied here were broadly distributed, and it may therefore be possible that only a fraction of the particles smaller than ~ 100 µm was internalised.

Hence, from the materials tested in this study with *D. magna*, the only suitable parameter that could serve as an indicator for ecotoxicity is release of toxic ions. This is considered in the cluster scheme proposal shown in Fig. 3, which suggests that the acute toxicity of inert materials towards *D. magna* is low, whereas high acute toxicity is to be expected for toxic ion-releasing materials. Particles that cannot be tested due to sedimentation, such as the silver flakes, are not considered.

## Conclusion

In total, 36 nano- and micromaterials differing in chemical identities, sizes, morphologies and complexity were analytically characterised and tested in the growth test with algae [42] as well as in the immobilisation test with daphnia [41]. The following conclusions were drawn regarding indicators for toxicity and clustering as well as prioritisation of the particles: (i) indicators for toxicity differ for algae and daphnia. Thus, materials of concern can differ for the organisms. (ii) *R. subcapitata*: properties such as chemical identity (toxic ion-releasing materials vs. other materials), morphology (elongated materials vs. compact materials), size (nm vs. µm range) and agglomeration behaviour must be considered. Toxic nanomaterials whose toxicity is not based on the release of toxic ions are not bioavailable as components in complex material compositions (e.g. substituted alloys). If the other components are nontoxic, the complex material is also nontoxic. (iii) *D. magna*: toxic ion-releasing materials are the only ones exerting toxicities ranging over several orders of magnitude in the acute 48 h assay. Neither shape nor size, complex chemical composition or additional physical–chemical properties such as reactivity or zeta-potential in the test medium influence the toxicity. Ingestion of materials does not give rise to concern. (iv) For both test organisms, charts for AdMa indicating the expected toxicity are suggested. (v) Clustering of particles releasing toxic ions is still limited, and further studies are

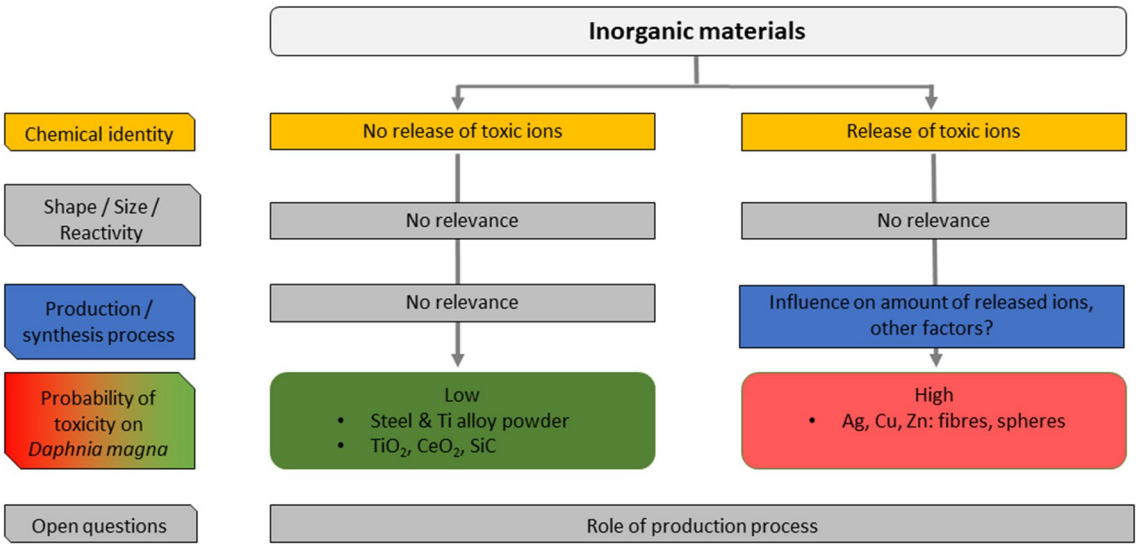



**Fig. 3** Clusters for particular (advanced) materials based on toxicity for *D. magna*

required to improve their assessment. For clustering the particles, the impacts of morphology, condition (powder, dispersion) and production process, including the chemicals used, must be investigated in studies with algae. For daphnia, the impacts of material production processes are still unknown.

In this study, we focused on assessment and clustering of the initial substances. For a comprehensive environmental risk assessment, it should be determined whether the effects of additional parameters, such as ageing or interactions with natural or anthropogenic substances, follow general principles and can be integrated into a clustering scheme for the particles.

## Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12302-022-00695-z.

**Additional file 1: Figure S1.** Principal component analysis (PCA) of materials. (A) PCA based on size, zeta-potential, solubility, and EC50 values in algae for releasing no or only nontoxic ions (named as stable) materials (red) and those releasing toxic ions (blue). (B) PCA of stable materials based on the values for size, zeta-potential, and $EC_{50}$ in algae. Colouring indicates the morphology of the material. (C) Same as in (B) but for materials releasing toxic ions. Here, solubility was taken into account. **Table S1** Material selection and underlying objective. **Table S2** Physicochemical properties measured to characterise the advanced materials and the corresponding detection method. **Table S3** Characterisation data for stable materials (additional data for fibres, see Table S5; used methods, see Table S2). **Table S4** Characterisation data for materials that release toxic ions (additional data for fibres, see Table S5; used methods, see Table S2). **Table S5** Size distributions for the fibres (lengths and diameters). **Table S6** Inhibition of R. subcapitata growth determined for materials tested with concentrations differing by a spacing factor of 10. **Table S7** Ag nano- and microparticles - ecotoxicity on algae and selected physicochemical properties sorted by producer and ecotoxicity (for ecotoxicities of the nanoGAP-materials, the effect at the highest test concentration with less than 100% is used). **Table S8** Ecotoxicity of inert nano- and micromaterials with different morphologies towards D. magna. The physical–chemical properties of the materials are listed in Table S1. **Table S9** Ecotoxicity and uptake of materials of complex composition compared to individual-component analogues in D. magna. The physical–chemical properties of the materials are listed in Table S1. **Table S10** Enlarged images showing the examples of agglomeration behaviour for particles and R. subcapitata presented in Figure 2A.

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All data generated during the current study are included in this published article.

## Declarations

**Ethics approval and consent to participate**
Not applicable.

**Consent for publication**
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**Competing interests**
The authors declare that they have no competing interests.

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