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Considerations about the relationship of nanomaterial's physical-chemical properties and aquatic toxicity for the purpose of grouping.

by

Fraunhofer Institute for Molecular Biology and Applied Ecology; Auf dem Aberg 1,
57392 Schmallenberg

Kerstin Hund-Rinke

In cooperation with

Institute of Energy and Environmental Technology (IUTA) e.V., Bliersheimer Straße 58-
60, 47229 Duisburg

Carmen Nickel

and

Helmholtz Centre for Environmental Research (UFZ), Permoserstr. 15, 04318 Leipzig

Dana Kühnel

On behalf of the German Environment Agency

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Abstract

The project aimed at the development of a concept for the grouping of engineered nanomaterials (NMs) with regard to their ecotoxicological effects on algae, daphnids and fish embryos. The project was structured into five steps. First, fourteen nanomaterials were selected which were different subtypes of Ag, ZnO, TiO₂, CeO₂ and Cu. In a second step, their physico-chemical properties were determined in water and in all relevant test media. Based on these results hypotheses regarding the expected ecotoxicity were formulated (third step). In a fourth step, the hypotheses were verified by testing the selected NMs in three aquatic ecotoxicological tests. Finally, step five consisted of the compilation of a grouping concept based on NM physico-chemical parameters which were identified as relevant for the emergence of a toxic effect in aquatic organisms.

Based on the results of the measurements and expert knowledge, morphology, stability (ion release, crystalline structure) were identified as relevant PC-parameters. A grouping scheme and procedure was proposed considering these parameters and the ecotoxicity of the chemical composition itself. It was realized that it is impossible to build meaningful grouping hypotheses based on one physico-chemical parameter. Rather, sets of parameters, and probably also additional physico-chemical parameters next to those investigated in this project need to be considered.

In order to further advance grouping concepts for regulatory testing, future developments with respect to research on grouping concepts should include (i) surface modifications which were excluded in the present project, (ii) the substitution of the fish embryo test which reveals to be of low sensitivity, (iii) adaptation of the methods for the determination of the reactivity as no relationship between ecotoxicity and the results of the applied methods on reactivity were observed, (iv) a detailed analysis of the kinetic of selected PC-parameters during the tests such as agglomeration behavior, zeta-potential, reactivity and solubility. Furthermore, (v) the number of effect values per NM and test organism has to be increased to achieve more robust datasets for statistical analyses.

Kurzbeschreibung

Projektziel war die Entwicklung eines Konzeptes, um Nanomaterialien (NM) hinsichtlich ihrer Ökotoxizität für Algen, Daphnien, und den Fischembryo zu gruppieren. Dabei wurden fünf Arbeitsschritte durchlaufen: (i) Auswahl von insgesamt 14 NM, die sich auf die Materialtypen Ag, ZnO, TiO₂, CeO₂, und Cu aufteilten; (ii) umfassende physikalisch-chemische Charakterisierung aller Materialien in Wasser und den drei Testmedien; (iii) Entwicklung von Hypothesen zur erwarteten Ökotoxizität; (iv) ökotoxikologische Testung aller NM in den drei ausgewählten Testsystemen; (v) Erprobung verschiedener Gruppierungsansätze auf Basis der physikalisch-chemischen Parameter (PC-Parameter), die als relevant für die aquatische Ökotoxizität identifiziert worden waren.

Als relevant wurden Morphologie, Stabilität (Ionenfreisetzung, Kristallstruktur) und die Ökotoxizität der chemischen Verbindung identifiziert und darauf basierend ein Schema zur Gruppierung vorgeschlagen. Es ist jedoch nicht auszuschließen, dass weitere Parameter zu berücksichtigen sind. Es zeigte sich weiterhin, dass keine sinnvolle Gruppierungshypothese auf einem einzelnen PC-Parameter beruhen kann. Für eine sinnvolle Gruppierung ist ein Set von Parametern notwendig.

Um das vorgeschlagene Gruppierungskonzept im Hinblick auf die regulatorische Anwendung zukünftig weiterzuentwickeln sind folgende Aspekte zu berücksichtigen: (i) gezielte Berücksichtigung von Oberflächenmodifikationen, die bewusst bei dem Projekt ausgeschlossen worden waren; (ii) Ersatz des Fischembryotests aufgrund seiner geringen Sensitivität; (iii) Anpassung der Methoden zur Bestimmung der Oberflächenreaktivität, da keine Übereinstimmung zwischen den entsprechenden Messwerten und der Ökotoxizität ermittelt wurde; (iv) die Kinetik ausgewählter PC-Parameter (Agglomerationsverhalten; Zeta-Potential, Reaktivität, Löslichkeit) im Test. Ferner wird eine größere Anzahl an EC-Werten benötigt, um die Aussagekraft der Statistik zu erhöhen.

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List of Abbreviations

ADaM	Aachener Daphnien Medium
BET	Brunauer-Emmett-Teller method
CNT(s)	Carbon nanotube(s)
DI	Deionized water
DLS	Dynamic Light Scattering
EC_x	Effect concentration, - indicating the concentration of a NM which exert x % of the maximum effect observed in a particular test organism and endpoint
ECETOC	European Centre for Ecotoxicology and Toxicology of Chemicals
ELS	Electrophoretic Light Scattering
EPR	Electron Paramagnetic Resonance Spectroscopy
ICP MS	Inductively coupled plasma mass spectrometry
NM(s)	Nanomaterial(s)
IEP	Isoelectrical point
ISO water	Zebrafish embryo rearing medium according to ISO 7346-3
OECD	Organisation for Economic Co-operation and Development
OECD medium	Medium for the test with algae according to OECD guideline 201
PC	Physico-chemical parameters
PDI	Polydispersity index
PNEC	Predicted non effect concentration
ROS	Reactive oxygen species
SEM	Scanning Electron Microscopy
Size_DLS / z.average	Average hydrodynamic diameter based on DLS measurements and method of cumulants
Size_DLS:D50	Median of the hydrodynamic diameter based on DLS measurements and Contin algorithm
“stable” NMs	In contrast to the NMs Ag, Cu and ZnO which release ions in significant amounts, for TiO ₂ and CeO ₂ “stable” is used as general term in this project.
Toxicity profile	Result of ranking the sensitivity of the three applied test organisms for a specific NM
Toxicity value	Effect concentration indicating the toxicity of a NM on a specific test organism and endpoint
ZP	Zeta potential

Summary

Manufactured nanomaterials (NMs) are being developed in many different variations such as size, shape, crystalline structure and surface modifications. So far the knowledge is still limited how the modifications affect ecotoxicity. To avoid the testing of each single nanomaterial modification, grouping and read across strategies for nanomaterials similar to classical chemicals are discussed. Grouping and read across aim to identify substance groups with analogous sets of properties or parameters that enable reasonable predictions of a NM hazard without additional testing. Whereas various approaches for grouping are proposed regarding human toxicity, the approaches and considerations regarding ecotoxicological grouping are limited.

The present project aimed at the development of a concept for the grouping of engineered NMs with regard to their ecotoxicological effects with focus on aquatic ecotoxicity. Following test organisms and test systems were selected by the German Environment Agency:

- ▶ Growth on green algae (OECD Guideline 201, 2011)
- ▶ Immobilization of daphnids (OECD Guideline 202, 2004)
- ▶ Fish embryo test (OECD Guideline 236, 2013)

The project was structured into five steps. First, fourteen nanomaterials were selected according to pre-defined criteria. The selected NMs were different subtypes of Ag, ZnO, TiO₂, CeO₂ and Cu. In a second step, their physico-chemical properties were determined in water and in all test media. Based on the results hypotheses regarding the expected ecotoxicity were formulated (third step). In a fourth step, the hypotheses were verified by testing the selected NMs in three ecotoxicological tests. Finally, step five consisted of the compilation of a grouping concept based on NM physico-chemical parameters which were identified as relevant for the emergence of a toxic effect in aquatic organisms.

Following nanomaterials had been selected together with the German Environment Agency. The list includes ion-releasing as well as "stable" NMs which differed in size and shape, crystalline structure, reactivity and zeta-potential. Specific surface modifications were excluded in order to avoid grouping dominated by surface specifics in this first attempt of grouping regarding ecotoxic effects.

Ag

- ▶ SRM 110525: wire, provided by the company „Rent a scientist“
- ▶ Batch 1340: nanowire, provided by the company „Rent a scientist“
- ▶ NM-300K: spherical nanomaterial, selected for the OECD Sponsorship Programme

TiO₂

- ▶ Doped with Eu (5 %), provided by IUTA
- ▶ Doped with Fe (5 %), provided by IUTA
- ▶ undoped, provided by IUTA

ZnO

- ▶ NM-110, selected for the OECD Sponsorship Programme
- ▶ NM-111, selected for the OECD Sponsorship Programme
- ▶ NM-113, selected for the OECD Sponsorship Programme

CeO₂

- ▶ Doped with Eu (5 %), provided by IUTA
- ▶ NM-211, selected for the OECD Sponsorship Programme
- ▶ NM-212, selected for the OECD Sponsorship Programme
- ▶ NM-213, selected for the OECD Sponsorship Programme

Cu

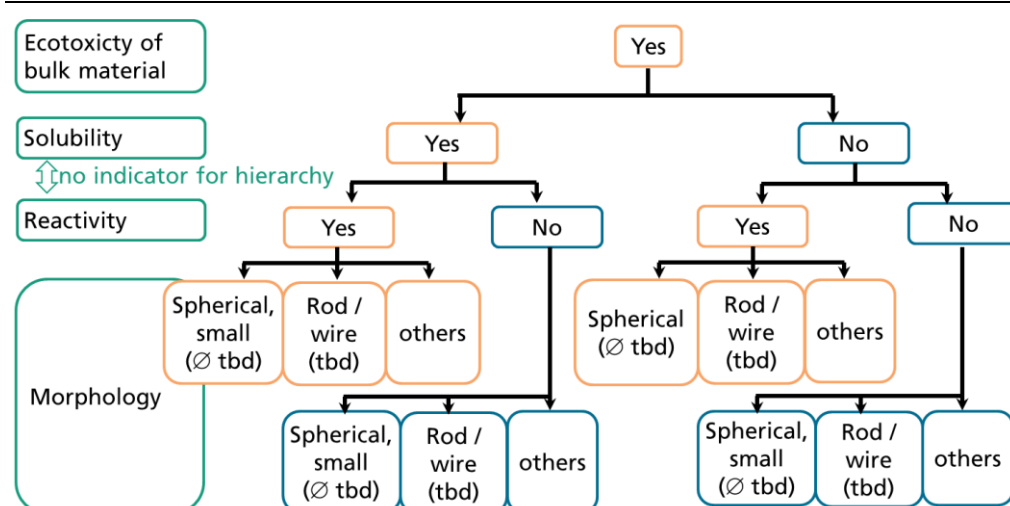
- ▶ Cu(0), provided by IUTA for comparison with other ion releasing NM with an expected high toxicity

Numerous statistical approaches were applied to the results of the comprehensive physico-chemical characterization as well as to a combination of these parameters and the EC50 values determined for the three test species. The combination of both was used to identify whether the grouping just on physico-chemical properties can be justified. If grouping with the two approaches (i) just on the PC-properties and (ii) on PC-properties and effect data result in the same groups, it can be concluded that the considered PC-parameters are the relevant ones.

Additionally, the NMs were grouped based on ecotoxicological data only by expert judgement.

A promising approach was a new flow-scheme, inspired by the ECETOC-approach (Arts et al., 2015; Arts et al., 2016). As the ECETOC approach was developed for the grouping regarding human toxicity, a specific scheme regarding ecotoxicity is required. In this scheme the physico-chemical parameters identified by the testing of the fourteen NMs are included. Additionally, as first step, the ecotoxicity of the bulk material or of NMs with the same chemical composition is considered. In the following the step-wise approach is presented for NMs with known ecotoxicological effect based on composition (Figure 1). The same scheme is applicable to NMs with known non-toxic chemical composition. If the information is missing, the NM has to be treated as toxic material.

Figure 1: Ecotox-scheme for the grouping of NMs (tbd = has still to be defined). The same scheme applies for materials with no toxicity of the bulk material.



In the following the findings and conclusions are summarized:

Grouping of the selected nanomaterials

- ▶ Based on the developed ecotox-scheme, six groups could be formed:
 - Ion releasing nanomaterials with DMPO/CPH reactivity and other morphology: Ag NM-300K, Cu.
 - Ion releasing nanomaterials with DMPO/CPH reactivity, wire: Ag, Batch 1340.
 - Ion releasing nanomaterials without DMPO/CPH reactivity, wire: Ag, SRM 110525.
 - Ion releasing nanomaterials without DMPO/CPH reactivity, other morphology: the three investigated ZnO nanomaterials.
 - Non-ion releasing nanomaterials, without DMPO/CPH reactivity and other morphology: the investigated four CeO₂ nanomaterials.
 - Non-ion releasing nanomaterials, with DMPO/CPH reactivity and other morphology: the investigated three TiO₂ nanomaterials.
- ▶ With exception of silver, the differences between the sub-types of the nanomaterials with same chemical composition were too small, to result in different groups. It seems that the differences between the nanomaterials of one chemical composition must be more pronounced than the selected ones to result in a significant different ecotoxicity.

Physico-chemical (PC)-parameters

- ▶ The results for the PC-parameters of the tested NMs differed between the various test media (water, ISO water for fish embryo test, OECD-medium for algae, ADaM for daphnids). Thus, for predictions of the ecotoxicity towards a specific organism only values determined in the relevant test medium are suitable.
- ▶ Due to the low evidence regarding the relationships between PC-parameters and ecotoxicity we conclude that the usually discussed parameters are not sufficient to explain or even predict the ecotoxicity. For example, in the case of this study the solubility of the NMs as sole indicator is not suitable. ZnO was completely dissolved within a period of 72 h, whereas the dissolved ratio of Ag was much smaller. Nevertheless the toxicity of ZnO was as expected to be lower compared to that of Ag, due to the different toxicity of the chemical composition (Okamoto et al., 2015). Additionally, a comparison within one chemical group like the silver NM indicates that the NM with the highest solubility did not show the highest effects. Therefore, the effect data of the NM with the highest solubility cannot be used as worst case approach. The toxicity of the chemical substance and further parameters such as shape have to be considered.
- ▶ The influence of the zeta potential in an ecotoxicological test seems to be comparably small. Further indicators seem to be of higher relevance. Examples are the results with ZnO. Negative and positive zeta potentials were determined but a relationship to ecotoxicity is not obvious. However, in the algae medium (OECD medium) we measured always high negative zeta potentials and observed always toxic effects. But especially in this test stability of the NMs seems to be of lower relevance as NMs and algae are in contact throughout the test due to extensive shaking.
- ▶ We assume *surface-reactivity* to be one reason for the observed ecotoxicity of the NMs TiO₂ and CeO₂. There is a need to identify or modify an existing method that the results correlate with the measured ecotoxicity which is assumed to be based on surface reactivity.
- ▶ The *morphology* of the NMs seems to be relevant. However, the differentiation in spherical and rods / wires is not sufficient (see also below: test species – daphnids). Threshold values have to be defined to indicate spheres and wires with a high toxic potential. A third group which comprises the remaining NMs is required.
- ▶ Several nanomaterials carried a *doping*. It is obvious that doping as sole information is not sufficient to indicate ecotoxicity. For TiO₂, doping modifies the crystalline structure followed by a modified ecotoxicity. For CeO₂ such a relationship is not obvious. The ecotoxicity of the E doped CeO₂ was comparable to the ecotoxicity of two non-doped CeO₂, but a third non-doped CeO₂ behaved differently.
- ▶ For NMs, obviously a set of parameters needs to be considered. The individual parameter has to be weighted taken the various test organisms and their behavior and the corresponding test media into account.
- ▶ Statistical analyses can be a useful tool to identify PC-parameters relevant for ecotoxicological effects. Statistical analyses provide only useful relationships if they are based on a large data set. The data set of this project is limited and has to be extended for robust conclusions.

Test species

- ▶ The test species behave differently regarding the ranking of the NMs. Therefore, they have to be treated separately and need to be considered separately in terms of reasoning for a certain grouping and read-across.
- ▶ The bioavailability of NM is linked to their behavior in the different test media. This actually means that data on toxicity of one NM in one test species cannot be used to forecast the toxicity of another NM of the same chemical substance for another test species.
- ▶ The *fish embryo test* turned out to be rather insensitive and for the assessment of NMs modifications such as dechoriation may be considered (e.g. (Bodewein et al., 2016); Henn

and Braunbeck (2011)). Otherwise, chronic fish tests have to be performed even if animal testing should be minimized. However, it has to be considered that a test period of 72 h was used in the project (instead of 96 h).

- ▶ *Algae* turned out to be most sensitive test species. We do not assume that shading due to turbid test dispersions is the reason. The effects were observed even in low test concentrations without significant turbidity.
- ▶ *Daphnids* are sensitive to thin and long Ag wire NMs. The range of critical dimensions still has to be investigated.

Ranking / Grouping based on statistical analyses

- ▶ Although various correlations between the selected PC-parameters (agglomerate size, primary particle size, zeta potential, BET surface, solubility, reactivity based on CPH and DMPO) could be calculated using various statistical approaches, the cluster analyses resulted in inconsistent results although it was performed independently for every test medium. The groups are not comprehensible regarding the ecotoxicity as ion releasing and “stable” NMs are mixed in the groups although obvious differences can be observed in the ecotoxicity. Besides a larger volume of data (robust data set) and an improved selection of PC-parameters, a reason might be the different conditions in the ecotoxicological testing and the determination of the PC-parameters. Influencing factors such as test organisms, their movement and exudates, illumination as well as turbulence such as shaking or stirring are not or to a lower extent considered in the determination of the PC-parameters. Up to now it is not possible to predict the ecotoxicity based on a routine statistical analysis of PC-properties. Expert-knowledge regarding the interaction of the various parameters is still required.

Ranking / Grouping based on ecotoxicological profiles

- ▶ Grouping / ranking based only on ecotoxicological data and profiles resulted in only rough categories. However, ecotoxicological profiles support the identification and weighting of PC-parameters relevant for ecotoxicity. Ion-releasing and “stable” NMs can be differentiated. For ion-releasing NMs of different chemical nature the difference in ecotoxicity can mainly be related to the toxicity of the chemical substance.
- ▶ However, grouping solely based on ecotoxicological data will not support the rationale for *read across*. For the aim of read-across, data on PC properties for comparison are needed to waive data acquisition on ecotoxicity of every member of one group.

Ranking / Grouping based on PC-parameters and ecotoxicity data

- ▶ The utilization of existing approaches to group NM is not possible with respect to environmental conditions. Specification and adaptations to ecotoxicological data would be required for the grouping of NMs.
- ▶ The flow chart developed for an ecotoxicological grouping within this project would need specification with regard to some parameters, e.g. solubility and reactivity levels. Additionally, the shape of the rods / wires resulting in increased ecotoxicity to daphnids has to be defined. For a hazard ranking scientifically based but also pragmatic threshold values are required. In any case, next to correlations between PC parameters and ecotoxicity, always expert judgment will be an essential element to judge on the grouping on a case by case basis.

Recommendations

Several recommendations shall support the further development of the grouping / read across approach regarding ecotoxicity:

- ▶ Adaptation of methods characterizing reactivity which relates to ecotoxicity of surface reactive NMs.
- ▶ Increased number of robust datasets for statistical analyses (effect values (recommended: EC50) for the same test systems; PC-parameters for the NMs in each medium)
- ▶ Improved measurement of the exposure concentration (better recovery)
- ▶ A detailed investigation of the effect of the morphology by using different NM types with the same morphology like ZnO or TiO₂ rods / wires
- ▶ A detailed characterization of the kinetics of solubility, zeta potential, reactivity and agglomeration is necessary, as no clear relationship between the measured PC-parameters and toxicity was observed. It seems possible that (i) the toxic response is the result of a combination of parameters (ii) not all relevant parameters important for the ecotoxicity were measured and (iii) measurements of PC-parameters without organisms and at one time point (as performed in this study) can be misleading, as indicated by the results of the soluble fraction in the tests with algae.
- ▶ In this project the zeta potential was identified by the statistical analyses as a potential parameter which can be related to the observed effect. However, in the test media most of the NMs had a zeta potential in the range indicating instable dispersions (-15 to +15 mV) with focus on negative values. Therefore, no clear correlation between the zeta potential and the effect could be identified. To determine if the zeta potential has an effect on the toxicity it would be necessary to test further NMs with a negative and positive zeta potential outside of this range.
- ▶ Verification of the presented ecotox-scheme with further NMs.

Zusammenfassung

Technisch hergestellte Nanomaterialien (NM) werden mit sehr variablen Eigenschaften in Bezug auf ihre Größe, Form, Kristallstruktur oder Oberflächenmodifikationen produziert. Bisher ist das Wissen über den Einfluss dieser Eigenschaften auf die ökotoxikologische Wirkung von NM begrenzt. Um den Testaufwand für diese Anzahl an einzelnen NM zu begrenzen, werden gegenwärtig Gruppierungs- bzw. *read across*-Ansätze in Analogie zur Chemikalienbewertung diskutiert. Sowohl die Gruppierung als auch *read across* zielen darauf ab, durch die Identifikation von bestimmten Eigenschaften oder Parametern eine verlässliche Vorhersage über Gefährdungen durch NM einer Gruppe ohne zusätzliche Testung zu erlauben und auf diese Art Übertragung von Daten von einem Mitglied einer Gruppe auf ein anderes zuzulassen (*read across*). Für eine Gruppierung von NM nach ökotoxikologischen Gesichtspunkten gibt es bisher nur wenige Ansätze, während für das Gebiet der Humantoxikologie bereits einige Gruppierungsvorschläge unterbreitet wurden.

Daher war es das Ziel dieses Projektes ein Gruppierungskonzept für technisch hergestellte NM mit Bezug zu ihren ökotoxikologischen Effekten und besonderem Fokus auf die aquatische Ökotoxikologie zu entwickeln. Die folgenden Testorganismen und Testsysteme wurden vom Umweltbundesamt (UBA) für diesen Zweck ausgewählt:

- ▶ Algeninhibitionstest (OECD Richtlinie 201)
- ▶ Akuter Immobilisierungstest mit Daphnien (OECD Richtlinie 202)
- ▶ Zebrafischembryo-Toxizitätstest (OECD Richtlinie 236)

Das Projekt bestand aus 5 Arbeitsschritten. Im ersten Schritt erfolgte nach definierten Kriterien die Auswahl von 14 NM. Die ausgewählten NM enthielten verschiedene Subtypen aus Ag, ZnO, TiO₂, CeO₂ sowie Cu. Im zweiten Schritt wurden die physikalisch-chemischen Eigenschaften aller NM sowohl in Wasser als auch in den jeweiligen Testmedien bestimmt. Basierend auf den bestimmten Eigenschaften wurden Hypothesen hinsichtlich der Ökotoxizität der 14 NM formuliert (Arbeitsschritt 3). In einem vierten Schritt erfolgte die Überprüfung der aufgestellten Hypothesen mittels der Durchführung der ökotoxikologischen Versuche. Im letzten Arbeitsschritt erfolgte die Erstellung eines Gruppierungskonzeptes basierend auf den physikalisch-chemischen Eigenschaften, welche für die Entstehung einer toxischen Wirkung in aquatischen Organismen als relevant identifiziert wurden.

Die nachfolgend aufgelisteten NM wurden für dieses Projekt vom Umweltbundesamt (UBA) ausgewählt und beinhalten sowohl Ionen-freisetzende als auch weitere NM, die sich jeweils in Größe, Form, Kristallstruktur, Reaktivität und Zeta-Potential unterschieden. Explizit ausgeschlossen waren NM mit Oberflächenmodifikationen.

Ag

- ▶ SRM 110525: Draht, zu Verfügung gestellt durch „Rent a scientist“
- ▶ Batch 1340: Nanodraht, zu Verfügung gestellt durch „Rent a scientist“
- ▶ NM-300K: sphärisches NM, aus dem OECD Sponsorship Programme

TiO₂

- ▶ Eu-dotiert (5 %), zu Verfügung gestellt von IUTA
- ▶ Fe-dotiert (5 %), zu Verfügung gestellt von IUTA
- ▶ undotiert, zu Verfügung gestellt von IUTA

ZnO

- ▶ NM-110, aus dem OECD Sponsorship Programme
- ▶ NM-111, aus dem OECD Sponsorship Programme
- ▶ NM-113, aus dem OECD Sponsorship Programme

CeO₂

- ▶ Eu-dotiert (5 %), zu Verfügung gestellt von IUTA
- ▶ NM-211, aus dem OECD Sponsorship Programme

- ▶ NM-212, aus dem OECD Sponsorship Programme
- ▶ NM-213, aus dem OECD Sponsorship Programme

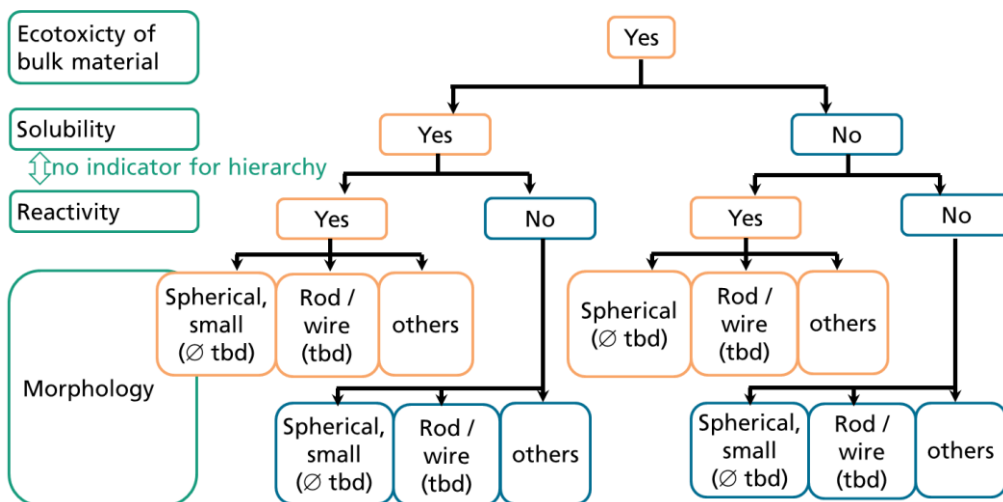
Cu

- ▶ Cu(0), zu Verfügung gestellt von IUTA, als ionenfreisetzendes Vergleichsmaterial mit einer erwarteten hohen Toxizität

Der umfassende Datensatz an physiko-chemischen Parametern wurde verschiedenen statistischen Verfahren unterzogen, um mögliche Gruppierungen zu identifizieren. In einem zweiten Schritt wurden die ermittelten Ökotoxizitätswerte in die statistische Auswertung einbezogen. Ergibt sich keine Veränderung in den auf Basis der physiko-chemischen Daten ermittelten Gruppierungen, kann dies als Indiz gewertet werden, dass die verwendeten physiko-chemischen Daten die Ökotoxizität bedingen.

Zusätzlich wurden die ökotoxikologischen Testergebnisse über Expertenbeurteilung gruppiert.

Als vielversprechender Ansatz wurde das unten stehende Flussschema entworfen, welches durch den ECETOC-Ansatz inspiriert ist (Arts et al., 2016). Da dieser Ansatz für eine Gruppierung hinsichtlich Humantoxikologie entwickelt wurde, waren spezifische Anpassungen bezüglich Ökotoxikologie erforderlich. Diese wurden im Schema basierend auf den durch die Testung identifizierten physikalisch-chemischen Parametern umgesetzt. Im ersten Schritt wird eine bekannte oder ermittelte Ökotoxizität entweder des NM oder des nicht-nanoskaligen Materials berücksichtigt. Unten dargestellt ist das Schema für NM deren ökotoxikologische Wirkung aufgrund des Ausgangsmaterials bereits bekannt ist. Das analoge Schema ist auch auf NM aus bekanntermaßen nicht-toxischen Materialien anwendbar. Liegt keinerlei Information zur Toxizität vor, wird das NM als toxisches Material behandelt.



Im Folgenden sind die wesentlichen Ergebnisse und Schlussfolgerungen zusammengefasst:

Gruppierung der ausgewählten Nanomaterialien

- ▶ Unter Verwendung des entwickelten Ökotox-Schemas ließen sich aus den untersuchten Nanomaterialien sechs Gruppen bilden:
 - Ionenfreisetzende Nanomaterialien mit DMPO/CPH-Reaktivität und anderer Morphologie: Ag NM-300K, Cu.
 - Ionenfreisetzende Nanomaterialien mit DMPO/CPH -Reaktivität, Draht: Ag, Batch 1340.
 - Ionenfreisetzende Nanomaterialien ohne DMPO/CPH -Reaktivität, Draht: Ag, SRM 110525.
 - Ionenfreisetzende Nanomaterialien ohne DMPO/CPH -Reaktivität, andere Morphologie: alle ZnO Nanomaterialien.
 - Nicht-ionenfreisetzende Nanomaterialien, ohne DMPO/CPH -Reaktivität und anderer Morphologie: alle CeO₂ Nanomaterialien.

- Nicht-ionenfreisetzende Nanomaterialien, mit DMPO/CPH -Reaktivität und anderer Morphologie: alle TiO₂ Nanomaterialien.
- ▶ Mit Ausnahme von Silber waren die Sub-Typen der einzelnen Nanomaterialien gleicher chemischer Zusammensetzung zu gering, um zu einer Trennung in verschiedene Gruppen zu führen. Es scheint, dass die Unterschiede zwischen den Nanomaterialien einer chemischen Zusammensetzung deutlich größer sein müssen als die in dem vorliegenden Projekt vorliegenden, um eine veränderte Ökotoxizität hervorzurufen.

Physikalisch-chemische (PC-)Parameter

- ▶ Die Ergebnisse zeigen, dass sich die PC-Parameter in den verschiedenen Testmedien stark unterscheiden (Wasser, ISO-Wasser für den Zebrafischembyrotest, OECD-Medium für den Algentest, ADaM für den Daphnientest). Für Vorhersagen hinsichtlich der Ökotoxizität für einen bestimmten Organismus können nur die Werte aus dem jeweiligen Testmedium herangezogen werden.
- ▶ Es wurden wenig Zusammenhänge zwischen PC-Parametern und ökotoxikologischen Wirkungen gefunden, was zu der Schlussfolgerung führte, dass die ermittelten Parameter nicht ausreichend sind, um eine ökotoxische Wirkung zu erklären oder sogar vorherzusagen. Zum Beispiel erwies sich die Löslichkeit eines NM als ungeeigneter alleiniger Indikator. ZnO-Partikel etwa lösten sich innerhalb von 72 h vollständig auf, für Ag hingegen war der gelöste Anteil wesentlich geringer. Trotzdem war aufgrund der unterschiedlichen Toxizität der chemischen Ausgangssubstanzen wie erwartet die Toxizität des ZnO geringer als die des Ag, (Okamoto et al., 2015). Zudem zeigte eine Betrachtung der Effekte innerhalb einer chemischen Gruppe, wie zum Beispiel der Silber-NM, dass das löslichste NM nicht unbedingt die höchsten Effekte hervorrufen muss. Das bedeutet, dass das löslichste NM nicht als Worst Case Ansatz herangezogen werden kann. Neben der Toxizität der Ausgangssubstanz sollten auch weitere Parameter wie beispielsweise die Form berücksichtigt werden.
- ▶ Der Einfluss des Zeta-Potentials scheint hingegen in ökotoxikologischen Tests vergleichsweise gering zu sein und andere Indikatoren haben folglich eine größere Relevanz. Beispielsweise haben die verschiedenen ZnO-Subtypen sowohl negative als auch positive Werte für das Zeta-Potential, die jedoch in keinem offensichtlichen Zusammenhang zu den ökotoxikologischen Effekten stehen. Eine Ausnahme stellt hier das Algen(OECD-)medium dar, in welchem immer hohe negative Werte gemessen wurden und immer toxische Effekte auftraten. Aber gerade in diesem Testsystem wird der Stabilität der NM im Medium eine geringere Bedeutung beigemessen, da Algen und NM während des gesamten Tests durch Schütteln in Kontakt bleiben.
- ▶ Die Oberflächenreaktivität wird als ein Grund für die beobachteten ökotoxischen Effekte durch die NM TiO₂ und CeO₂ vermutet. Die Messmethode für die Reaktivität bedarf aus unserer Sicht einer näheren Betrachtung. Es ist eine Methode zu identifizieren oder eine bestehende Methode anzupassen, die mit einer Ökotoxizität korreliert, von der erwartet wird, dass sie auf Oberflächenreaktivität beruht.
- ▶ Die Morphologie der NM wird ebenfalls als relevant betrachtet. Eine Differenzierung in sphärische Partikel und Stäbchen/Drähte allein wird jedoch als nicht ausreichend betrachtet. (siehe auch unter: Testspezies – Daphnien). Schwellenwerte sind festzulegen, die besonders toxische sphärische und stäbchenförmige NM von einer dritten Gruppe abtrennen, in die die übrigen NM fallen könnten.
- ▶ Einige NM-Subtypen waren dotiert. Die Ergebnisse zeigen deutlich, dass eine Dotierung alleine keinen Aufschluss über eine mögliche ökotoxische Wirkung gibt. Für TiO₂ modifiziert eine Dotierung die Kristallstruktur und damit auch die ökotoxische Wirkung. Für CeO₂ wurde dieser Zusammenhang nicht beobachtet, da die Effekte für Eu-dotiertes CeO₂ vergleichbar zu zwei der

nicht-dotierten CeO₂ NM waren, während ein drittes nicht-dotiertes CeO₂ andere Effekte zeigte.

- ▶ Damit müssen für NM offenbar Kombinationen aus verschiedenen Parametern berücksichtigt werden. Der einzelne Parameter muss gewichtet werden, wobei die verschiedenen Testorganismen, die Testmedien und ihr Verhalten darin berücksichtigt werden müssen.
- ▶ Statistische Verfahren können nützlich sein, um physiko-chemische Parameter zu identifizieren, die die Ökotoxizität beeinflussen. Derartige Verfahren können jedoch nur bei einem großen Datensatz zielführend eingesetzt werden. Der Datensatz, der in diesem Projekt erhoben wurde und der den Auswertungen zugrunde lag, war limitiert und muss für robuste Aussagen erweitert werden.

Testspezies

- ▶ Die Testspezies verhalten sich hinsichtlich der Rangordnung der NM unterschiedlich. Sie müssen daher separat behandelt werden (auch im Sinne der Möglichkeiten und Grenzen der Datenübertragung im Falle von *read across*).
- ▶ Die Bioverfügbarkeit von NM ist mit ihrem Verhalten in den verschiedenen Testmedien verknüpft. Daher können Daten zur Toxizität eines Nanomaterials für einen bestimmten Organismus nicht ohne Weiteres verwendet werden, um die Toxizität für das gleiche Nanomaterial aber einen anderen Testorganismus abzuleiten.
- ▶ Der *Zebrafischembryo*-Test erwies sich als eher unempfindlich und für die Bewertung von NM sollten Modifikationen wie Dechorionierung in Betracht gezogen werden ((Bodewein et al., 2016); Henn and Braunbeck (2011)). Andernfalls müssen chronische Fischuntersuchungen durchgeführt werden, auch wenn Tierversuche minimiert werden sollten. Einschränkend muss jedoch gesagt werden, dass der Fischembryotest im vorliegenden Projekt nur mit einer Inkubationszeit von 72 h anstelle von 96 h durchgeführt wurde.
- ▶ *Algen* erwiesen sich als empfindlichste Testart. Wir gehen nicht davon aus, dass Abschattung aufgrund von trüben Testdispersionen der Grund ist. Die Effekte wurden auch bei niedrigen Testkonzentrationen ohne signifikante Trübung beobachtet.
- ▶ *Daphnien* sind empfindlich gegenüber dünnen und langen Ag-Drähten. Der Bereich der besonders kritischen Dimensionen muss noch untersucht werden.

Ranking / Gruppierung nach statistischen Analysen

- ▶ Obwohl verschiedene Korrelationen zwischen den ausgewählten PC-Parametern (Agglomeratgröße, Primärteilchengröße, Zeta-Potential, BET-Oberfläche, Löslichkeit, Reaktivität auf Basis von CPH und DMPO) unter Verwendung verschiedener statistischer Ansätze berechnet werden konnten, führten die Clusteranalysen zu inkonsistenten Ergebnissen, obwohl sie unabhängig für jedes Testmedium durchgeführt wurden. Die gebildeten Gruppen sind hinsichtlich der Ökotoxizität nicht plausibel, da Ionen freisetzende und nicht-ionenfreisetzende NM in den Gruppen gemischt werden, obwohl offensichtliche Unterschiede in der Ökotoxizität beobachtet werden können. Neben einer größeren Datenbasis (robustes Datenset) und einer verbesserten Auswahl von PC-Parametern können die Gründe in unterschiedlichen Bedingungen in der ökotoxikologischen Prüfung und der Bestimmung der PC-Parameter liegen. Einflussfaktoren wie Testorganismen, deren Bewegung und Ausscheidungsprodukte, Beleuchtung sowie Turbulenzen wie Schütteln oder Rühren wurden bei der Bestimmung der PC-Parameter nicht berücksichtigt. Bisher ist es nicht möglich, die Ökotoxizität auf Basis einer routinemäßigen statistischen Analyse vorherzusagen. Fachwissen über die Interaktion der verschiedenen Parameter ist noch erforderlich.

Ranking / Gruppierung nach ökotoxikologischen Profilen

- ▶ Gruppierung / Ranking, die nur auf den ökotoxikologischen Daten und Profilen basiert, führte zu nur groben Kategorien. Ökotoxikologische Profile unterstützen jedoch die Identifikation und Gewichtung der für die Ökotoxizität relevanten PC-Parameter. Ionenfreisetzende und weitere NM können unterschieden werden. Für die Ionen freisetzenden NM kann die unterschiedliche Ökotoxizität der chemischen Substanz erkannt werden.
- ▶ Gruppierung nur auf Basis von ökotoxikologischen Daten stellt keine Option für read-across dar, da Sub-Materialien einer chemischen Zusammensetzung nicht differenziert werden können.

Ökotox-Schema für Gruppierung

- ▶ Gruppierung auf Basis eines physiko-chemischen Parameters ist nicht möglich. Es müssen mehrere Parameter berücksichtigt werden. Ein Ansatz ähnlich dem für Humantoxizität (ECETOC Schema) wurde für Ökotoxizität entwickelt.
- ▶ Das für eine ökotoxikologische Gruppierung in diesem Projekt entwickelte Flussdiagramm würde eine Spezifikation in Bezug auf einige Parameter erfordern, z.B. Löslichkeit und Reaktivität. Zusätzlich muss die Form der Stäbe / Drähte, die zu einer erhöhten Ökotoxizität gegenüber Daphnien führen, definiert werden. Für eine Gefährdungsabschätzung sind wissenschaftlich fundierte, aber auch pragmatische Grenzwerte erforderlich. Neben Korrelationen von physiko-chemischen Parametern und Ökotoxizität ist jedoch immer noch Expertenwissen notwendig, um die Gruppierung in einer Einzelfallentscheidung zu verifizieren.

Ranking / Gruppierung nach PC-Parametern und Ökotoxischen Daten

- ▶ Die Anwendung existierender Gruppierungsansätze (zumeist bezogen auf Humantoxizität) für NM ist nicht direkt ohne Anpassungen auf ökotoxikologische Daten auf Umweltfragestellungen übertragbar.
- ▶ Einige Parameter (z.B. Löslichkeit und Reaktivität) des Fließschemas, das im Rahmen dieses Vorhabens entwickelt worden ist, müssen noch präzisiert werden. Weiterhin sind die Form der Stäbchen bzw. Drähte, die zu einer gesteigerten Ökotoxizität für Algen führen, zu definieren. Für ein Ranking des Gefährdungspotentials sind Schwellenwerte wissenschaftlich basiert, aber dennoch pragmatisch festzulegen. Über eine reine Korrelation von PC-Parametern und Ökotoxizität hinaus, wird immer Expertenwissen ein wesentliches Element bei der Gruppierung im Rahmen einer Einzelfallentscheidung benötigt werden.

Empfehlungen

Mehrere Empfehlungen sollen die Weiterentwicklung des Gruppierungs- / *read across* Ansatzes in Bezug auf Ökotoxizität unterstützen:

- ▶ Anpassung von Methoden, die die Reaktivität charakterisieren, die sich auf die Ökotoxizität oberflächenreaktiver NM bezieht.
- ▶ Erhöhte Anzahl robuster Datensätze für statistische Analysen (Effektwerte (empfohlen: EC50-Werte) für die gleichen Testsysteme, PC-Parameter für die NM in jedem Medium).
- ▶ Verbesserte Messung der Expositionskonzentration (bessere Wiederfindung).
- ▶ Eine detaillierte Untersuchung der Wirkung der Morphologie durch Verwendung verschiedener NM-Typen mit der gleichen Morphologie wie ZnO- oder TiO₂-Stäben / Drähte.
- ▶ Eine detaillierte Charakterisierung der Kinetik von Löslichkeit, Zeta-Potential, Reaktivität und Agglomerationsverhalten wird als notwendig erachtet, da keine eindeutige Beziehung zwischen den gemessenen PC-Parametern und der Toxizität beobachtet wurde. Es ist nicht auszuschließen, dass (i) die toxische Reaktion das Ergebnis einer Kombination von Parametern ist, (ii) nicht alle für die Ökotoxizität relevanten Parameter gemessen wurden, und (iii)

Messungen von PC-Parametern ohne Organismen und nur zu einem Zeitpunkt irreführend sein könnten, wie durch die Ergebnisse der löslichen Fraktion in den Tests mit Algen angezeigt. Daher ist eine detaillierte Charakterisierung der Kinetik von Löslichkeit, Zeta-Potential, Reaktivität und Agglomeration erforderlich.

- ▶ In diesem Projekt wurde das Zeta-Potential durch die statistischen Analysen als ein potentieller Parameter identifiziert, der mit dem beobachteten Effekt in Zusammenhang stehen kann. In den Testmedien hatten die meisten NM jedoch ein Zeta-Potential im Bereich von instabilen Dispersionen (-15 bis +15 mV) mit Fokus auf negativen Werten. Daher konnte keine eindeutige Korrelation zwischen dem Zeta-Potential und dem Effekt festgestellt werden. Um zu bestimmen, ob das Zeta-Potential einen Einfluss auf die Toxizität hat, wäre es notwendig, weitere NM mit einem negativen und positiven Zeta-Potential außerhalb dieses Bereichs zu testen.
- ▶ Überprüfung des Ökotox-Schemas mit weiteren Nanomaterialien.

1. Background

Manufactured nanomaterials (NMs) are being developed in many different variations such as size, shape, crystalline structure and surface modifications. So far the knowledge is still limited how the modifications affect ecotoxicity. To avoid the testing of each single nanomaterial modification, grouping and read across strategies for nanomaterials similar to classical chemicals are discussed. Grouping and read across aim to identify groups of substances/forms of a substance with specific sets of properties or parameters that enable reasonable predictions of a NM hazard without additional testing. For grouping and read across of NMs it is mainly discussed whether and how it can be performed for NMs of one chemical composition to address the various modifications. This approach is required in the scope of regulations such as REACH (European Union, 2006). There, grouping and read across can be used as instruments to fill data gaps within the regulatory information requirements. According to Annex XI of REACH it is possible to summarize substances which feature structural similarity based on a high consistency or a common pattern regarding their physical-chemical, toxicological and ecotoxicological properties into one substance group or use a substance as analog for another. Such a structural similarity needs to be proven by e.g. the chemical structure (e.g. functional groups) or other common properties like common source materials or degradation products. In addition, established patterns featuring equal changes of the properties and effects of group members can help to decide on a substance group. In its guidances on information requirements and chemical safety assessment (ECHA, 2008), the European Chemicals Agency (ECHA) included guidances on the requirements related to grouping and read across. These mainly relate to a guidance given by OECD (OECD, 2007, 2014). Both, OECD and ECHA identified the need to review the process of grouping based on the concept of structural similarity for NMs.

In 2014, the US Environmental Protection Agency hosted an OECD Expert Meeting in which various categorization strategies in a risk assessment framework were discussed (OECD, 2016a). Physicochemical characterization, fate, exposure, ecotoxicity, human health, exposure assessment as well as risk assessment and risk management were addressed. In 2016, there was a follow-up workshop held in Brussels (OECD, 2016b). The conclusion of the workshop, which dealt especially with the OECD Guidance on Grouping of chemicals, was that the existing grouping and read across strategy applies also for NMs. However, it was stated that there is a need to consider additional parameters, standardized methods, guidance and case studies to develop knowledge on how to connect physicochemical properties of NMs with behavior and effects intended to be predicted in the grouping and read-across exercise. Additionally, the industry initiated activities on this topic. An ECETOC Task Force published a concept for the grouping and safety assessment of nanomaterials with focus on inhalation toxicology (Arts et al., 2015; Arts et al., 2016). At the EU level, projects such as ITSnano in the scope of the 7th framework, developed basic strategies and identified subjects for a grouping which have to be further developed (Stone et al., 2014). Further approaches are already published such as those of Godwin et al. (2015) and Lynch et al. (2014). Based on the example CNT a decision-tree approach is proposed. However, also this approach focuses on human toxicology. It is pointed out in Godwin et al. (2015) that no single categorization strategy is likely to work for all classes of NMs in all regulatory situations. However, it may be possible to develop a general framework that can be adapted and customized for specific NM compositions and specific regulatory contexts. Lynch et al. (2014) postulate that the toxicity of a specific NM is caused by at least one of four mode-of actions (release of toxic chemical constituents from NMs; direct effects from physical contact with NMs; inherent properties of the material such as photochemical properties; capacity of NMs to act as vectors for the transport of other toxic chemicals). They assume that it should be possible to characterize the toxicity by three categories of parameters (intrinsic factors, extrinsic properties, composition) which include many individual parameters. Their approach should be the basis for the development of QNARs (quantitative nanomaterial activity relationship). General considerations on grouping approaches are also published by Sellers et al. (2015) and Oomen et al. (2015). In a recently

published document existing information on bridging data gaps between and grouping of nanoforms of the same substance is summarized with focus on the use by registrants to predict the hazard properties of (an)other form(s) of the same substance (European Chemicals Agency et al., 2016). Additionally, the ECHA has already published a draft version of a guidance documents with recommendations for nanomaterials on QSARs and grouping (ECHA, 2017). Within the discussion of grouping and read across of NMs it is generally accepted that the chemical identity of a NM alone is not sufficient to decide on groups of or read across between different (nano)forms of one substance. Additional parameters, such as intrinsic (such as size, morphology, surface modification) and extrinsic properties (such as dissolution rate, dispersion stability) as well as specific reactivities needs to be considered for a decision on the similarity of different nanoforms or nanoforms and bulk form. However, within this discussion the relevance of the single parameters for grouping remain unclear, especially as they depend on the nanoforms under investigation, the endpoint in question, and the influence of the surroundings. Furthermore, the evidence is hampered by the mutual influence of the different parameters in regards to ecotoxicity and kinetics.

Additionally, grouping is discussed in a broader sense where NMs of different chemical compositions are considered (e.g. approaches to rank and prioritize NMs).

For the prediction of effects from NM associated PC-parameters various models were developed. Most of them focus on human toxicity, (Kuempel et al., 2012; Low-Kam et al., 2015; Oomen et al., 2015; Winkler, 2016; Zhang et al., 2012), but also, QSAR-approaches are developed (Jagiello et al., 2016; Winkler, 2016; Wyrzykowska et al., 2016) or approaches are tested in which numerous PC-parameters are included without identifying the relevant ones (Tamm et al., 2016). In other approaches selected PC-parameters are linked with ecotoxicological tests which are not relevant in the scope of regulation such as microbial tests (Patel et al., 2014) and the transferability of the identified parameters to regulatory relevant ecotoxicological tests is unknown.

Specific proposals for relationships between physicochemical properties and ecotoxicological behavior are still missing. A step towards a specification is currently also done in the German national project nanoGRAVUR funded by the Federal Ministry of Education and Research (BMBF). In this project the not only hazard assessment but all aspects of risk assessment of a NM is considered and criteria catalogues for a grouping of nanomaterials according to the respective potentials for exposure, hazard and risk are developed. The ecotoxicological approach in the particular project is based on ecotoxicity profiles which are developed from a literature review conducted during this project. Based on these ecotoxicity profiles, relevant physicochemical properties are identified and a grouping of selected nanomaterials is proposed. The identified groups are verified by aquatic and terrestrial ecotoxicological testing using selected nanomaterials which were not considered for the development of the grouping approach. In nanoGRAVUR the applied NMs are already in commerce. Fifteen different chemical compositions of NMs are considered in the whole project and several surface modifications are included.

The approach applied in the present project differs from the strategy applied in nanoGRAVUR. In the project sponsored by the German Federal Ministry for the Environment, Natural Conservation, Building and Nuclear Safety, and coordinated by UBA NMs with specific properties were chosen to test the influence of the modifications on the ecotoxicity. In total fourteen NMs had been selected. They relate to five chemical compositions (Ag, ZnO, CeO₂, TiO₂, Cu) with three to four sub-types (except Cu with only one sub-type). The sub-types per nanomaterial differed in parameters such as size, shape, crystalline structure, solubility, reactivity. NMs featuring specific surface coatings were not considered for the grouping to get in a first step a basic grouping concept. A coating of a NM will hamper the direct transferability to other NMs with the same chemical composition or coating, as the coating may superimpose the effects due to other properties of the NMs and thereby may results in grouping results based on coating properties only.

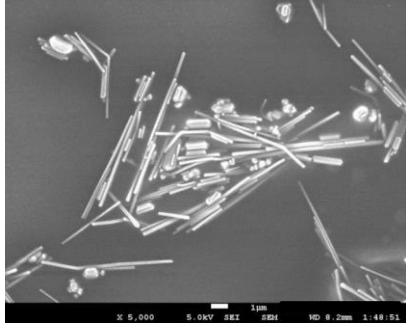
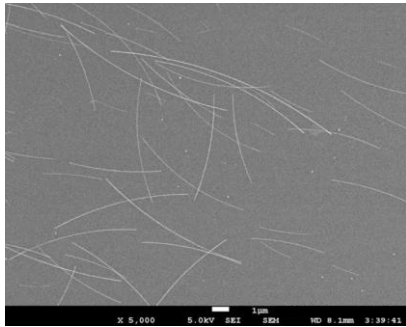
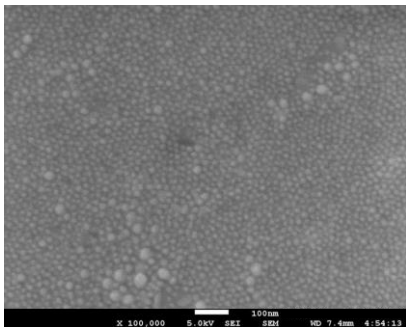
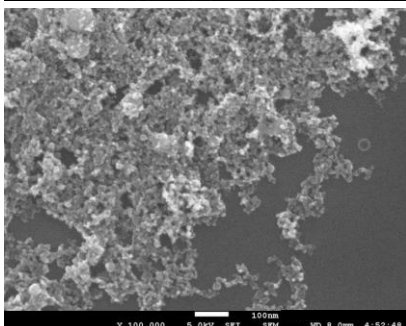
Two approaches to identify relevant physicochemical parameters for each of the five chemical compositions were pursued (i) grouping based on the physicochemical properties of the NMs (NM perspective) (ii) ranking of NMs based on the expected sensitivity of the three test organisms (organism perspective). With this approach focusing on ranking and prioritization could be achieved to identify NMs which have to be investigated with higher priority. Therefore the NMs and their behavior in the test media were comprehensively characterized and the ecotoxicity of the 14 NMs was tested with algae, daphnids and fish embryo following the OECD test guidelines No. 201, 202 and 236. The developments on the adaptation of the test guidelines regarding the testing of nanomaterials were considered. In a second step differences between the hypotheses and test results were discussed and considered in read-across and grouping. Furthermore, a grouping concept including a combination of ecotoxicity and physicochemical parameters of the NMs was developed.

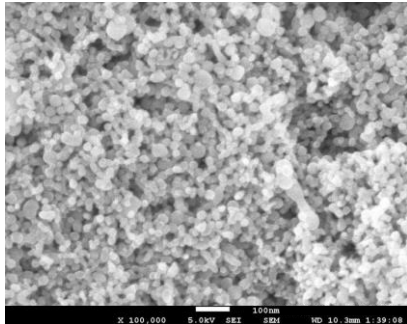
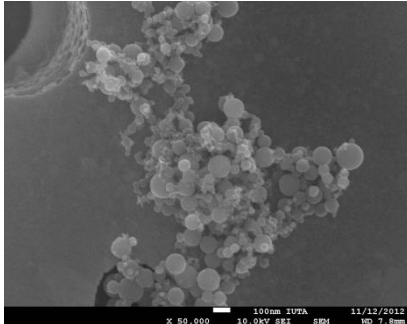
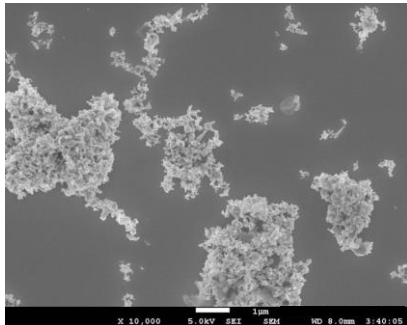
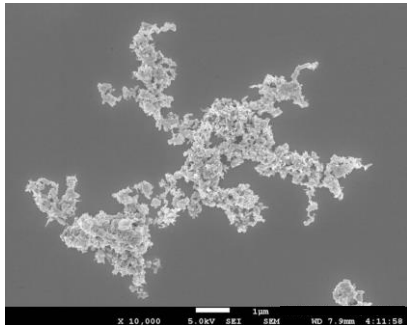
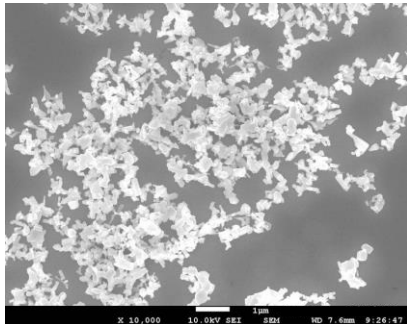
2 Characterization of the selected nanomaterials

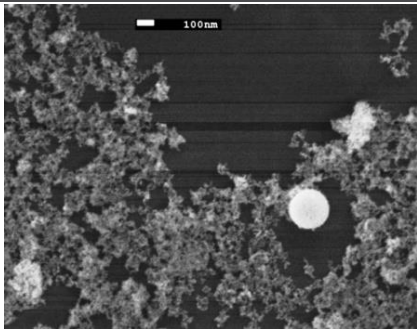
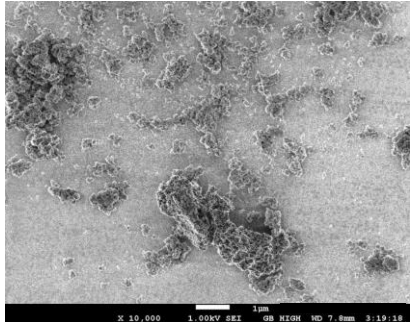
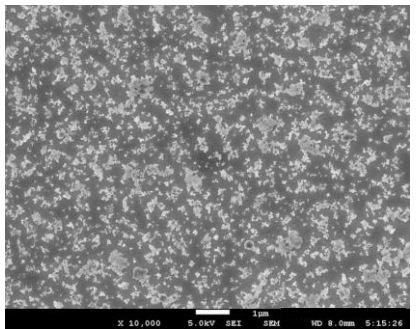
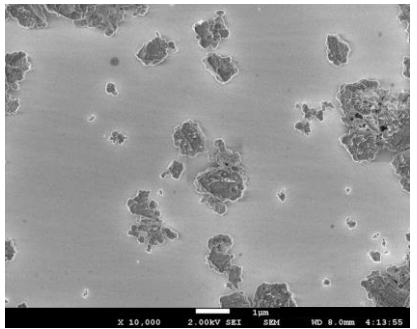
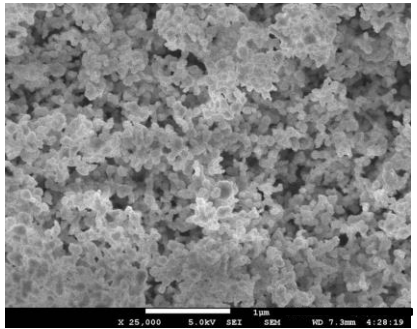
2.1 Selected NM

The nanomaterials listed in Table 1 had been selected with the contracting agency of this project (UBA).

Table 1: NMs selected for the project

Nanomaterial type	Nanomaterial subtype	Company/Provider	SEM
Ag	SRM 110525	Rent a scientist	
	Batch 1340	Rent a scientist	
	NM-300K	selected from the NM repository used for the OECD Sponsorship Programme	
TiO ₂	Doped with Eu (5%)	IUTA e.V.	

Nanomaterial type	Nanomaterial subtype	Company/Provider	SEM
ZnO	Doped with Fe (5%)	IUTA e.V.	
	Undoped	IUTA e.V.	
	NM-110	selected from the NM repository used for the OECD Sponsorship Programme	
	NM-111	selected from the NM repository used for the OECD Sponsorship Programme	
	NM-113	selected from the NM repository used for the OECD Sponsorship Programme	

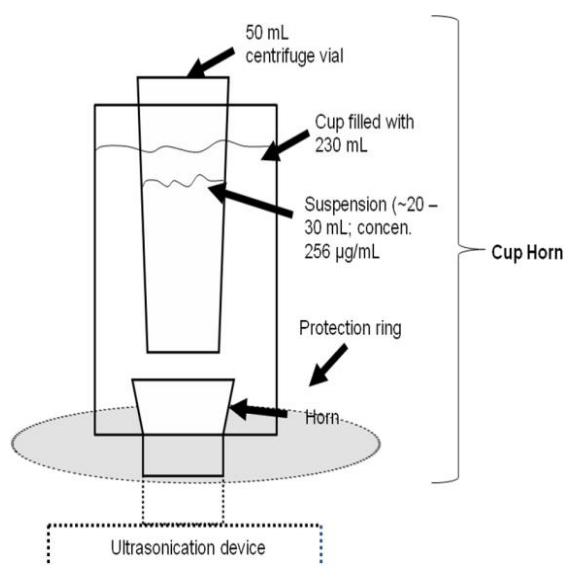
Nanomaterial type	Nanomaterial subtype	Company/Provider	SEM
CeO ₂	Doped with Eu (5%)	IUTA e.V.	
	NM-211	selected from the NM repository used for the OECD Sponsorship Programme	
	NM-212	selected from the NM repository used for the OECD Sponsorship Programme	
	NM-213	selected from the NM repository used for the OECD Sponsorship Programme	
Cu	Cu(0)	IUTA e.V.	

2.2 Suspension preparation

For the application of the nanomaterials a suspension has to be prepared to guarantee a homogeneous and comparable application of the nanomaterial to the test system.

As a first step a stock suspension of the NMs was prepared in deionized water (DI) water using the following standard operation procedure. For the preparation of the stock suspension 40 mg ± 3 mg of the powder of the nanomaterials or 40 µL of the NM-300K suspension was mixed with 40 mL DI water to reach a concentration of 1 g/L or 10 g/L for the silver NMs, respectively. The suspension was sonicated for 10 minutes using a cup horn (Figure 2), and a pulse of 2 (0.2 s / 0.8 s). The suspension preparation procedure was established in the EU SIINN project nanOxiMet (www.nanOxiMet.eu). The other two silver nanomaterials (SRM and 1340) were available as suspension and were not sonicated to avoid any structural defects on the wires. For the stock suspension preparation 200 µL of these materials were applied to 20 mL DI water and mixed using a vortex shaker (2000 rpm) for 1 min.

Figure 2: Suspension preparation procedure using a cup horn (www.nanOxiMet.eu)



After preparation of the stock suspension a specific amount of the media was applied to DI water and the test media to reach the target concentration. For the physicochemical characterization of the NM in suspension a concentration of 100 mg/L was used. The physicochemical parameters of the suspended nanomaterials were measured in DI water and the three test media, Algae – OECD medium (according to OECD Guideline 201 (2011)), Daphnids -ADaM (Aachener Daphnien-Medium, according to Klüttgen et al. (1994)) and Fish embryo –ISO water (according to ISO 7346-3 (1996)). In Table 2 the composition of the media are listed.

Table 2: Composition of the media used for algae, daphnids and fish embryo ecotoxicity tests

Component	ADaM (<i>Daphnia magna</i>) [mM]	ISO water (Fish – <i>Dania rerio</i>) [mM]	OECD medium (Algae) [mM]
pH	7.6±0.2	7.6±0.2	8.1
CaCl ₂	1.84	1.34	0.122
MgSO ₄		0.50	0.0609
NaHCO ₃	0.66	0.75	0.595
SeO ₂	0.000013		
Sea salt	333 mg/L		
KCl		0.074	
KH ₂ PO ₄			0.00919

MgCl ₂			0.0590
NH ₄ Cl			0.280
FeCl ₃			0.000237
Na ₂ -EDTA			0.000269
H ₃ BO ₃			0.00299
MnCl ₂			0.00210
ZnCl ₂			0.0000220
Na ₂ MoO ₄			0.0000289
CoCl ₂			0.00000630
CuCl ₂			0.00000006

2.3 Methods

Based on the actual literature some physicochemical parameters of NMs are discussed as important drivers for their possible ecotoxicological effects. These parameters are therefore important candidates for a grouping and were analyzed in this project if not provided by the manufacturer. The parameters were directly measured after spiking of the media from afore prepared stock suspensions as described in section 2.2 with one concentration (100 mg/L) and without contact to the organisms or from the powder of the NM. The chosen parameters as well as the used detection methods are summarized in Table 3 and methods are described below.

Table 3: Physicochemical parameters measured to characterize the nanomaterials and the corresponding detection method.

Physicochemical parameter	State of matter	Method
Surface chemistry	Powder	provided by the manufacturer
Surface area	Powder	BET (Brunauer-Emmett-Teller method) - provided by the manufacturer
Crystalline structure	Powder	provided by the manufacturer
Morphology	Powder / suspension	SEM- Scanning electron microscopy
Primary particle size	Powder / suspension	SEM
hydrodynamic diameter (z.average)	Suspension	DLS / SEM- Dynamic light scattering
Zeta potential	Suspension	ELS- Electrophoretic light scattering
Isoelectric point	Suspension	ELS
Solubility (rate)	Suspension	ICP MS- Inductively Coupled Plasma– Mass Spectrometry
Reactivity (CPH)	Suspension	EPR - Electron Paramagnetic Resonance Spectroscopy
Reactivity (DMPO)	Suspension	EPR

Dynamic light scattering (DLS)

Colloidal particles dispersed in a liquid show an undirected movement due to Brownian motion. By using Dynamic Light Scattering (DLS) the effective hydrodynamic diameter of particles in a suspension can be measured based on fluctuation of light, which is scattered by a liquid dispersion after radiation with a laser. The signal fluctuation is detected via a time correlation function - the method of cumulants (Koppel, 1972) and an average particle size (z.average) and a polydispersity index (PDI) can be calculated. Alternatively, the correlation can be numerically analyzed in terms of the particles size distribution. In this project the CONTIN Algorithm was used. This method was developed for the characterization of stable more or less spherical homogeneous monomodal samples. Problems may occur if the samples are unstable, multimodal, show a high background of natural occurring particles

or if non spherical particles were measured. Here the values have to be interpreted with care. Further information can be found elsewhere (Nickel et al., 2014).

Electrophoretic light scattering (ELS)

The zeta potential (ZP) is a characteristic parameter of the electric double layer, which is formed at any charged surface in a liquid. It is defined as the electric potential at the shear plane, which separates the mobile oppositely charged counter-ions (ion cloud) from solvent molecules and ions that adhere to the particle surface. By imposing a relative motion between bulk solvent and particle e.g. induced by an electric field, the ZP can be detected (Delgado et al., 2007). The velocity of the electrophoretic motion is proportional to the strength of the electric field and to the ZP. The particle size has only a second order impact. In this study the ZP was measured with a DELSA-NANO C (Beckmann Coulter). This instrument measures the phase shift (Doppler effect) of a light signal that is scattered at all moving particles. From the spread of the phase shift one can derive an intensity weighted distribution of the ZP.

Electron Paramagnetic Resonance (EPR) Spectroscopy

The detection of particle induced reactive oxygen species (ROS) and/or "surface reactivity" was done by spin trap/probe based electron paramagnetic resonance (EPR) spectroscopy technique (EPR Spectrometer Mini Scope 400, Fa Magnostech, Berlin). Two different complementary approaches were used a) sensitive to metal (Fenton-like) induced hydroxyl radical generation ($\text{OH}\cdot$) and b) kind of surface reactivity (redox-activity). Additionally to these two approaches the photo catalytic activity of TiO_2 nanomaterials was studied as impact of UV irradiation by EPR spectroscopy.

Reactivity measured with spin trap DMPO

In the presence of hydrogen peroxide (H_2O_2) and 5,5-dimethyl-1-pyrroline-N-oxide (DMPO) hydroxyl radicals ($\text{OH}\cdot$) generated via Fenton-type reactions are detected (Shi et al., 2003). Briefly, 50 μL of the particle suspension was mixed with 100 μL DMPO (0.05 M) and 50 μL of H_2O_2 (0.5 M), incubated in a dark, shaking water bath for 15 min at 37 °C before analyzed by EPR.

Reactivity measured with spin probe CPH

A possible (surface) reactivity of the material, caused by particle surfaces bound components and / or physicochemical particle properties, was established by measurements using the spin probe 1-hydroxy-3-carboxy-2,2,5,5-tetramethylpyrrolidine hydrochloride (CPH) mixed with the chelator desferrioxamine (0.1 mM) (Driessen et al., 2015; Papageorgiou et al., 2007). The (surface) reactivity is expressed by splitting of the H^+ of the CPH molecule or by generating an electron delocalization via binding. This effect is driven probably by directly active surfaces of the material. The preparation was done by mixing 50 μL of particle suspensions with 50 μL CPH (1 mM) and incubating for 10 min at 37 °C before analyzing by EPR.

Reactivity measured with spin trap DMPO after UV irradiation (photo catalytic activity)

Hydroxyl radical generation after UV-irradiation was measured according to Lipovsky et al. (2009) and Lipovsky et al. (2012) in the presence of 5,5-dimethyl-1-pyrroline-N-oxide (DMPO). This method is especially sensitive for the detection of hydroxyl radicals ($\text{OH}\cdot$) after UV-irradiation. For the measurement 30 μL of the particle suspension (final conc. 5 mg/L) is mixed with 30 μL DMPO (final conc. 0.05 M) and analyzed by EPR after irradiation with UV-light (Exo Terra natural light 25 Watt E27) for 10 min.

Scanning electron microscopy (SEM)

Scanning electron microscopy (a JEOL 7500F with a nominal resolution of 2 nm was used) was used to determine the morphology and size of the primary particles as well as their agglomeration status in media and size for selected materials. Either single crystalline silicon substrates or nucleopore

membrane filters were applied as substrates. The particles were applied onto the substrates by using a defined amount of a particle suspension which was dried prior to the SEM investigations. In case of the nucleopore filters a gold layer of approximately 10 nm had to be evaporated onto the filters to make them electrically conductive. The nucleopore filter was used if the particles/agglomerate size (distribution) of the NM in the media should be analyzed. For this, the suspension was filtered with a vacuum pump to minimize the contraction of the NM during the preparation.

The particle and agglomerate morphology was investigated by SEM images obtained at different magnifications. Size analysis was conducted on a series of images obtained at the same (high) magnification by means of an image analysis tool (ImageJ v1.41). Size analysis of the primary particles (near spherical particles) was conducted by measuring the particle diameter for 500 particles. For the agglomerate size the diameter was measured in two nearly perpendicular axes roughly representing the particle area (i.e. for the maximum diameter a Feret diameter is used whereas the minimum diameter of the perpendicular axis does not represent the minimum Feret diameter). These measurements were done for 300 agglomerates per material. The obtained data for both primary particle size and agglomerate size were statistically analyzed with regard to the mean and mode value of the size distribution.

Solubility experiments

The NM was weighted in a vial with a target concentration of the suspension of 1 mg/mL and 10 mg/mL for the silver NMs with a volume of at least 40 mL. This resulted in a test sample of 40 mg ($\pm 1\%$) of the solid NM and 40 mL ($\pm 1\%$) of the medium. The mixture was shaken for a defined time period (24 h, 72 h) using an overhead shaker at 60 revolutions / minute. The room temperature was recorded (1 hour-values), since the temperature also influences the solubility. After shaking, the sample was immediately centrifuged for 45 minutes at 5.000 G. After centrifugation the vials were carefully removed from the centrifuge and transported in an upright position until filtration, to minimize a mixing of the solid and aqueous fraction. For the following filtration the supernatant (3 x 10 mL) of each sample was taken with a 10 mL pipette and filled into a disposable syringe (B. Braun Inject 10 mL), equipped with a nylon syringe filter (pore size of 0.22 μm). The filtered supernatant was filled in a labelled vial for quantification of the soluble fraction.

The total concentration was detected by ICP-MS in the supernatant without filtering.

2.4 Sampling of the test media

Algae and daphnids

Beside the above mentioned "basic" characterization of the PC parameters which was measured directly from the powder or as suspension without organisms the nanomaterial concentration in the water phase was measured at the end of the tests to define the actual exposure concentration. Therefore a subsample (10 mL) of the test media was separated using a pipette from the middle of the test vials at the end of the tests (after 48 h for daphnids,). In the test with algae 10 mL samples were collected at test start and at the end of the tests (after 72h). The samples were analyzed after microwave digestion with 1.5 mL HNO_3 using ICP-MS. Randomly, some samples (algae after 72h) with soluble NM were also filtered (0.4 μm nylon filter) and the filtrate was analyzed for the released ions.

Fish embryo

Instead of the concentration in the ISO water the fish eggs or the recently hatched fish larvae including the egg shell were collected and analyzed via ICP-MS at the end of the tests (after 72 h). As fish eggs lay on the bottom of the test vessel during the test, due to this the embryos will be exposed to sedimented NMs, this gives a more realistic exposure assessment. For the analysis five fish eggs / or hatched fish larvae were pooled and analyzed together to guarantee that the measured concentration was above the detection limit of the instrument. Therefore, five fish eggs or hatched fish larvae including the egg

shell were digested with 1 mL HNO₃ (63 %) and 0.2 mL H₂O₂ (30 %) and sonicated for 20 min at 50°C in a sonication water bath. The solution was then analyzed with ICP-MS.

2.5 Results

The intrinsic NM properties are listed in Table 4, followed by the media dependent PC-parameters determined in water (Table 5), ISO medium (fish embryo test; Table 6), ADaM (test with daphnids; Table 7), and OECD medium (Table 8).

The measurements values for the solubility tests of TiO₂ or CeO₂ can be affected by the sample preparation (pore size of the filter), as no ion release of this two materials is expected. Therefore these values were not included in the evaluation of the results.

Table 4: Intrinsic properties of the selected nanomaterials. The detection method is mentioned in brackets.

Nanomaterial	Surface chemistry	Surface area [m ² /g] (BET)	Crystalline structure	Morphology (SEM)	Primary particle size [nm]
Ag – SRM 110525	Uncoated	n.d. NM provided as suspension	No information	Wires	Length: 2423; diameter: 241 (no NM according to EU definition)
Ag – 1340	Uncoated	n.d. NM provided as suspension	No information	Wires	Length: 3797; diameter: 44
Ag – NM300K	Uncoated	n.d. NM provided as suspension	No information	Spherical ¹	15 ¹
TiO ₂ undoped	Uncoated	78	91% Anatase, 9% Rutile	Spherical	19 (BET)
TiO ₂ Eu doped (5%)	Uncoated	148	Predominantly Rutile	Spherical	10 (BET)
TiO ₂ Fe doped (5%)	Uncoated	63	Predominantly Rutile	Spherical	23 (BET)
ZnO – NM110	Uncoated	12 ²	Hexagonal zincite ²	Cubical ⁴	41 (XRD) – 151 (SEM) ²
ZnO – NM113	Uncoated	6 ²	Hexagonal zincite ²	Cubical ⁴	42 (XRD) – 892 (SEM) ²
ZnO – NM111	Triethoxycaprylsilane (1-4%)	15 ²	Hexagonal zincite ²	Cubical ⁴	34 (XRD) – 141 (SEM) ²
CeO ₂ Eu doped (5%)	Uncoated	71	No information	Spherical	12 (BET)
CeO ₂ – NM211	Uncoated	66 ³	Cubic ceronite ³	spherical ³	10 (XRD) ³
CeO ₂ – NM212	Uncoated	27 ³	Cubic ceronite ³	polyhedral ³ - spherical ⁴	33 (XRD) ³ - 35 (SEM) ⁴
CeO ₂ – NM213	Uncoated	4 ³	Cubic ceronite ³	polyhedral ³	33 (XRD) - 615 (SEM) ³
Cu	Uncoated	Not measured	No information	Spherical	76 (SEM)

¹ Klein et al. (2011); ² Singh et al. (2011); ³ Singh et al. (2014), ⁴ MARINA (2013)

Table 5: PC - parameters of the different Nanomaterials in DI water, NM concentration 100 mg/L, for solubility experiments 1g/L, 10 g/L NM-300K; n ≥ 2.

Nanomaterial	Agglomerate size – z.average [nm] (±SD)	PDI	Zeta potential [mV]	pH	IEP	Solubility 24h [µg/L]	Solubility 72h [µg/L]	Reactivity CPH (sample to blank ratio)	Reactivity DMPO (sample to blank ratio)	Reactivity DMPO irradiation (sample to blank ratio)
Ag – SRM 110525	3570 ± 378	1.1	-34.16	5.3	/	182 ± 0.39	101 ± 98	1.11 ± 0.39	1.22 ± 0.08	- ¹
Ag – 1340	1522	0.4	-0.62	6.6	/	15633 ± 777	13325 ± 5334	1.77 ± 0.38	1.11 ± 0.1	- ¹
Ag – NM300K	149	0.2	-15.7 ± 2.4	7.4	< 4	436667 ± 133167*	496667 ± 167432*	9.97 ± 3.42	1.64 ± 0.2	- ¹
TiO ₂ undoped	743 ± 859	0.3	-21.6 ± 2.4	7.3	< 4	/	/	0.93 v 0.14	0.76± 0.03	1.65 ± 0.12
TiO ₂ Eu doped (5%)	440 ± 27	0.2	-24.6 ± 2.3	7.6	6-5	/	/	0.99 ± 0.18	0.9 ± 0.0	1.58 ± 0.1
TiO ₂ Fe doped (5%)	333 ± 32	0.2	-22 ± 3.6	8.0	5.5	/	/	1.4 ± 0.13	1.1 ± 0.0	3.08 ± 0.07
ZnO – NM110	1415 ± 230	0.4	-13.9 ± 15.1	7.4	7.5-8.5	633 ± 246	2267 ± 569	1.15 ± 0.17	1.4 ± 0.1	- ¹
ZnO – NM113	2192 ± 907	0.6	-16.3 ± 12	8.0	7-8	943 ± 787	4650 ± 2509	1.04 ± 0.21	1.29 ± 0.07	- ¹
ZnO – NM111	577 ± 378	0.3	-24.7 ± 3.8	7.5	6-5	188 ± 63	2725 ± 1486	0.91 ± 0.11	1.47 ± 0.03	- ¹
CeO ₂ Eu doped (5%)	822 ± 40	0.3	29.4 ± 2.8	6.3	6-6.5	Not determined	Not determined	Not determined	Not determined	- ¹
CeO ₂ – NM211	593 ± 348	0.2	-15.3 ± 7.6	6.1	6-5	/	120	0.86 ± 0.14	1.0 ± 0.01	- ¹
CeO ₂ – NM212	1201 ± 430	0.4	-19.4 ± 3	7.9	6.5-5.5	/	0.66	0.89 ± 0.02	1.26 ± 0.14	- ¹
CeO ₂ – NM213	967 ± 170	0.4	-26.6 ± 1.2	7.3	6-5	/	140	0.78 ± 0.21	1.65 ± 0.98	- ¹
Cu	1423	0.5	-19.8	7.8	9.5	13 ± 6.3	220 ± 147	285 ± 146	25.7 ± 7.17	-

/ = measured but not detected; - = not measured; * stock concentration 10 g/L; ¹ Photocatalytic activity of the chemical composition described in the literature, but not measured in the project due to the limited budget; a determination was not considered absolutely necessary as no peculiar results had been obtained such as the missing, but expected difference in ecotoxicity between the two crystalline forms of TiO₂ (anatase, rutile).

Table 6: PC - parameters of the different Nanomaterials in ISO water used for the zebrafish embryo test, NM concentration 100 mg/L, for solubility experiments 1g/L, 10 g/L NM-300K; n ≥ 2.

Nanomaterial	Agglomerate size – z.average [nm] (±SD)	PDI	Zeta potential [mV]	pH	IEP	Solubility 24h [µg/L]	Solubility 72h [µg/L]	Reactivity CPH (sample to blank ratio)	Reactivity DMPO (sample to blank ratio)	Reactivity DMPO irradiation (sample to blank ratio)
Ag – SRM 110525	2247	0.7	-14.7	7.5	/	37 ± 5	26 ± 13	1.07 ± 0.19	1.12 ± 0.08	- ¹
Ag – 1340	2059 ± 1027	0.6	-1.6	7.1	/	2967 ± 1626	1626 ± 848	1.37 ± 0.29	1.04 ± 0.05	- ¹
Ag – NM300K	307 ± 284	0.2	-8.4 ± 4.2	7.7	/	400000 ± 157162*	436667 ± 138684*	8.9 ± 1.03	1.32 ± 0.23	- ¹
TiO ₂ undoped	3291	0.2	-5.7 ± 2.8	7.0	/	-	< 0.5	1.01 ± 0.44	0.76 ± 0.07	1.6 ± 0.16
TiO ₂ Eu doped (5%)	6211 ± 5090	0.7	-5.2 ± 1.1	7.3	> 9	-	< 0.5	1.080 ± 0.56	0.7 ± 0.1	1.7 ± 0.12
TiO ₂ Fe doped (5%)	5006 ± 799	0.8	-6.1 ± 1.2	7.4	/	-	< 0.5	0.82 ± 0.1	1.0 ± 0.1	1.4 ± 0.08
ZnO – NM110	2547 ± 500	0.6	0.0 ± 4.3	7.7	7-6	2300 ± 2090	2620 ± 1469	0.88 ± 0.16	0.9 ± 0.1	- ¹
ZnO – NM113	2493 ± 729	0.7	4.3	7.6	<4	2550 ± 835.2	3050 ± 1473	0.88 ± 0.17	0.92 ± 0.06	- ¹
ZnO – NM111	1077 ± 549	0.4	-12.9 ± 6.9	7.6	6-5	2007 ± 1601.3	3400 ± 1667	0.84 ± 0.19	1.05 ± 0.05	- ¹
CeO ₂ Eu doped (5%)	2422 ± 438	0.6	-7.5 ± 1.4	7.5	/	Not determined	Not determined	Not determined	Not determined	- ¹
CeO ₂ – NM211	1604 ± 896	0.5	-9.1 ± 1.7	7.2	/	-	< 0.5	0.83 ± 0.29	0.89 ± 0.05	- ¹
CeO ₂ – NM212	1450 ± 220	1.3	-9.5 ± 1.5	7.8	< 4	-	< 0.5	0.73 ± 0.13	0.99 ± 0.21	- ¹
CeO ₂ – NM213	1154 ± 402	0.4	-10.9 ± 1.6	7.4	/	-	< 0.5	0.76 ± 0.20	1.0 ± 0.07	- ¹
Cu	1713 ± 875	0.5	-11 ± 10.3	7.5	> 10	161 ± 94.5	297 ± 298	300 ± 111	21.8 ± 5.9	-

/ = measured but not detected; - = not measured; * stock concentration 10 g/L; * stock concentration 10 g/L; ¹ Photocatalytic activity of the chemical composition described in the literature, but not measured in the project due to the limited budget; a determination was not considered absolutely necessary as no peculiar results had been obtained such as the missing, but expected difference in ecotoxicity between the two crystalline forms of TiO₂ (anatase, rutile).

Table 7: PC - parameters of the different Nanomaterials in ADaM used for the test with daphnids, NM concentration 100 mg/L, for solubility experiments 1g/L, 10 g/L NM-300K; n ≥ 2.

Nanomaterial	Agglomerate size – z.average [nm] (±SD)	PDI	Zeta potential [mV]	pH	IEP	Solubility 24h [µg/L]	Solubility 72h [µg/L]	Reactivity CPH (sample to blank ratio)	Reactivity DMPO (sample to blank ratio)	Reactivity DMPO irradiation (sample to blank ratio)
Ag – SRM 110525	9023 ± 688	1.2	-16.3	7.3	/	39 ± 7	12 ± 9	0.91 ± 0.02	1.10 ± 0.04	- ¹
Ag – 1340	1382 ± 57	0.4	-1.2 ± 2.9	7.2	/	4033 ± 1007	1500 ± 245	1.63 ± 0.06	1.10 ± 0.09	- ¹
Ag – NM300K	257 ± 40	0.2	-7.4 ± 1.8	8.0	/	420000 ± 112694*	466333 ± 123423*	3.84 ± 1.55	1.15 ± 0.22	- ¹
TiO ₂ undoped	2583 ± 157	0.3	-9.4 ± 12.0	8.1	/	-	< 0.5	0.44 ± 0.14	0.73 ± 0.06	1.92 ± 0.19
TiO ₂ Eu doped (5%)	3294 ± 1304	0.6	-5.2 ± 2.7	7.5	> 9	-	< 0.5	0.37 ± 0.06	0.7 ± 0.1	1.64 ± 0.03
TiO ₂ Fe doped (5%)	4078 ± 3298	0.6	-3.4 ± 1.8	8.2	/	-	< 0.5	0.53 ± 0.04	1.0 ± 0.1	3.9 ± 0.03
ZnO – NM110	1790 ± 628	0.6	1.7 ± 1.6	7.6	/	357 ± 212.2	1150 ± 1226	0.81 ± 0.39	1.0 ± 0.2	- ¹
ZnO – NM113	1858 ± 388	0.5	5.9 ± 5.1	7.6	/	643 ± 346.7	1328 ± 1018	0.73 ± 0.35	0.97 ± 0.16	- ¹
ZnO – NM111	709 ± 78	0.3	-15.6 ± 8.3	7.5	/	2427 ± 188.2	2135 ± 2734	0.67 ± 0.23	1.06 ± 0.22	- ¹
CeO ₂ Eu doped (5%)	2617 ± 636	0.6	-6.7 ± 0.1	7.4	/	Not determined	Not determined	Not determined	Not determined	- ¹
CeO ₂ – NM211	1345 ± 275	0.4	-8.0 ± 2.5	7.5	/	-	< 0.5	0.46 ± 0.1	0.86 ± 0.08	- ¹
CeO ₂ – NM212	1526 ± 203	0.5	-3.5 ± 6.0	7.8	/	-	< 0.5	0.37 ± 0.11	0.93 ± 0.15	- ¹
CeO ₂ – NM213	1774 ± 1354	0.7	-12.6 ± 4.2	7.6	7-6	-	< 0.5	0.38 ± 0.1	0.94 ± 0.15	- ¹
Cu	1979 ± 751	0.6	-11.2 ± 10.6	7.6	> 10	140 ± 26	11 ± 10.1	173 ± 38	18.1 ± 12.7	-

/ = measured but not detected; - = not measured; * stock concentration 10 g/L; * stock concentration 10 g/L; ¹ Photocatalytic activity of the chemical composition described in the literature, but not measured in the project due to the limited budget; a determination was not considered absolutely necessary as no peculiar results had been obtained such as the missing, but expected difference in ecotoxicity between the two crystalline forms of TiO₂ (anatase, rutile).

Table 8: PC - parameters of the different Nanomaterials in OECD medium used for the test with algae, NM concentration 100 mg/L, for solubility experiments 1g/L, 10 g/L NM-300K; n ≥ 2.

Nanomaterial	Agglomerate size – z.average [nm] (±SD)	PDI	Zeta potential [mV]	pH	IEP	Solubility 24h [µg/L]	Solubility 72h [µg/L]	Reactivity CPH (sample to blank ratio)	Reactivity DMPO (sample to blank ratio)	Reactivity DMPO irradiation (sample to blank ratio)
Ag – SRM 110525	4531 ± 2355	0.9	-23.7 ± 2.6	7.2	/	49 ± 10	38 ± 12	1.14 ± 0.07	1.07 ± 0.09	- ¹
Ag – 1340	1354 ± 41	0.4	-2.8 ± 2.2	7.0	/	1020 ± 849	849 ± 263	1.46 ± 0.13	1.12 ± 0.15	- ¹
Ag – NM300K	146 ± 95	0.2	-13.5 ± 6.5	7.6	/	413333 ± 118462*	453333 ± 170392*	6.53 ± 2.03	1.08 ± 0.08	- ¹
TiO ₂ undoped	2664 ± 1089	0.4	-22.8 ± 3.3	7.0	6-7	-	< 0.5	0.66 ± 0.15	0.71 ± 0.09	1.48 ± 0.11
TiO ₂ Eu doped (5%)	1612 ± 384	0.4	-23.1 ± 1.5	7.2	6-5	-	0.7	0.85 ± 0.30	0.7 ± 0.1	1.41 ± 0.09
TiO ₂ Fe doped (5%)	1866 ± 106	0.4	-21.3 ± 1.5	7.2	/	-	9.1	0.83 ± 0.11	1.0 ± 0.1	1.38 ± 0.07
ZnO – NM110	1975 ± 427	0.5	-18.9 ± 3.4	7.4	/	2050 ± 50	2675 ± 1520	0.72 ± 0.07	1.0 ± 0.2	- ¹
ZnO – NM113	1920 ± 610	0.5	-21.4 ± 1.3	7.4	8-7	1777 ± 225	2750 ± 686	0.87 ± 0.21	0.99 ± 0.11	- ¹
ZnO – NM111	445 ± 245	0.2	-25.2 ± 1.8	7.5	7.5-6.5	1433 ± 322	1667 ± 503	0.79 ± 0.20	1.01 ± 0.18	- ¹
CeO ₂ Eu doped (5%)	1251 ± 32	0.4	-21.0 ± 0.9	7.7	/	Not determined	Not determined	Not determined	Not determined	- ¹
CeO ₂ – NM211	442 ± 85	0.3	-19.8 ± 1.5	7.3	5-6	-	3.9	0.78 ± 0.17	0.81 ± 0.05	- ¹
CeO ₂ – NM212	831 ± 209	0.3	-20.4 ± 1.9	7.4	7-6	-	< 0.5	0.74 ± 0.23	0.96 ± 0.15	- ¹
CeO ₂ – NM213	1042 ± 178	0.4	-25.9 ± 2.4	7.4	/	-	0.67	0.85 ± 0.07	1.07 ± 0.12	- ¹
Cu	1789 ± 964	0.6	-16.9 ± 2.5	7.4	7.4	363 ± 5.8	142 ± 93	307 ± 46	3.5 ± 1.8	-

/ = measured but not detected; - = not measured; * stock concentration 10 g/L; * stock concentration 10 g/L; ¹ Photocatalytic activity of the chemical composition described in the literature, but not measured in the project due to the limited budget; a determination was not considered absolutely necessary as no peculiar results had been obtained such as the missing, but expected difference in ecotoxicity between the two crystalline forms of TiO₂ (anatase, rutile).

3 Hypothesis regarding the ecotoxicological behavior of the selected nanomaterials

3.1 Preliminary considerations

A group is characterized by NMs with comparable PC-properties resulting in comparable ecotoxicity. Currently there is no information, which variability for each PC-property can be accepted. Current attempts to group nanomaterials are mostly based on expert judgement. However, it is still unknown how to weight the impact of the different influencing physico-chemical parameters on ecotoxicity. This shall be illustrated with two examples using the NMs selected in this project:

▶ Ag NMs

There were one spherical silver NM and two silver wires. The solubility of the three forms differed by many orders of magnitude. Two groups could be formed with high focus on the shape (one group with the spherical NM and one group including the two wires) or the three NMs could be sorted in three groups due to the large differences in solubility. The solubility of the two wires differed by a factor of 100.

▶ ZnO

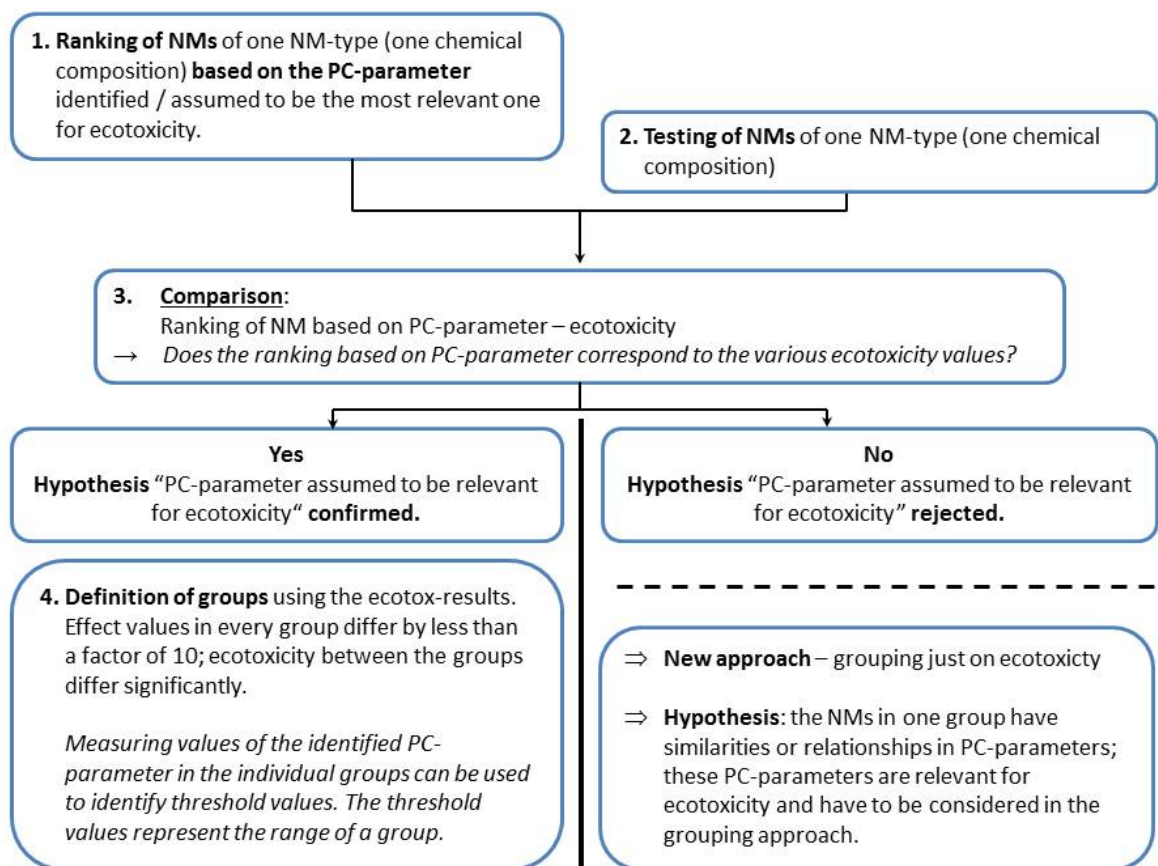
Based on the solubility and reactivity the three NMs would be grouped together. Taking the standard deviation into account there would be no difference between the three NMs. Based on the zeta-potential in the test medium used for daphnids (ADaM) and the fish embryo (ISO water), NM-111 differed from NM-110 and -113. Therefore, one as well as two groups could be justified. However, if it is not important whether the zeta-potential is nearly neutral or negative for daphnids, as the organisms are also exposed to sedimented NMs (NMs with a neutral ZP) one group is still justified.

Due to these difficulties in grouping, a ranking of the NMs based on selected PC-parameters was considered as appropriate approach. The ranking was performed on the PC-parameter which was considered to be the most essential one. If the ranking based on the PC-parameter correspond to a ranking based on the ecotoxicity determined in the experiments, grouping can be based on the identified PC-parameter. In this case, the NMs with a comparable ecotoxicity can be grouped. The effect values of a group should differ by less than a factor of 10. The factor of 10 is chosen pragmatically in the scope of grouping as a factor of 10 is often applied in the assessment of chemical substances (e.g. categories for substances hazardous to the aquatic environment: acute toxicity - category 1: $EC_{50} \leq 1$ mg/L; category 2: $EC_{50} > 1$ but ≤ 10 mg/L; category 3: $EC_{50} > 10 \leq 100$ mg/L) (United Nations, 2015). Based on the groups, the threshold values for the relevant PC-property can be defined.

If the ranking based on the PC-parameters does not correspond to a ranking based on the ecotoxicity, the hypothesis, that the selected PC-parameter is the relevant one, has to be rejected and a grouping based on ecotoxicity to identify the threshold values for PC-parameters is not possible. However, in this case, grouping just on ecotoxicity can be used to identify the relevant PC-parameter(s). All NMs in a group should show similarities or relationships in one or more PC-parameters.

The considerations are visualized in Figure 3.

Figure 3: Approach for grouping based on ranking results.



In the project various NMs and various test organisms were used. Therefore, the ranking was performed with two perspectives:

- ▶ Perspective “nanomaterial”
The perspective “nanomaterial” describes the expected ecotoxicity of the various nanomaterials in one specific test system. For this perspective, the physicochemical properties of the nanomaterials are of relevance. The parameters considered to be most relevant for each test organism are used for the prediction of the NMs effects.
- ▶ Perspective “test organisms”
The perspective “test organisms” describes and ranks the ecotoxicity of one NM in the three test systems algae, daphnids and fish embryo. The focus is on the sensitivity of the selected test systems compared to each other.

With regard to the interaction of the selected test organisms with any given NM, some basic assumptions had been made, which are summarized in Table 9. Apart from the physicochemical properties of the NMs the bioavailability of the NM has to be considered. For each organism, way of living, life stage, feeding behavior and conditions during the test have to be taken into account to decide on the likelihood of direct contact of organism and NM and whether the NM is internalized or not. In the Table 9 stable and unstable dispersions are addressed. For NMs threshold values for the zeta potentials exceeding +15 mV and below -15 mV are discussed as rough indicator for stable dispersions without consideration of any time-dependency. As a general principle, algae do not take up NM, but due to constant agitation during the test, they will be in contact with the NM irrespective of the agglomeration status. Daphnids are filter feeders and reside mainly in the water phase. They are hence considered to internalize NM and it is assumed that upon sedimentation the direct contact and

accordingly uptake is reduced but still possible. During the test zebrafish embryo reside statically without moving around on the bottom of the test vessels, which is why exposure to sedimented NM is higher. Embryonic stages of zebrafish are protected by the eggshell (chorion) from external influences. Direct uptake of both ions and NM is possible due to the presence of pores with diameter of around 0.6-0.7 μm . However, the uptake of NMs through the chorion is limited to small enough NMs (Böhme et al., 2015). In case of ion releasing NM, the availability and uptake of ions by all organisms is assumed.

Table 9: Summary of basic assumptions on the dependence of NM-organism interaction on stability of the NM in dispersion.

Organism	Algae			Daphnia			Zebrafish embryo			
	Dispersio n stability	Stable	Unstabl e / Sedime ntation	Release of ions	Stable	Unstabl e / Sedime ntation	Release of ions	Stable	Unstabl e / Sedime ntation ¹	Release of ions
Bioavaila bility / Direct contact	X	X	X	X	Limited	X	Limited	X	X	X
Uptake by organism	-	-	X	X	Limited	X	-	(x)	X	X

¹ Due to the protective role of the chorion little uptake of sedimented NM is expected, however passage of small particles through the chorion is possible.

3.2 Ion releasing nanomaterials: Ag, ZnO, Cu

To avoid duplications in the derivation of the hypotheses and identification of PC-parameters relevant for ecotoxicity and grouping, the ion-releasing NMs were considered together.

Nano-silver, nano-zinc and nano-copper release ions and are at least partly soluble during the incubation period of the test. Many studies demonstrate that released ions are one driver of the ecotoxicity (Notter et al., 2014). Additionally, particular effects and unknown mechanisms of toxicity are also discussed (Gagne et al., 2013). DNA or RNA-profiling showed different expression patterns in organisms upon exposure to NMs and dissolved metal salts, supporting the hypothesis of additional toxicity mechanisms for NMs apart from the sole toxicity of the ions alone (Garcia-Reyero et al., 2014). These differences between NMs and ions may be driven by different uptake mechanisms or toxicokinetics (Novo et al., 2015).

The solubility of the NMs was determined in the three pure test media without test organisms. It cannot be excluded that the test organisms themselves or their exudates affect the solubility. As the characterization of NMs is usually performed without the influence of test organisms, in the scope of this project the hypotheses were also based on the values resulting from this approach. Table 10 (solubility) and Table 11 (morphology) summarize the parameters as basis for the hypotheses for the three ion-releasing nanomaterial-types.

Table 10: Mean value of the solubility with standard deviation and percentage of ion releasing NM compared to total amount in the three test media, 1g/L for all NM, except NM-300K 10 g/L (incubation period: 72 h) for the ion-releasing nanomaterials, n ≥ 2.

Nanomaterial	ISO water (fish embryo)		OECD-medium (algae)		ADaM (daphnids)	
	[µg/L]	[%] ¹	[µg/L]	[%] ¹	[µg/L]	[%] ¹
Ag: SRM 110525	26 ± 13	< 0.01	38 ± 12	< 0.01	12 ± 9	< 0.01
Ag: Batch 1340	1626 ± 848	0.01	849 ± 263	< 0.01	1500 ± 245	0.02 ± 0.00
Ag: NM-300K	436667 ± 138684	4.4 ± 1.4	453333 ± 170392	4.5 ± 1.7	466333 ± 123423	4.6 ± 1.2
ZnO: NM-110	2620 ± 1469	0.3 ± 0.12	2675 ± 1520	0.3 ± 0.17	1150 ± 1226	0.12 ± 0.15
ZnO: NM-113	3050 ± 1473	0.3 ± 0.16	2750 ± 686	0.3 ± 0.03	1328 ± 1018	0.13 ± 0.12
ZnO: NM-111	3400 ± 1667	0.4 ± 0.17	1667 ± 503	0.6 ± 0.74	2135 ± 2734	0.22 ± 0.33
Cu	297 ± 298	0.03 ± 0.00	142 ± 93	0.01 0.01	11 ± 10.1	< 0.01

¹ [%] solubilized NM compared to total amount

A low solubility was observed for the tested silver, zinc and copper NMs. A low solubility of the ZnO NM was also observed in the JRC report dealing with the PC-parameters of the NM-110, -111 and -113 (Singh et al., 2011) and in the project nanoOxiMet for the ZnO NM NM-110 (www.nanoximet.eu).

Apart from solubility, further specific physicochemical parameters could affect the ecotoxicity for the various material types.

Differences in the morphology are obvious for the selected silver NMs. Apart from the spherical NM NM-300K two wires had been selected. It is assumed that the availability for uptake by organisms affects significantly the toxicity. The general possibility to internalize Ag nanowires was already shown by Sohn et al. (2015).

Further parameters such as reactivity (e.g. the formation of reactive oxygen species, ROS (Ma et al., 2013)) or the zeta potential are indicators for reactivity and dispersion stability / agglomeration behavior (e.g. (Meißner et al., 2010; Nickel et al., 2015; von der Kammer et al., 2010)) and therefore, the availability of the NMs for the test organisms can affect the outcome of the tests.

Table 11: Morphology and size of the three silver nanomaterials

Nanomaterial	Morphology	Length, REM [nm]	Diameter, REM [nm]
Ag: SRM 110525	Wire	2423	241
Ag: Batch 1340	Wire	3797	44
Ag: NM-300K	Spherical	---	15

3.2.1.1.1 Perspective “nanomaterial”

Based on the dissolution, following order regarding the ecotoxicity of the sub-nanomaterials for the nanomaterial-types was expected:

- ▶ Silver
 NM-300K > Batch 1340 > SRM 110525
 Based on the shape we expected that the nanomaterials can be separated in two groups, one

group consisting of the spherical NM-300K and the second one consisting of the two wires. The reactivity which is comparably high for NM-300K supports this grouping, whereas the impact of the zeta-potential seems to be small.

▶ ZnO

NM-111 > NM-110, NM-113

However, if we consider the standard deviation identified for dissolution of these three forms the differences in ecotoxicity for ZnO associated to the solubility should be small. The same should be valid for the impact of the reactivity as there are no obvious differences between the various sub-NMs.

Only one Cu nanomaterial was available and therefore, no ranking was possible.

3.2.1.1.2 Perspective “test organism”

Based on the dissolution in the three different test media, following order regarding the sensitivity of the test organisms was expected:

▶ Silver

NM-300K, SRM 110525: no significant difference between the solubility in the various test media. Therefore:

daphnids ~ algae ~ fish embryo

Batch 1340: daphnids > fish embryo > algae

If there is an uptake of the nanowires resulting in a locally increased silver concentration in the gut of daphnids, an increased sensitivity of daphnids compared to the other organisms is possible.

▶ ZnO

Based on the dissolution (mean value) there was a different order of the test organisms for every sub-nanomaterial.

NM-110: fish embryo ~ algae > daphnids

NM-113: fish embryo > algae > daphnids

NM-111: fish embryo > daphnids > algae

If we consider the standard deviations, the differences in the sensitivity of the test organisms should be small.

If we had considered the zeta potential with the consequences for agglomeration and stability in the test dispersion as well as the exposure conditions for the test organisms (increased exposure concentration of fish embryos and reduced exposure concentration for daphnids in case of sedimentation) a modified ranking of the toxicity based on nominal concentrations could result.

▶ Cu

Based on the dissolution, even if the standard deviations had been considered, we expected following order:

fish embryo > algae > daphnids

3.3 “Stable” nanomaterials: CeO₂, TiO₂

To avoid duplications in the derivation of the hypotheses and identification of PC-parameter relevant for ecotoxicity and grouping, the NMs which do not release significant amounts of ions are considered together.

For “stable” nanomaterials size, zeta potential and surface area are in general discussed as essential parameters regarding ecotoxicity. Additionally, for CeO₂ Booth et al. (2015) mentions the formation of ROS in the test medium according to OECD Guideline 201 (2011). In contrast, other studies have

reported that CeO₂ NMs exhibit a scavenging ability and can reduce oxidative stress (Amin et al., 2011). For the selected CeO₂ NMs, differences between the test media were observed only for the zeta potential. In the medium for algae, zeta potentials of about -20 mV were determined for all CeO₂ nanomaterials. Thus a higher stability and lower agglomeration potential of the nanomaterials in the test dispersions compared to the behavior in the test media for daphnids (-12.6 - -7.5 mV) and fish embryos (-10.9 - -7.5 mV) were expected. However, sedimentation in the fish embryo test can result in higher exposure concentrations of the fish eggs. The surface area is also discussed as relevant parameter as a high surface area possibly is the basis for a reactive contact area. For the surface area the BET values were considered. They were showing differences between the three materials with the lowest BET for NM213 and the highest for NM211. However, the differences in the BET surface were not reflected in the reactivity.

For TiO₂ photocatalytic properties have to be considered. The crystalline structure anatase shows a substantially higher photoreactivity than rutile (Xu et al., 2011), but mixed-phase titanium catalysts can show an even greater photoreactivity (Hurum et al., 2003). In contrast to the two doped TiO₂ nanomaterials, which contain 100 % rutile, the undoped TiO₂ is a rutile/anatase mixture and can cause photocatalytic induced ecotoxicity in the presence of suitable wavelengths. In contrast to the incubation conditions in the tests with daphnids and fish embryo, illumination is an unavoidable test condition in the algae test. However, in preliminary experiments it was demonstrated that apart from the crystalline structure, also the choice of the test medium significantly affected the photocatalytic activity. As presented in Figure 4, none of the nanomaterials showed photocatalytic activity in the test media used for the fish embryo and the algae test whereas the same lightning conditions resulted in photocatalytic activation in the Daphnia medium ADaM. Obvious differences between irradiated and non-irradiated samples were determined for the undoped TiO₂ and for the Fe doped TiO₂ nanomaterial in this test medium.

Apart from the crystalline structure, the differences between the various TiO₂ nanomaterials in size, zeta potential and reactivity without irradiation are not significant. However, regarding the zeta potential and the stability in dispersion a higher agglomeration behavior is expected in the Daphnia medium ADaM. Therefore, based on nominal concentrations the ecotoxicity in the daphnid test could be lower.

A summary of the parameters primary particle size, zeta potential and reactivity are listed in Table 12 and Table 13.

Table 12: Mean value of primary particle size and zeta potential with standard deviation for the NMs TiO₂ and CeO₂, in a suspension with a concentration of 100 mg/L directly after preparation; n ≥ 2.

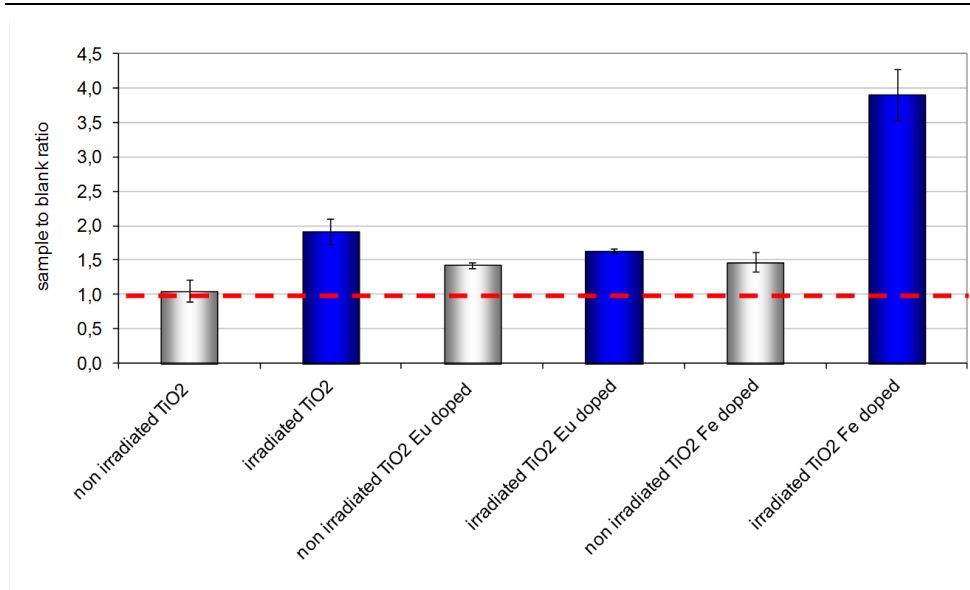
Nanomaterial	Primary particle Size [nm]	Zeta Potential in ISO water (fish embryo) [mV] (pH)	Zeta Potential in OECD-medium (algae) [mV] (pH)	Zeta Potential in ADaM (daphnids) [mV] (pH)
CeO ₂ : Eu doped	12-17	-7.5 ± 1.4 (pH 7.5)	-21.0 ± 0.9 (pH 7.7)	-6.7 ± 0.1 (pH 7.4)
CeO ₂ : NM-211	10	-9.1 ± 1.7 (pH 7.2)	-19.8 ± 1.5 (pH 7.3)	-8.0 ± 2.5 (pH 7.5)
CeO ₂ : NM-212	33	-9.5 ± 1.5 (pH 7.8)	-20.4 ± 1.9 (pH 7.4)	-3.5 ± 6.0 (pH 7.8)
CeO ₂ : NM-213	33	-10.9 ± 1.6 (pH 7.4)	-25.9 ± 2.4 (pH 7.4)	-12.6 ± 4.2 (pH 7.6)
TiO ₂	19	-6.1 ± 1.2 (pH 7.0)	-22.8 ± 3.3 (pH 7.0)	-9.4 ± 12.0 (pH 8.1)
TiO ₂ : Eu (5%)	10	-5.7 ± 2.8 (pH 7.3)	-22.8 ± 3.3 (pH 7.2)	-5.2 ± 2.7 (pH 7.5)
TiO ₂ : Fe (5%)	23	-5.2 ± 1.1 (pH 7.4)	-21.3 ± 1.5 (pH 7.2)	-3.4 ± 1.8 (pH 8.2)

Table 13: Mean value of reactivity (sample to blank ratio) with standard deviation for the NMs TiO₂ and CeO₂, without irradiation. Only values above 1.3 are defined as reactive in this context; n ≥ 2.

Nanomaterial	ISO water (fish embryo)		OECD-medium (algae)		ADaM (daphnids)	
	CPH	DMPO	CPH	DMPO	CPH	DMPO
CeO ₂ : Eu (5%)	n.d. ¹	n.d.	n.d.	n.d.	n.d.	n.d.
CeO ₂ : NM-211	0.83 ± 0.03	0.89 ± 0.05	0.78 ± 0.17	0.81 ± 0.05	0.46 ± 0.1	0.86 ± 0.08
CeO ₂ : NM-212	0.73 ± 0.13	0.99 ± 0.21	0.74 ± 0.23	0.96 ± 0.15	0.37 ± 0.11	0.93 ± 0.15
CeO ₂ : NM-213	0.76 ± 0.2	1.0 ± 0.07	0.85 ± 0.07	1.07 ± 0.12	0.38 ± 0.1	0.94 ± 0.15
TiO ₂ :	1.01 ± 0.44	0.76 ± 0.07	0.66 ± 0.15	0.71 ± 0.09	0.44 ± 0.14	0.73 ± 0.06
TiO ₂ : Eu (5%)	1.08 ± 0.56	0.7 ± 0.1	0.85 ± 0.3	0.7 ± 0.1	0.37 ± 0.06	0.7 ± 0.1
TiO ₂ : Fe (5%)	0.82 ± 0.1	1.0 ± 0.1	0.83 ± 0.11	1.0 ± 0.1	0.53 ± 0.04	1.0 ± 0.1

¹ n.d. = not determined

Figure 4: Effect of irradiation on the photocatalytic activity (DMPO) of three TiO₂ nanomaterials in ADaM used for tests with daphnia; n =3.



3.3.1.1.1 Perspective “nanomaterial”

► CeO₂ and TiO₂

If the chemical composition results in toxic effects at all, due to the small differences between the relevant parameters such as size, zeta potential and reactivity in the individual tests following ecotoxicity is expected:

CeO₂: NM-211 ~ NM-212 ~ NM-213 ~ EU doped

TiO₂: Eu doped ~ Fe doped ~ undoped (if dark? test conditions are considered)

3.3.1.1.2 Perspective “test organism”

Apart from the toxicity of the chemical composition, toxicity is only observed if the chemicals are bioavailable. As neither CeO₂ nor TiO₂ release substantial amounts of ions, the bioavailability of the particles will be mainly driven by the stability of the particles in the medium (see Table 9).

- ▶ CeO_2
If the chemical composition results in toxic effects at all, due to the higher dispersion stability in the test medium used for the test with algae, following order based on nominal concentrations is expected:
algae > daphnids ~ fish embryo
- ▶ TiO_2
If the chemical composition results in toxic effects at all, due to the higher dispersion stability in the test medium used for the tests with algae and fish embryo, following order based on nominal concentrations is expected:
algae > daphnids ~ fish embryo

4 Ecotoxicological test results and verification of the hypothesis on ecotoxicity

4.1 General remarks

The discussions on grouping in this report are based on mean values for the analytical results of PC properties and EC values for the ecotoxicological data. The raw data and further information on the test performance are summarized in comprehensive individual reports addressing the (i) characterization of the selected nanomaterials, and the ecotoxicity testing applying (ii) algae (OECD Guideline 201, 2011), (iii) daphnids (OECD Guideline 202, 2004) and (iv) fish embryos (OECD Guideline 236, 2013). These reports are available by the German Environment Agency on request.

In the scope of this project ecotoxicity characterized by EC-values and PC-parameters are compared. First of all, some general subjects shall be addressed.

4.1.1 Verification of nominal test concentrations

Regarding the verification of the test concentrations, all samples had been sent to IUTA for chemical analyses. By this approach, differences in analytical procedures and systematic errors were avoided. As the main focus was on total metal content the different time points of sampling and analyses were accepted. In the scope of this project only the determination at test end and exemplarily at test start was feasible. The calculation of time-weighted averages regarding the test concentration was not possible.

For the verification of the nominal concentration a sub-sample of the initial sample was used. It cannot be excluded that by sorption to the walls of the vials and sedimentation during storage the concentration in the aqueous phase was reduced. In retrospect, due to the low recovery rates, the use of the total sub-samples would have been to be preferred.

4.1.2 ECx-values

In order to characterize the ecotoxicity of chemical substances, effect concentrations (EC) were derived from modeled concentration effect curves. EC10 or EC50 values represent the concentration exerting 10 or 50 % effect. EC10 values are used for precautionary hazard assessment of chemicals and for the calculation of PNEC values (ECHA, 2008). EC50 values are in the middle of the slope of the concentration-effect curves. If the conclusions regarding grouping based on EC10 or EC50 values are comparable, the following considerations can be performed only on one kind of value. Therefore, first the comparability of the statements either based on EC10 or on EC50 values had to be checked. Additionally, it had to be decided whether nominal or analytical concentrations were the best choice. Since the test organisms and test systems differed, the considerations were performed for every test system in a separate chapter.

4.1.3 Test on algae

To characterize the test dispersion 10 mL-samples were collected at the start of the test of every test concentration without algae. At the end of the tests the samples were taken from the vessels incubated with algae. All samples were sent to IUTA for chemical analyses. Due to the continuous shaking during the incubation period, we assume a homogenous dispersion

The nanomaterial itself and the test concentration affect the analytical recovery as shown in Table 14. For most of the NMs the recovery is low at test end - even at the higher test concentrations. Only for two ZnO NMs (NM-110, NM-113) and for Cu nominal and analytical concentrations differ by less than 30 % in the higher test concentrations. The quality control of the chemical instrument shows no significant deviation, due to this we assume that the recovery was affected by sampling. We expected a heterogeneous distribution of NM due to agglomeration, which may cause an inhomogeneous distribution of NM in the subsamples if different numbers or sizes of NM are randomly sampled.

Furthermore, it cannot be excluded that the low recovery was due to sorption of the NMs to the glass vessels during the test or to the Falcon tubes during shipping and storage. For NM-300 K this has been already shown (Hoppe, pers. communication). In sub-samples of the NMs taken at test start a significantly higher recovery was determined (nominal 150 µg/L: 93 %) and supports the assumption of sorption and sedimentation during the incubation period. However, for the Europium doped CeO₂-NM at test start also a low recovery was determined (nominal 10000 µg/L: 28 %). If the analytical concentrations differ by more than 20 % from the nominal concentrations, the analytical concentrations have to be used for the calculation of the effect data according to the test guidelines for the three applied test systems. However, the range applies for soluble chemicals. Additionally, we cannot exclude that the analytical concentrations are less reliable (see chapter 4.1.1). It is not obvious whether the nominal or the analytical concentration are more suitable to represent the observed effects, although there are indications that the nominal concentrations are more suitable at least for the silver NM Batch SRM 110525. For the two highest test concentrations different inhibitory values were calculated which is consistent with the two different nominal concentrations. The two analytical concentrations are comparable to each other, although the effect values differ significantly. Besides the total concentration additionally the dissolved concentration (released ions) are listed in Table 14.

Table 14: Relationship of test concentration (nominal and analytical) at test end and inhibition of algal growth.

Nanomaterial	Nominal concentration [µg/L]	Analytical concentration [µg/L]	Recovery [%]	Inhibition [%] ¹
Ag: Batch SRM 110525	17	4.5	26	-4.5
	50	4.8	10	-5.6
	150	10	7	7.9
	450	28	6	5.2
	1350	650	16	22.3
	4050	650	48	76.2
Ag: Batch 1340	1.9	0.1	5	-0.4
	5.6	0.5	9	4.4
	17	2.5	15	14.5
	50	12	24	100
	150	40	27	100
Ag: NM-300K	1.85	30	1622*	1.8
	5.5	0.83	15	-1.7
	16.6	1.2	7	3.7
	50	18	36	11.8
ZnO: NM-110	150	68	45	100
	62.5	138.58	222	25.0
	125	214.16	171	77.7
	250	377.94	151	100
	500	629.89	126	100
	1000	1070.82	107	100

Nanomaterial	Nominal concentration [µg/L]	Analytical concentration [µg/L]	Recovery [%]	Inhibition [%] ¹
ZnO: NM-113	11	41.57	378	-0.3
	34	50.39	148	0.5
	100	119.68	120	42.8
	300	352.74	118	100
	900	919.64	102	100
ZnO: NM-111	10	37.79	378	0.7
	100	54.17	54	1.9
	1000	214.16	21	77.7
	10000	1284.98	13	100
	100000	8566.54	86	100
CeO ₂ : EU-doped	120	22.4	19	0.2
	370	74.6	20	1.3
	1110	49.7	4	4.3
	3330	1243.4	37	53.9
	10000	3481.6	35	100
CeO ₂ : NM-211	1024	683.9	67	0.37
	2560	1865.1	73	7.57
	6400	4973.7	78	20.1
	16000	20267.9	127	98.2
	40000	19646.2	49	100
CeO ₂ : NM-212	625	236.3	38	-1.0
	1250	373.0	30	10.9
	2500	969.9	39	18.3
	5000	2735.5	55	33.9
	10000	5346.7	53	100
CeO ₂ : NM-213	2560	136.8	5	-2.1
	6400	261.1	4	1.8
	16000	1989.5	12	6.0
	40000	5595.4	14	24.8
	100000	7833.6	8	100
TiO ₂ : Eu	70	37.62	54	-7.5
	200	111.15	56	-1.4
	670	427.50	64	23.7
	2000	854.99	43	95.0
	6000	3932.97	66	100
TiO ₂ Fe	1200	427.50	36	17.1

Nanomaterial	Nominal concentration [µg/L]	Analytical concentration [µg/L]	Recovery [%]	Inhibition [%] ¹
TiO ₂	3700	1077.29	29	48.4
	11000	5129.96	47	100
	33000	9917.93	30	100
	100000	24452.82	24	100
	70	51.30	73	0.9
	200	124.83	62	18.6
	670	427.50	64	79.3
	2000	1795.49	90	98.4
Cu	6000	3419.97	57	100
	1	5.5	550*	-0.9
	4	8	200	-0.7
	12	16	133	11.9
	37	38	103	92.6
	111	85	77	100
	333	430	129	100
	1000	930	93	100

¹ negative values indicate higher growth compared to the control. * measuring error, the quality control of the chemical analysis shows no significant deviation.

In Table 15 the EC10 and EC50 values are listed. The values are based on nominal and analytical verified concentrations. Analytical EC values were calculated by plotting concentration-response curves based on the analytical values.

Table 15: Ecotoxicological tests with algae (growth rate): EC10 and EC50 values based on nominal and analytical total concentrations; values in brackets: confidence interval (95 %)

Nanomaterials	Nominal: EC ₁₀ [mg/L]	Nominal: EC ₅₀ [mg/L]	Analytical: EC ₁₀ [mg/L]	Analytical: EC ₅₀ [mg/L]
Ag: Batch SRM 110525	0.881 (0.637-1.094)	2.37 (2.07 – 2.71)	0.0365 (0.0024-0.5450)	0.6383 (0.0199-17.9663)
Ag: Batch 1340;	0.0162 (n.d.) ²	0.0217 (n.d.) ²	0.00238 (0.00-n.d.) ²	0.00335 (0.00-n.d.) ²
Ag: NM-300K;	0.012 (0.007-0.017) ¹ 0.049 (n.d.) ²	0.081 (0.067-0.099) ¹ 0.062 (n.d.) ²	0.0178 (0.00-n.d.) ²	0.0233 (0.00-n.d.) ²
ZnO: NM-110	0.05 (0.04-0.05)	0.09 (0.08 - 0.10)	0.12 (0.11-0.12)	0.17 (0.16-0.18)
ZnO: NM-113	0.07 (0.04-0.11)	0.11 (0.05 - 0.21)	0.08 (0.06-0.11)	0.13 (0.09-0.18)
ZnO: NM-111	0.20 (0.14-0.27)	0.55 (0.38 - 0.80)	0.08 (0.07-0.10)	0.15 (0.12-0.19)
CeO ₂ : EU- doped	1.59 (1.37-1.84)	3.16 (2.64 – 3.76)	0.94 (0.00 -n.d.)	1.22 (0.0 – n.d.) ²
CeO ₂ : NM-211	1.28 (1.24-1.32)	8.5 (7.7 – 9.3)	4.22 (3.92-4.54)	7.34 (6.34-8.45)

Nanomaterials	Nominal: EC ₁₀ [mg/L]	Nominal: EC ₅₀ [mg/L]	Analytical: EC ₁₀ [mg/L]	Analytical: EC ₅₀ [mg/L]
CeO ₂ : NM-212	4.25 (3.02-5.97)	5.6 (3.0 – 10.4)	2.50 (0.00-n.d.) ²	2.92 (0.00-n.d.) ²
CeO ₂ : NM-213	37.19 (0.00-n.d.) ²	43.8 (n.d.) ²	4.41 (0.00-n.d.) ²	5.83 (0.00-n.d.) ²
TiO ₂ : Eu - doped	0.50 (0.43-0.57)	0.91 (0.75-1.10)	0.35 (0.32-0.39)	0.52 (0.46-0.59)
TiO ₂ : Fe - doped	1.31 (1.01-1.71)	3.6 (2.6-4.8)	0.36 (0.35-0.36)	1.08 (1.05-1.10)
TiO ₂	0.15 (0.13-0.17)	0.38 (0.33-0.43)	0.09 (0.08 - 0.11)	0.24 (0.20-0.28)
Cu	0.0114 (0.0109- 0.0119)	0.0198 (0.0189 – 0.0208)	0.0154 (0.0149- 0.0159)	0.0235 (0.0227- 0.0244)

¹ Results determined in the national joint project UMSICHT (partner UBA), funded by the German Federal Ministry of Education and Research (BMBF); ² n.d. = not determinable due to mathematical reasons or inappropriate data (explanation according to the applied calculation programme ToxRat (ToxRat Solutions GmbH)).

For algae, the statements regarding the toxicity ranking of the various nanomaterials based on nominal EC₅₀ values and on nominal EC₁₀ are comparable. For example, the order of the increasing toxicity for ZnO nanomaterials was NM-110 > NM-113 > NM-111, independent whether EC₁₀ or EC₅₀ are used. This also applies for Ag, CeO₂ and TiO₂.

Due to the partly low recovery, the EC values differ substantially depending on whether they were calculated based on nominal or analytical concentrations. In Table 16 the quotients of the EC₅₀ values based on nominal and analytical value are shown. A factor exceeding 1 indicates higher toxicity based on analytical concentrations and values below 1 indicate lower toxicity based on analytical concentrations. For silver, CeO₂ and TiO₂ the EC₅₀ based on analytical values indicate higher toxicity compared to the nominal values, whereas for ZnO and Cu it was vice versa. In 50 % of the cases the nominal and analytical values differ by less than a factor of 2.

For the scope of this project it is essential whether the statements on the ranking of the toxicity and hence the grouping differ if EC₁₀ or EC₅₀ and analytical or nominal values are used. For these statements the order of the NMs regarding their toxicity is essential and not the absolute values. There is no difference whether the statements on toxicity of the various Ag NMs are compared to each other based on the nominal or analytical concentrations. The same applies for the tested TiO₂ NMs. For ZnO the order of the EC₅₀ values based on nominal and analytical values differs. The hydrophobic NM-111 shows a higher ecotoxicity based on analytical concentrations. Due to difficulties in the preparation of the test dispersion, it is assumed that the analytical value is more suitable to indicate the toxicity of this NM. Based on analytical values the toxicity of the three ZnO NMs is comparable.

Differences were also observed for one of the CeO₂ NMs (NM-213). There is a factor of 10 between the EC₅₀ value based on nominal and analytical concentrations, with a higher toxicity based on the latter ones. Such a difference between the EC values is not obvious for the other three CeO₂ NMs. Neither the characterization data determined in this project nor the data reported by Singh et al. (2014) justify the lower toxicity of NM-213 compared to the other CeO₂ NMs if the nominal concentrations are used for the EC calculation. On the other hand, the surface area of NM-213 is smaller by a factor of about 10 compared the other three NMs, which may affect the toxicity. Minor differences can be observed in the order of the EC values for ZnO and CeO₂ between EC₁₀ or EC₅₀ (nominal and analytical), but considering the confidence intervals (95 %) the differences are considered to be negligible.

Table 16: Quotient of nominal and analytical EC₅₀ values (EC₅₀ nominal / EC₅₀ analytical) for algae¹

Nanomaterials	Test with algae
Ag: Batch SRM 110525	3.7
Ag: Batch 1340;	6.5
Ag: NM-300K	2.7
ZnO: NM-110	0.5
ZnO: NM-113	0.8
ZnO: NM-111	3.7
CeO ₂ : EU-doped	2.6
CeO ₂ : NM-211	1.2
CeO ₂ : NM-212	1.9
CeO ₂ : NM-213	7.5
TiO ₂ : Eu-doped	1.8
TiO ₂ : Fe-doped	3.3
TiO ₂	1.6
Cu	0.8

¹ values above 1 indicate higher toxicity based on analytical concentrations; values below 1 indicate lower toxicity based on analytical concentrations

From the considerations above, it can be concluded that it is negligible for the test with algae whether analytical or nominal, EC₁₀ or EC₅₀ values are used for the further considerations on grouping. Only for the ZnO NM-111 and the CeO₂ NM-213 differences have to be considered. For ZnO NMs, Cu and the Ag NM Batch SRM 110525 increasing concentrations of soluble ions fit to increasing effects.

4.1.4 Test on daphnids

The analytical concentration for each NM after the end of exposure was determined (Table 17). For this purpose, medium was taken from the water column without prior agitation, in order to mirror the exposure conditions in the water phase for daphnids. In case of Ag NMs tested, despite low recovery of the two silver wires a clear dose-response relationship of toxic effects on daphnids was observed. In addition to dissolution, also sedimentation may account for high silver loss from the media, as all subtypes of silver showed a low absolute zeta potential value, indicating low stability (Batch SRM 110525 showed the highest zeta potential of -16mV).

For the subtypes of ZnO, the recovery rate was inversely proportional to the nominal concentration, leading the interesting observation of quite constant analytical concentrations irrespective of initial concentration (~2.5 – 8 mg/L). Still, as in the case of silver, clear dose-response relationships for toxic effects in daphnids were observed. All subtypes of ZnO showed low absolute zeta potential values in ADaM, indicating instability. Strong sedimentation was observed visually already 10 min after end of sonication for all subtypes.

None of the sub-types of CeO₂ and TiO₂ exerted any toxicity on daphnids and for all of the materials a very low or no recovery was obtained. Accordingly, either the materials were not available for the daphnids due to sorption to the test vessels, or due to sedimentation or the chemical composition is non-toxic for the organisms. Sedimentation of NM was visually observed during the tests. Both TiO₂ and CeO₂ are unstable in Daphnia medium (ADaM) due to low absolute values of the zeta potential.

With a recovery of 60 % of the initial Cu concentration in the Daphnia medium Cu showed a low reduction of the exposure concentration. Interestingly, this observation is made despite the Cu-NPs are relatively unstable with a zeta potential of ~ -11 mV.

In fact, none of the NM showed a clear dose-dependency for the analytically determined concentration values. Probably this is due to the generally high instability followed by sedimentation of the selected NM in the ADaM medium. Hence, a meaningful calculation of concentration-response-curves for deriving analytical EC₁₀ and EC₅₀ values was not possible. In addition, it was considered as not meaningful to just recalculate the single analytical EC_x values from the respective nominal EC_x values. Accordingly, all further conclusions are drawn from the nominal values, keeping in mind the fact that high losses of NM from the exposure media were observed. Therefore, in Table 18 the EC₁₀ and EC₅₀ values based on nominal concentrations are listed. There is no obvious difference in the ranking of the NMs and the subsequent conclusions between EC₁₀ or EC₅₀ values. Therefore, it was decided to use in the following chapters exclusively the EC₅₀ values.

Table 17: Nominal and analytical test concentrations and % recovery at test end.

Nanomaterial	Nominal concentration [µg/L]	Analytical concentration [µg/L]	Recovery [%]	Remark
Ag: Batch SRM 110525	1	<0.1	0	Almost no recovery. irrespective of initial concentration
	44	0.58	0.01	
	133	1.2	0.01	
	400	1.3	0	
Ag: Batch 1340	1.6	0.88	0.55	Almost no recovery. irrespective of initial concentration
	4.9	1.1	0.22	
	14.8	1.8	0.12	
	44.4	<0.1	0	
	400	33	0.08	
Ag: NM-300K	n.d.	n.d.	n.d.	---
ZnO: NM-110	1200	1312.5	109.4	Recovery inversely proportional to concentration. resulting in real concentrations ~ 3 mg/L
	3700	2125	57.34	
	11000	3062	27.85	
	33000	2750	8.33	
	100000	3417	3.4	
ZnO: NM-113	1200	1625	135.42	Recovery inversely proportional to concentration. resulting in real concentrations ~ 3 mg/L
	3700	2000	54.05	
	11000	2875	26.14	
	33000	3375	10.23	
	100000	3208	3.21	
ZnO: NM-111	1200	962.5	80.21	Recovery inversely proportional to concentration. resulting in real concentrations ~ 3 – 8 mg/L
	3700	4125	111.5	
	11000	2938	26.7	
	33000	2875	8.7	
	100000	8000	8	
CeO ₂ : EU- doped	11000	22.2	0.27	Very low to no recovery
	33000	4.66	0.33	

Nanomaterial	Nominal concentration [µg/L]	Analytical concentration [µg/L]	Recovery [%]	Remark
CeO ₂ : NM-211	100000	2.66	0.08	Very low to no recovery
	11000	11.1	0.01	
	33000	35.52	0.00	
CeO ₂ : NM-212	100000	14.43	0.06	Very low to no recovery
	11000	6.44	0.00	
	33000	14.43	0.01	
CeO ₂ : NM-213	100000	7.1	0.20	Very low to no recovery
	3700	2.77	0.01	
	33000	109.9	0.11	
TiO ₂ : Eu-doped	100000	266.4	0.10	Very low to no recovery
	3700	16.53	0.09	
	33000	18.37	0.06	
TiO ₂ Fe-doped	100000	90.18	0.45	Very low to no recovery
	11000	81.83	0.04	
	33000	31.73	0.10	
TiO ₂	100000	36.74	0.74	Very low to no recovery
	11000	7.01	0.02	
	33000	718.1	2.18	
Cu	100000	18.37	0.06	Medium to high recovery. ~ 60 % for all exposure concentrations
	4.9	2.9	59.2	
	44	30	68.2	
	148	10	6.76	
	150	91.5	61	
	400	245	61.25	

n.d. - not determined

Table 18: Ecotoxicological tests with daphnids (immobilization): EC10 and EC50 values based on nominal concentrations; values in brackets: confidence interval (95 %). Derivation of EC10 and EC50 values based on analytical concentrations was not possible (see text above)

Nanomaterials	Nominal: EC ₁₀ [mg/L]	Nominal: EC ₅₀ [mg/L]
Ag: Batch SRM 110525	0.0003 (0.0001 – 0.0020)	0.0085 (0.002 – 0.015)
Ag: Batch 1340	0.0015 (0.0014 – 0.0018)	0.0016 (0.00 - 0.04)
Ag: NM-300K	0.027 (0.022-0.032) ¹	0.043 (0.038-0.047) ¹
ZnO: NM-110	0.90 (0.64 – 1.24)	3.43 (2.85 – 4.02)
ZnO: NM-113	0.89 (0.37 – 2.25)	8.25 (4.70 – 11.80)
ZnO: NM-111	0.81 (0.39 – 1.91)	5.63 (3.26 – 8.00)

Nanomaterials	Nominal: EC ₁₀ [mg/L]	Nominal: EC ₅₀ [mg/L]
CeO ₂ : EU-doped	---	> 100 mg/L
CeO ₂ : NM-211	---	> 100 mg/L
CeO ₂ : NM-212	---	> 100 mg/L
CeO ₂ : NM-213	---	> 100 mg/L
TiO ₂ : Eu-doped	---	> 100 mg/L
TiO ₂ : Fe-doped	---	> 100 mg/L
TiO ₂	---	> 100 mg/L
Cu	0.0046 (0.0034 – 0.0064)	0.0132 (0.0007 – 18.1792)

¹ Results determined in the national joint project UMSICHT (partner UBA), funded by the German Federal Ministry of Education and Research (BMBF); n.d. - not determined

4.1.5 Test on zebrafish embryos (ZFE)

In contrast to the tests with algae or daphnids, it is expected that the fish eggs which contain the embryos will be exposed to a higher NM concentration if the NMs are not stable in the water phase as the fish eggs lay on the bottom of the test vessel during the tests. To analyse a relevant exposure concentration for the fish embryos not the supernatant but the embryos were transferred without prior washing to Eppendorf tubes and shipped to IUTA for further analyses at the end of the test (deviating from the Test Guideline OECD 236 72 h after fertilization). The analytical concentrations were obtained as mass of the element associated to five fish embryos per test concentration. In order to allow the comparison to the nominal concentrations in µg/L, the volume of the embryo (approx. 700 nL) was taken into account to obtain volume-based analytical concentrations per embryo, irrespective of whether embryos were hatched or not. We considered this procedure as reasonable, because most of the NMs were visually observed attaching to the chorion. For the chosen NM exerting no toxic effects in ZFE (ZnO, CeO₂, TiO₂), only samples from the highest exposure concentration (100 mg/L assuming the highest exposure situation due to highest instability and thus strongest sedimentation) were analyzed to gain rough insight into the association of these NM to the embryo. For the NM exerting toxic effects in ZFE (Cu, Batch SRM 110525, Batch 1340), only viable organisms from the respective test concentrations were sampled for analyses.

Table 19: Nominal and analytical test concentrations and calculated enrichment factor at test end.

Nanomaterial	Nominal concentration [µg/L]	Analytical concentration [µg/fish]	Calculated analytical concentration [µg/L] ¹	Enrichment factor
Ag: Batch SRM 110525	3700	0.033	47 142.86	12.74
	11000	0.21755	310 785.71	28.25
Ag: Batch 1340	200	0.0035	5 000	25
Ag: NM-300K	n.d.	n.d.	n.d.	
ZnO: NM-110	100000	2.15	3 071 428.57	30.71
ZnO: NM-113	100000	2.4	3 428 571.43	34.29
ZnO: NM-111	100000	0.575	821 428.57	8.21

Nanomaterial	Nominal concentration [µg/L]	Analytical concentration [µg/fish]	Calculated analytical concentration [µg/L] ¹	Enrichment factor
CeO ₂ : EU- doped	100000	1.9	2 714 285.71	27.14
CeO ₂ : NM-211	100000	7.1	10 142 857.1	101.43
CeO ₂ : NM-212	100000	1.3	1 857 142.86	18.57
CeO ₂ : NM-213	100000	4.5	6 428 571.43	64.29
TiO ₂ : Eu-doped	100000	0.19	271 428.57	2.71
TiO ₂ Fe-doped	100000	0.09	128 571.43	1.29
TiO ₂	100000	0.038	54 285.71	0.54
Cu	300	0.072	102 857.14	342.86
	600	0.036	51 428.57	85.71

n.d. - not determined, ¹ for the calculation of analytical concentrations, a volume of 700 nL per embryo was assumed

As obvious from the calculated volume based concentrations, all NM showed an accumulation on the zebrafish embryo, except TiO₂. By dividing the calculated concentrations by the nominal concentrations, the enrichment of NM at the chorion was calculated. The lowest enrichment was observed for the subtypes of TiO₂. The silver and zinc subtypes showed enrichments factors between 8 and 34 fold, the highest enrichment was observed for copper and the CeO₂ subtypes. Interestingly, for the CeO₂ large differences in enrichment were observed (but no differences in toxicity). As only viable embryos were subjected to analyses of metal content, it was not possible to analyze embryos from all test concentrations, hence it was impossible to calculate analytical EC values. Therefore, in Table 20 only the nominal EC10 and EC50 values are listed. The analytical results relate to the concentration of NMs found to associate to the zebrafish embryo after the end of exposure (72 h after fertilization) (Table 19). As there were no obvious differences in the ranking of the NMs and in the conclusions resulting from applying EC10 or EC50 values in the following chapters exclusively the EC50 values were used.

Table 20: Ecotoxicological tests with fish embryos (development): EC10 and EC50 values based on nominal concentrations; values in brackets: confidence interval (95 %). Derivation of EC10 and EC50 values based on analytical concentrations was not possible (see text above)

Nanomaterials	Nominal: EC ₁₀ [mg/L]	Nominal: EC ₅₀ [mg/L]
Ag: Batch SRM 110525	7.282 (3.318 – 14.391)	20.4 (10.06 - 30.80)
Ag: Batch 1340;	0.155 (0.143 – 0.173)	0.203 (0.19 - 0.22)
Ag: NM-300K	0.174 (0.093-0.228) ¹	0.292 (0.221-0.352) ¹
	0.739 (0.502-0.912) ¹	1.668 (1.433-2.043) ¹
	0.71 (0.65-0.76) ²	1.03 (0.99-1.07) ²
ZnO: NM-110	---	> 100 mg/L
ZnO: NM-113	---	> 100 mg/L
ZnO: NM-111	---	> 100 mg/L

Nanomaterials	Nominal: EC ₁₀ [mg/L]	Nominal: EC ₅₀ [mg/L]
CeO ₂ : EU- doped	---	> 100 mg/L
CeO ₂ : NM-211	---	> 100 mg/L
CeO ₂ : NM-212	---	> 100 mg/L
CeO ₂ : NM-213	---	> 100 mg/L
TiO ₂ : Eu-doped	---	> 100 mg/L
TiO ₂ : Fe-doped	---	> 100 mg/L
TiO ₂	---	> 100 mg/L
Cu	0.2489 (0.1785 – 0.3490)	0.4735 (0.3895 – 0.5574)

¹ Results determined in the national joint project UMSICHT (partner UBA), funded by the German Federal Ministry of Education and Research (BMBF); incubation period 48 h; ² Results determined in the scope of a master thesis; incubation period 72 h (Brüggemann, 2015); n.d. = not determinable

In general, zebrafish embryo was the least sensitive test organism and the lowest or no toxicity was observed for the selected NM. This may be a result of the low availability of NM to the embryo due to the protective role of the chorion. With regard to the stability, all subtypes of TiO₂ and CeO₂ had low absolute values (~ 10 mV) of zeta potential in ISO water, indicating a high tendency to sedimentation. A strong sorption of particles to the zebrafish chorion was observed. Likewise, all subtypes of the other NM, except Ag SRM and ZnO NM 111 showed low absolute zeta potentials.

4.1.6 PC-parameters

Measurement values of the different PC-parameters have to be discussed differently regarding ecotoxicity on the various test organisms with their different behavior. It is expected that high values for solubility and reactivity result in high toxicity, independent on the test organisms. However, high values for z-average indicating high agglomeration and sedimentation should result in higher bioavailability for fish embryos due to sedimentation, if the NM can pass the pores in the chorion. This means that the higher availability due to sedimentation is only relevant if the NM can pass the shell. However, despite a chorion pore size of ~ 200 nm, interaction of metal with the protein structure of the chorion may prevent passage of NPs (Bohme et al., 2017). For daphnids the exposure should be reduced in case of high z-average values as these organisms swim in the water with reduced uptake of sedimented particles. In the algae test with continuous stirring, the effect of sedimentation on exposure should be minimized compared to the other two test organisms. Zeta potentials with absolute values above 15 mV are discussed as indicators for stable dispersions resulting in higher exposure concentrations for daphnids, whereas lower exposure is expected for fish embryos.

4.2 Ag

The EC₅₀ values determined in the ecotoxicological tests with algae, daphnids and fish embryo are listed in Table 21. Additionally, the solubility of the NMs in the test media is included. According to the considerations described in chapter 3.2, the solubility is considered to be the most essential parameter and was determined in the test media without test organisms. However, the presence of test organisms may alter the dissolution of ions due to changes in media pH or the availability of other molecules (excretion products) which may absorb to the surface of the NM and may reduce or catalyse the solubility as it was shown for humic substances (Misra et al., 2012). Hence, in all assays pH was recorded at the beginning and end of the test (see Annex for details). For the three test organisms, only slight changes in pH were recorded. In Figure 5 besides the EC₅₀-values and the solubility, also the PC-parameters z.average, zeta potential, and reactivity are presented. The profiles for every parameter comprising the data of the three NMs can be compared among each other. The profiles vary significantly: Exemplarily, for algae, the lowest EC₅₀ values indicating highest toxicity were calculated for Batch 1340, followed by NM-300K and SRM 110525. However, the z.average decrease with the order SRM 110525 < Batch 1340 < NM-300K, the solubility increases in the same order whereas the highest negative zeta potential was determined for Batch 1340, followed by NM-300K and SRM 110525. Last, but not least, the reactivity of the three NMs was comparable. Additionally, the profiles of the parameters differed between the three test media.

As already addressed in chapter 3.1, for every parameter, the height of the values (shown as height of the pillars) can affect the ecotoxicity differently. Exemplarily, a high value (high pillar) of solubility is expected to result in high toxicity independent of the test organism, whereas a high pillar in the hydrodynamic diameter indicates high agglomeration potential resulting in sedimentation and therefore a high exposure concentration for the fish embryo, but a lower exposure concentration for daphnids.

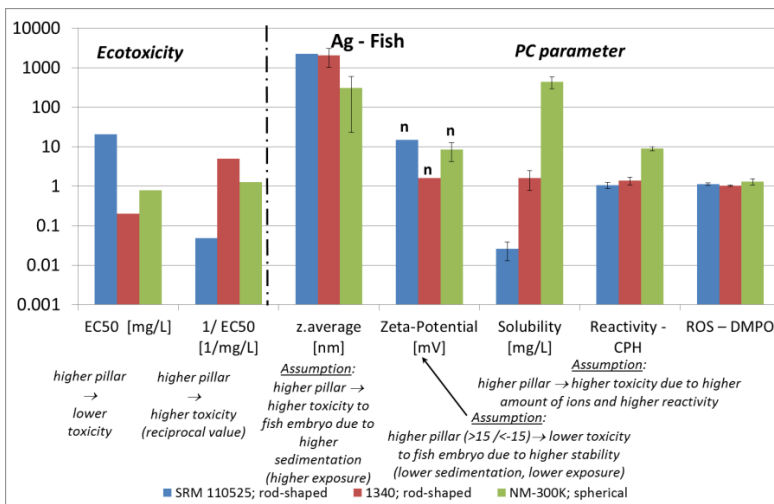
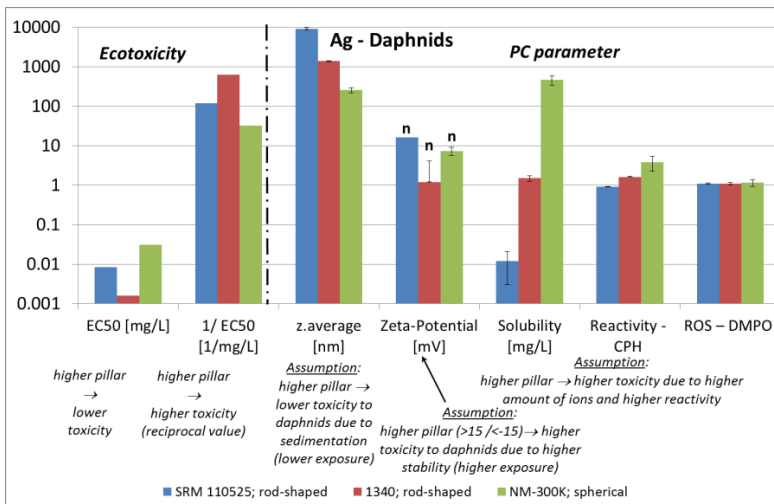
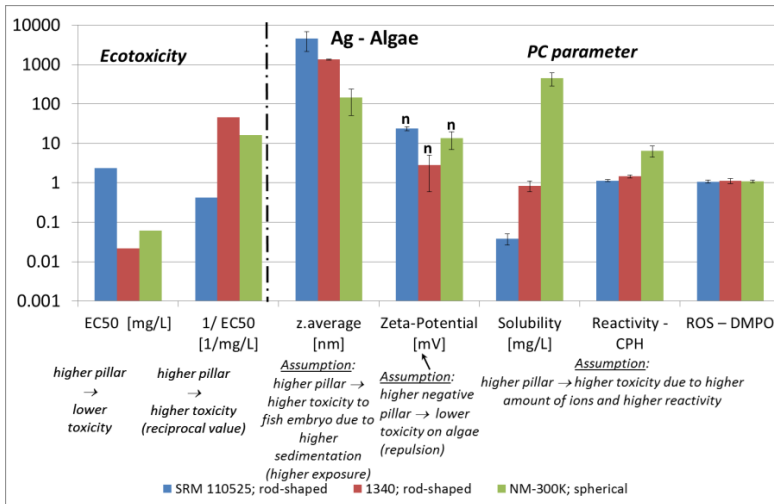
Table 21: Ag-NMs - EC₅₀-values (basis: nominal concentrations) and solubility in the test media

Ag NMs	Algae (OECD TG 201):		Daphnids (OECD TG 202)		Fish embryo (OECD TG 236)	
	EC ₅₀ [mg/L]	Solubility 72h [µg/L]	EC ₅₀ [mg/L]	Solubility 72h [µg/L]	EC ₅₀ [mg/L]	Solubility 72h [µg/L]
SRM 110525	2.37 (2.07 – 2.71)	38 ± 12	0.0085 (0.002 – 0.015)	12 ± 9	20.4 (10.06 - 30.80)	26 ± 13
Batch 1340	0.0217 (n.d.) ³	849 ± 263	0.0016 (- 0.04 - 0.04)	1500 ± 245	0.203 (0.19 - 0.22)	1626 ± 848
NM-300K	0.015 – 0.081 ¹ 0.062 (n.d.)	453333 ± 170392	0.031 – 0.043 ¹	466333 ± 123423	0.292 - 0.78 ¹ 1.1 ²	436667 ± 138684

¹ Results determined in the joint project UMSICHT (partner UBA) funded by the German Federal Ministry of Research and Education; ² results of IME in the scope of a master thesis (72 h incubation)

³ replicates are close together and on the curve; additionally, the slope of the curve is steep; therefore, the applied calculation programme ToxRat was not able to calculate a confidence interval.

Figure 5: Toxic effect of three Ag NMs (basis: nominal concentrations) on algae, daphnids and fish embryos and results for selected PC-parameters in the three different test media (consider the additional reciprocal value of the EC-values to make the relationships more obvious.)
 n = zeta potential is negative in the test medium (due to the logarithmic scale negative values cannot be presented)



4.2.1 Verification of the hypotheses

In Table 22 the expected and determined toxicity rankings are listed. The hypotheses were not confirmed.

Table 22: Perspective "nanomaterial" and "test organism" – expected and determined ecotoxicity of the selected NMs (basis: nominal concentrations)

Perspective	Expected	Determined
Nanomaterial	Basis solubility: NM-300K > Batch 1340 > SRM 110525	Test with algae and fish embryos: Batch 1340 ~ NM-300K > SRM 110525
Test organism	NM-300K, SRM 110525: daphnids ~ algae ~ fish embryo Batch 1340: fish embryo > daphnids > algae If there is an uptake of the nanowires with a locally increased ion concentration in the gut of daphnids, an increased sensitivity of daphnids compared to the listed orders is possible	Test with daphnids: Batch 1340 > SRM 110525 > NM-300K NM-300 K: algae ~ daphnids > fish embryos Batch 1340; SRM 110525: daphnids > algae > fish embryos

It is discussed that the surface area of NMs is relevant for the determined ecotoxicity. Therefore, in an additional approach the EC50 values were calculated based on the surface instead of mass (Table 23). The NMs were provided in a suspension and the surface could not be determined by BET. Therefore, the surface was calculated based on the primary particle size of the NMs. Following calculation steps were performed:

1. The volume and the surface were calculated.
2. Based on the specific density of silver (10.49 g/cm³ or 10.49 mg/mm³) and the volume of a NM, the mass of a single NM was calculated.
3. With the mass of a single NM and the surface, the EC50 on mass basis was converted into an EC50 based on surface area per L.

Table 23: Ag-NMs- EC50 values referring to surface area

NM	Step 1 ¹	Step 1 ¹	Step 2 ¹	Step 3 ¹	Step 3 ¹	Step 3 ¹
	Volume [nm ³]	Surface area [nm ²]	Mass per NM [mg]	EC50 - algae [m ² /L]	EC50 - daphnids [m ² /L]	EC50 - fish embryo [m ² /L]
SRM 110525	110,473,256	1,924,768	1.1589E-09	0.0039	0.000014	0.034
Batch 1340	5,867,987	532,096	6.1555E-11	0.00019	0.000014	0.0018
NM-300K	1767	707	1.8536E-14	0.0024	0.0012	0.042

¹ The steps refer to the steps described in the text

The EC50 based on surface area for the three NMs differ from each other and it can be concluded that the surface area is not the single parameter responsible for the ecotoxicity. It is obvious that the toxicity of Batch 1340 differs for algae and fish embryo from the toxicity of SRM 110525 and NM 300K. Batch 1340 is about a factor of 10 more toxic than SRM 110525 and NM-300K. For daphnids, both wires seem to exert a similar toxicity which is 100 times higher compared to NM-300K.

The agglomeration potential / sedimentation of the silver subtypes in the different media affect availability for organisms. For fish embryo, the Ag subtype with the highest stability (SRM, -15 mV zeta potential) was the least toxic, compared to the less stable subtypes, for which a higher sedimentation and hence bioavailability was expected. However, the same situation is true for the daphnids, where a higher bioavailability from the water phase is expected.

The reactivity (measured by CPH) and the solubility are comparable in ADaM and ISO water, but seem not to be driver of toxicity, as NM-300K shows the highest reactivity, but toxicity is comparable to Batch 1340.

Microscopic investigations provided additional insights in the ecotoxicity mechanism of the investigated Ag-NMs.

High toxicity of Batch 1340 on algae

Microscopic images indicated mechanical effects by Batch 1340 in contrast to SRM 110525 and NM-300K (Figure 6). As the NM wires of Batch 1340 have a very low diameter, they were shown to get in close contact with the algae. Probably, the nanowires injure the algae cells, a process which may be increased by the constant agitation during exposure. During the microscopic observation, the complexes of NMs and algae moved together which indicated a fixed connection of NMs and test organisms. In contrast such a connection was not obvious for the other two NMs and the algae. It seems to be unlikely that the wires of Batch 1340 skewer the algae due to the mechanical treatment. The required forces cannot be achieved by the shaking procedure. Therefore, we assume that the wires orient themselves upright to the surface by electrostatic forces followed by a local damage of the cell wall due to an increased ion concentration. Then the wires penetrate the algae cells.

► High toxicity of the Ag wires on daphnids

The high toxicity of the wires on daphnids compared to the spherical NM NM-300K could be explained by an uptake of the wires and a limited excretion due to steric reasons resulting in a high concentration of released ions in the gut and therefore a high exposure concentration for the daphnids. The uptake of the wires by daphnids is presented in Figure 7. In addition, silver wires may exert external physical effects on daphnids. However, as visualized in Figure 7, there was little attachment of nanowires to the carapace.

Figure 6: Algae and NMs under test conditions (OECD TG 201)

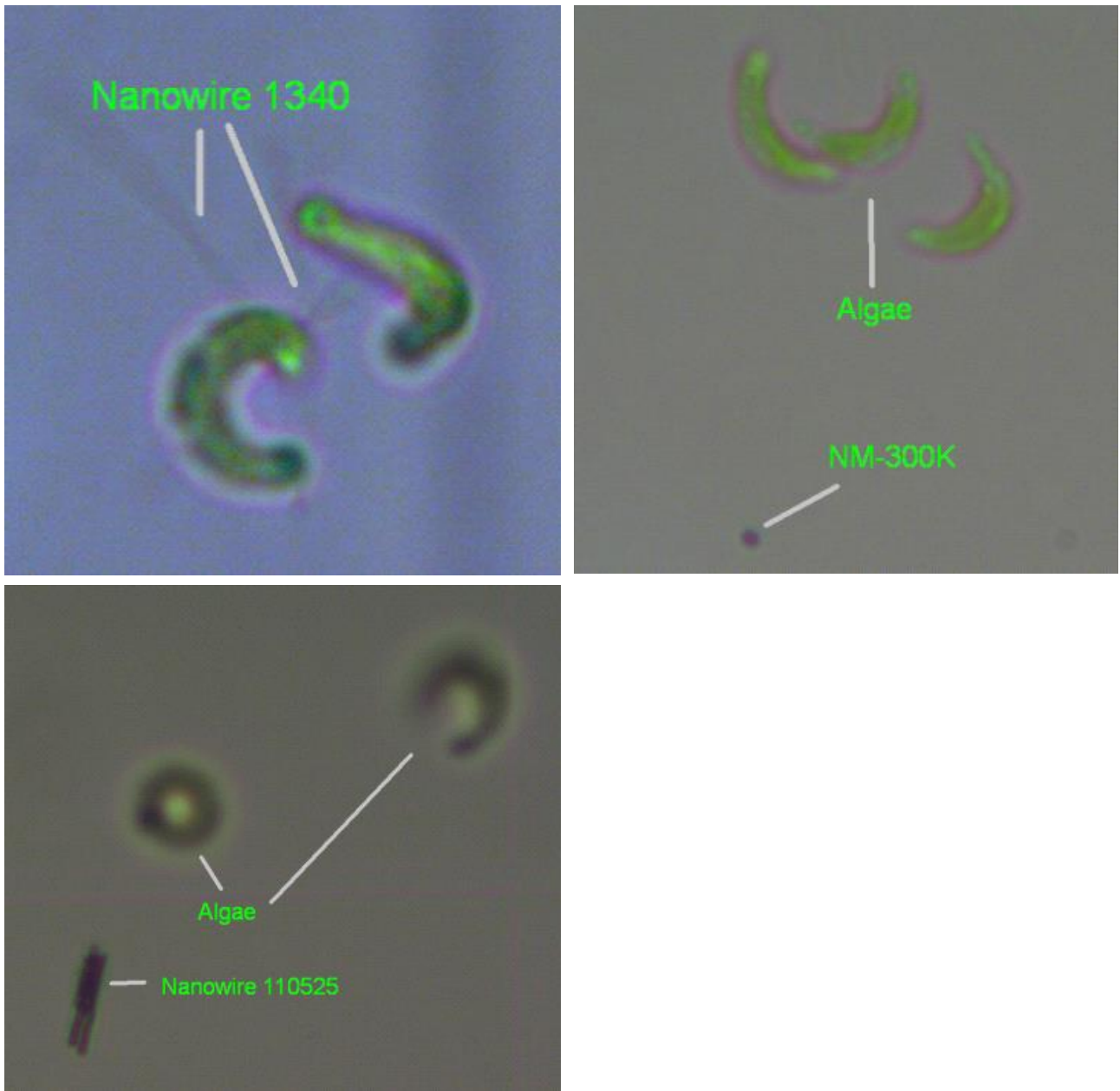
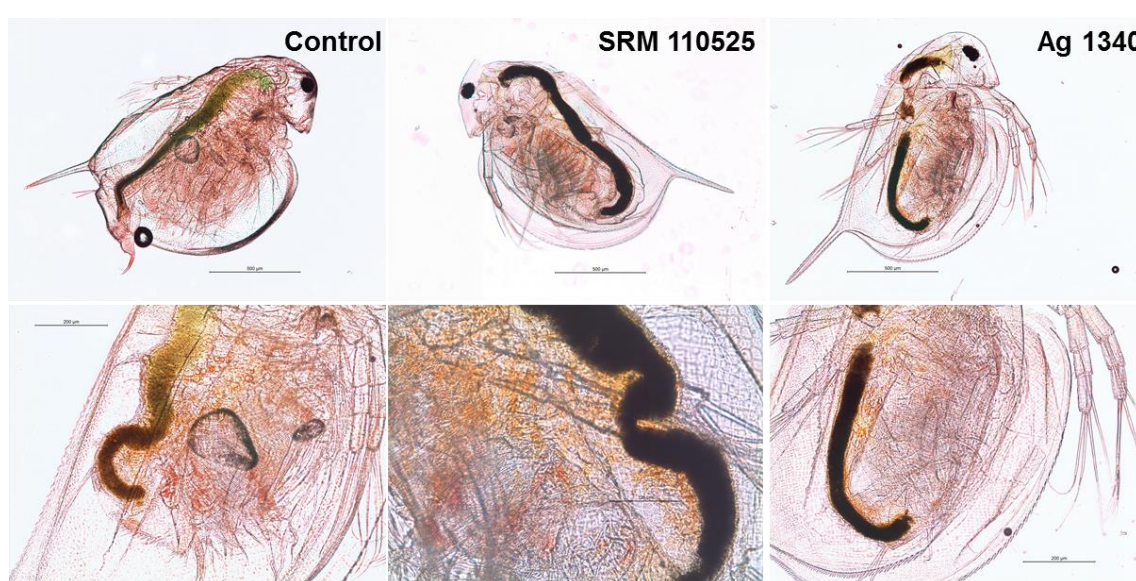


Figure 7: Daphnids exposed to 0.8 µg/L Ag wires for 48 h. Silver wires did not attach to the daphnids carapace, but an uptake into the gut was observed, as indicated by the black staining. In comparison, guts of control animals show a green staining stemming from algae food.



4.2.2 Conclusion

The results show that only one PC-parameter like the dissolution as indicator on toxicity is not sufficient. Further parameters have to be considered. Parameters such as morphology as well as test organism-specific issues and bioavailability of NM in the test system (see chapter 3.1; Table 9: parameters such as sedimentation in the test; uptake by daphnids, direct contact to algae; exposure of fish embryos to sedimented particles; protection of fish embryos by the chorion) seem to be of relevance.

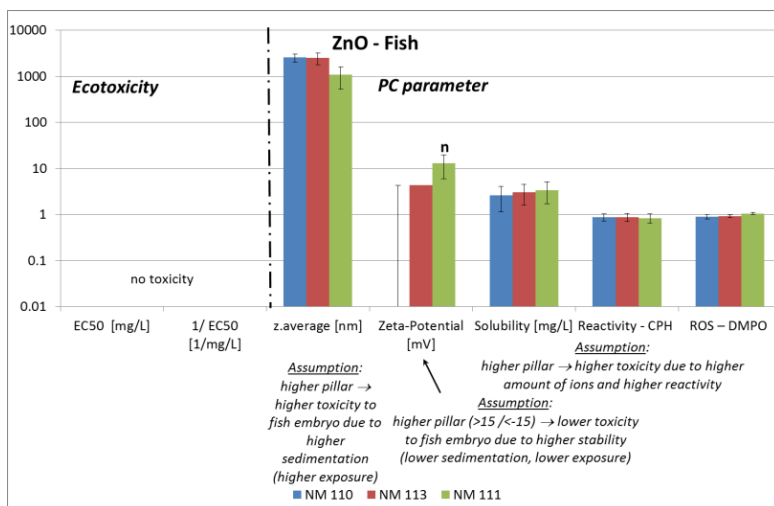
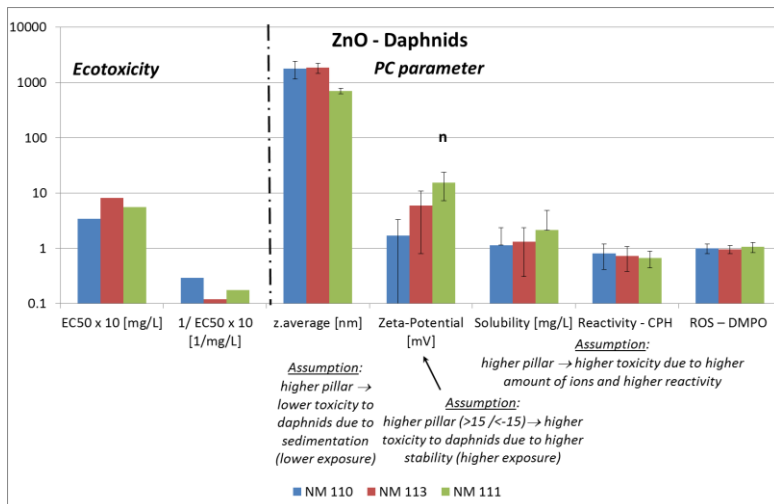
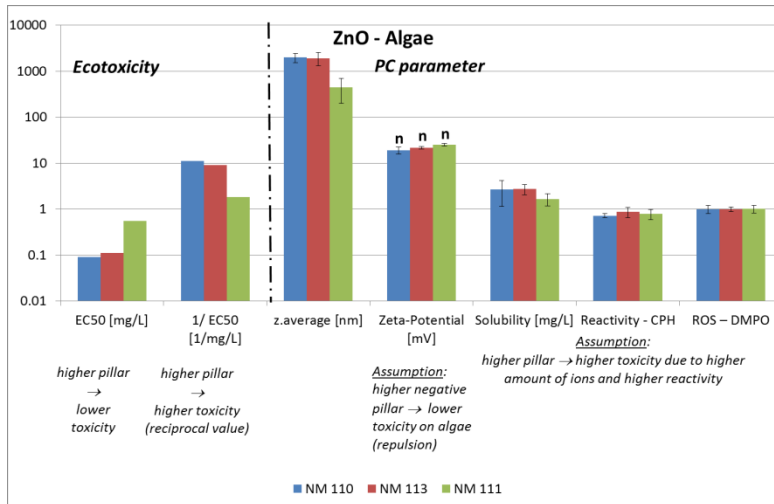
4.3 ZnO

The EC50 values determined in the ecotoxicological tests with algae, daphnids and fish embryo are listed in Table 24. Additionally, the solubility of the NMs in the test media (as measured in the stock dispersion), which is most essential parameter according to the considerations described in chapter 3.2, is included. In Figure 8 besides the EC50-values and the solubility, also the PC-parameters z.average, zeta potential, and reactivity are presented. The profiles for every parameter comprising the data of the three NMs can be compared among each other. The profiles vary significantly.

Table 24: ZnO-NMs - EC50-values (basis: nominal concentrations) and dissolution in the test media

ZnO NMs	Algae (OECD TG 201):		Daphnids (OECD TG 202)		Fish embryo (OECD TG 236)	
	EC ₅₀ [mg/L]	Solubility 72h [µg/L]	EC ₅₀ [mg/L]	Solubility 72h [µg/L]	EC ₅₀ [mg/L]	Solubility 72h [µg/L]
NM-110	0.09 (0.08 - 0.10)	2675 ± 1520	3.43 (2.85 - 4.02)	1150 ± 1226	> 100 mg/L	2620 ± 1469
NM-113	0.11 (0.05 - 0.21)	2750 ± 686	8.25 (4.70 - 11.80)	1328 ± 1018	> 100 mg/L	3050 ± 1473
NM-111	0.55 (0.38 - 0.80)	1667 ± 503	5.63 (3.26 - 8.00)	2135 ± 2734	> 100 mg/L	3400 ± 1667

Figure 8: Toxic effect of three ZnO NMs (basis: nominal concentrations) on algae, daphnids and fish embryos and results for selected PC-parameters in the three different test media (consider the additional reciprocal value of the EC-values to make the relationships more obvious.)
 n = zeta potential is negative in the test medium (due to the logarithmic scale negative values cannot be presented)



Comparable to silver, also for ZnO the EC50 values were calculated based on surface area (Table 25). For algae NM-111 is the least toxic NM based on the surface area also. For daphnids, the order of the NMs with respect to toxicity based on the surface area changes. However, it has to be considered that the differences of the three EC50 values for this organism based either on mass or surface area are small and therefore, the discussion about differences in the EC50 values for daphnids is questionable.

Table 25: ZnO-NMs - EC50 values referring to surface area compared to EC50 values on mass.

NM	Surface area [m ² /g]	EC50 - algae [mg/L]	EC50 - algae [m ² /L]	EC50 - daphnids [mg/L]	EC50 - daphnids [m ² /L]	EC50 - fish embryo [mg/L]	EC50 - fish embryo [m ² /L]
NM-110	12.0	0.09	0.0011	3.43	0.041	>100	>1.2
NM-113	6.0	0.11	0.0007	8.25	0.050	>100	>0.6
NM-111	15.0	0.55	0.0083	5.63	0.084	>100	>1.5

4.3.1 Verification of the hypotheses

In Table 26 the expected and determined toxicity rankings are listed. The hypotheses were mainly not confirmed.

Table 26: Perspective “nanomaterial” and “test organism”– expected (basis: nominal concentrations) and determined ecotoxicity of the selected NMs.

Perspective	Expected	Determined
Nanomaterial	Basis solubility: NM-111 > NM-110;-113 With consideration of the standard deviation: NM-111 ≥ NM-110 ≥ NM-113	Test with algae (mass, surface, solubility): NM-110;-113 > NM-111 Considering the difficulties in the preparation of the test dispersion of NM-111 and therefore using the analytical values as basis for the calculation of the EC50 value: NM-110 ~ NM-113 ~ NM-111 (see chapter 0) Test with daphnids (mass, surface): NM-110 ≥ NM-111 ≥ NM-113 No toxicity to fish embryos.
Test organism	NM-110: fish embryo ~ algae > daphnids NM-113: fish embryo > algae > daphnids NM-111: fish embryo > daphnids > algae With consideration of the standard deviation: fish embryo ~ algae ~ daphnids	The results for the NMs didn't differ: algae > daphnids > fish embryos

4.3.2 Conclusion

The small differences in the dissolution and the comparable morphology (spherical NMs) seem to be reflected by the comparable results on toxicity. This supports the hypothesis shown for silver that the shape can be used as indicator for the extent of an effect and has to be considered in grouping and read-across. However, the hypothesis of shape as influencing factor on toxicity has still to be verified for ZnO.

4.4 CeO₂

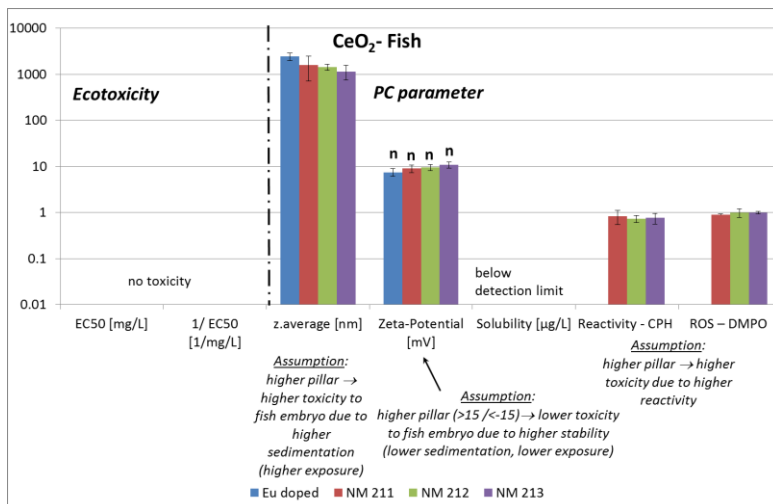
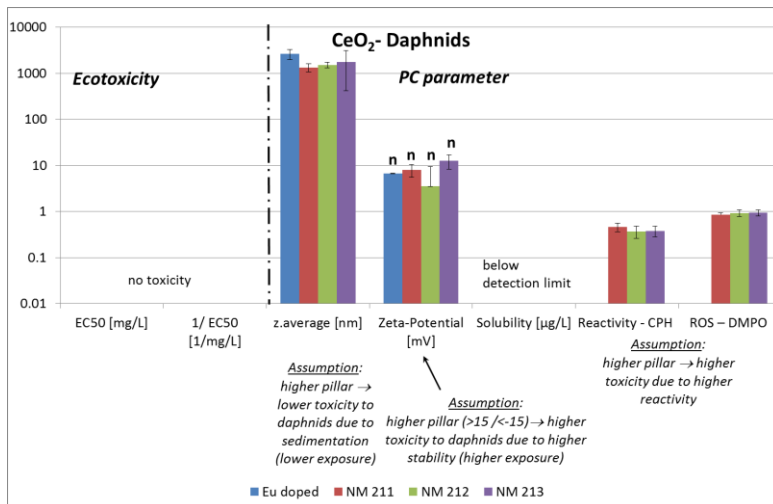
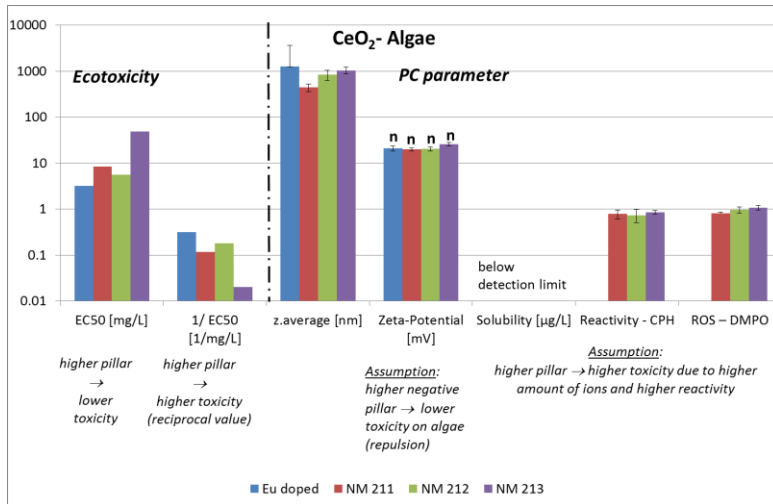
The EC50 values determined in the ecotoxicological tests with algae, daphnids and fish embryo are listed in Table 27. No further PC-parameter is listed as the differences between the NMs are small and no relevant parameter could be identified (see chapter 3.3). In Figure 9 besides the EC50-values and the solubility, also the PC-parameters z-average, zeta potential, and reactivity are presented. The profiles for every parameter comprising the data of the three NMs can be compared among each other. The toxicity profile formed by the effect values of the four NMs differs significantly from the toxicity profiles formed based on the assumed influence of PC-parameters. The NMs were only toxic to algae. Due to the low effect values a toxic effect based on shading is considered unlikely. The differences in the PC-parameters are negligible, whereas based on nominal concentrations NM-213 is less toxic to algae than the other three NMs. The differences disappear based on the measured concentrations (see chapter 4.1.3).

Table 27: CeO₂-NMs - EC50-values (basis: nominal concentrations) for algae, daphnids and fish embryos

CeO ₂ NMs	Algae (OECD TG 201) EC ₅₀ [mg/L]	Daphnids (OECD TG 202) EC ₅₀ [mg/L]	Fish embryo (OECD TG 236) EC ₅₀ [mg/L]
EU doped	3.2 (2.6 – 3.8)	> 100 mg/L	> 100 mg/L
NM-211	8.5 (7.7 – 9.3)	> 100 mg/L	> 100 mg/L
NM-212	5.6 (3.0 – 10.4)	> 100 mg/L	> 100 mg/L
NM-213	43.8 (n.d.)	> 100 mg/L	> 100 mg/L

n.d. not determinable

Figure 9: Toxic effect of four CeO₂ NMs (basis: nominal concentrations) on algae, daphnids and fish embryos and results for selected PC-parameters in the three different test media (consider the additional reciprocal value of the EC-values to make the relationships more obvious.)
 n = zeta potential is negative in the test medium (due to the logarithmic scale negative values cannot be presented)



In Table 28 the EC50 values for algae based on the surface area are listed. The differences in EC50 values based on surface area between the NMs are smaller supporting the hypothesis on ecotoxicity with the perspective of the nanomaterial.

Table 28: CeO₂-NMs - EC50 values for algae referring to surface area compared to EC50 values on mass

NM	Surface area [m ² /g]	EC50 - algae [mg/L]	EC50 - algae [m ² /L]
Eu doped	71	3.2	0.22
NM-211	66	8.5	0.56
NM-212	28	5.6	0.16
NM-213	4	43.8	0.18

4.4.1 Verification of the hypotheses

In Table 29 the expected and determined toxicity rankings are listed. The hypotheses were not confirmed using nominal mass as metric. Based on the PC-parameters, such as agglomeration, zeta potential, and reactivity the significant ecotoxicity to algae was not expected. Potential reasons for the observed effects are discussed in the following:

- ▶ Using the surface area as metric (based on nominal values) the hypothesis with the perspective of the nanomaterial (if toxicity at all: similar toxicity of the four NMs) is supported. The surface area of NM-213 is about a factor of 10 smaller than the surface areas of the other three CeO₂ NMs. Based on the surface areas, the differences in ecotoxicity between the four CeO₂ NMs are much smaller (Table 28). It cannot be excluded that a surface dependent effect which is not detected by the applied physiochemical methods induces the ecotoxicity. Under this assumption, the surface area has a significant influence on the toxicity of a NM.
- ▶ The difference in ecotoxicity between the four CeO₂ NMs is reduced and the hypothesis on the ecotoxicity ranking confirmed, however, only in case the analytical values are applied for the calculation of the EC₅₀ (Table 15). The recovery of NM-213 with 3 to 11 % is lower compared to the three other NMs (recovery mainly between 30 and 60 %). However, only aliquots had been used for the chemical analysis and the recovery varied significantly for the three NMs. Therefore, it cannot be doubtlessly concluded that the analytical values provide a better basis for the EC₅₀-values than the nominal values.
- ▶ From the organism perspective, it cannot be excluded that the required constant illumination (about 100 μmol m⁻² s⁻¹; corresponding to the requirement of about 100 μE m⁻² s⁻¹ mentioned in the guideline) in the algae test affects the NMs and hence the ecotoxicity. During daphnid and zebrafish embryo exposure a less intensive illumination is used. Data on reactivity in the presence of illumination are not available. In this context it has to be considered that for TiO₂ the data on photoreactivity in the presence of illumination did not correspond to the expected reactivity for the anatase/rutile NM (TiO₂ undoped). Therefore, a limited suitability of the applied procedure cannot be excluded.
- ▶ Another explanation regarding the insensitivity of daphnids and fish embryos to CeO₂ can be the difference between single cell organisms (algae) and multicellular organisms (daphnids, fish embryos). Arts et al. (2016) showed reactivity of CeO₂ using the *in vitro* alveolar macrophage assay. Macrophages are also single cells and a reaction with the cell surface could be more pronounced using single cells compared with multicellular organisms. But we have to consider the specific function of macrophages here, which actively take up NM because they (mis)take it for bacteria or cell debris, and destroy it by generation of intracellular ROS.

Table 29: Perspective "nanomaterial" and "test organism"— expected (basis: nominal concentrations) and determined ecotoxicity of the selected NMs

Perspective	Expected	Determined
Nanomaterial	NM-211~NM-212 ~ NM-213 ~ EU doped	Test with algae: Nominal - mass: Eu doped, NM-211;-212 > NM-213: NM-211 ~-212 > NM-213 Measured – mass; nominal – surface area: Eu doped ~ NM-211 ~-212 ~ NM-213 No toxicity to daphnids and fish embryos.
Test organism	If the chemical composition results in toxic effects at all, due to the higher dispersion stability in the test medium used for the test with algae, following order based on nominal concentrations is expected: algae> daphnids ≥ fish embryo	The results of the NMs didn't differ: algae >> daphnids, fish embryos (no toxicity to daphnids and fish embryos).

4.4.2 Conclusion

The significant ecotoxicity of CeO₂ NMs to algae in comparison to missing effects on daphnids and fish embryos was not expected based on the considered PC-parameters. Based on the surface area (based on nominal values) as well as on the analytical values the hypothesis with the perspective of the nanomaterial is supported. We assume that a surface dependent effect different from the measured reactivity is involved.

4.5 TiO₂

The EC₅₀ values determined in the ecotoxicological tests with algae, daphnids and fish embryo are listed in Table 30. No further PC-parameter is reported in the table as the differences between the NMs are small and no relevant parameter could be identified (see chapter 3.3). In the test medium used for toxicity testing of daphnids illumination resulted in photocatalytic activity (see chapter 3.3).

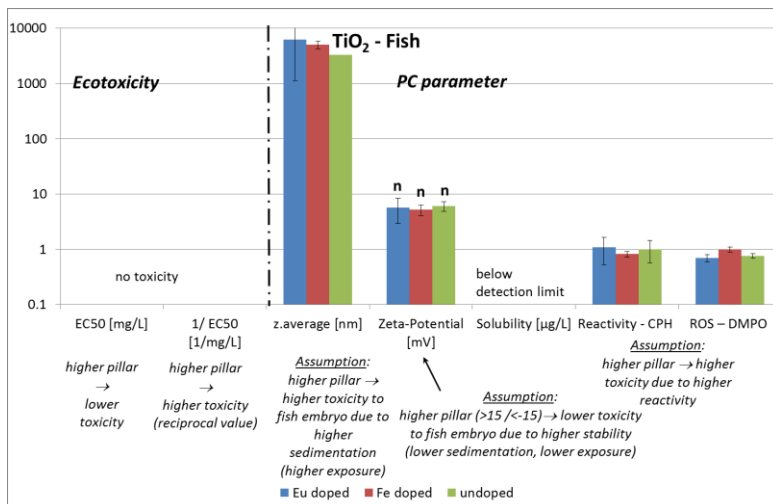
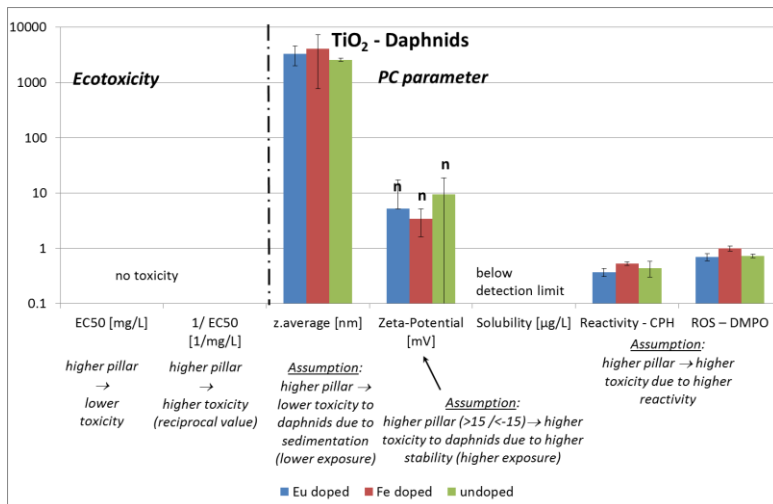
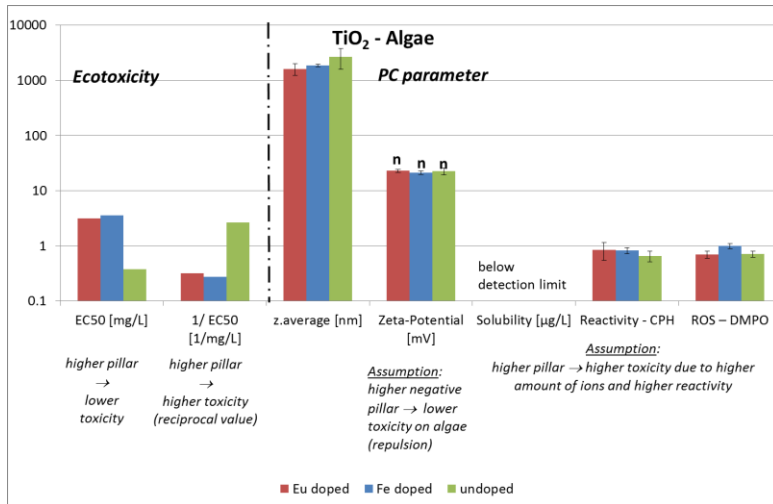
Therefore, the test with daphnids was additionally performed according to the guideline with diffuse light and with an illumination providing photocatalytic conditions. But ecotoxicity to daphnids was not observed independent of the test conditions (Wyrwoll et al. (2016)), however, observed increased toxicity of three different TiO₂ (NM100, NM101 and NM102) towards *Daphnia* when exposure was carried out under light. Probably, the crystal structure, being 100 % anatase for the three TiO₂-NM enhances the reactivity of the TiO₂ used in the Wyrwoll study compared to the TiO₂ used in this project. The NMs were only toxic to algae. Due to the low effect values a toxic effect based on shading only is considered unlikely.

In Figure 10 besides the EC₅₀-values and the solubility, also the PC-parameters z.average, zeta potential, and reactivity are presented. The profiles for every parameter comprising the data of the three NMs can be compared among each other. The toxicity profile formed by the effect values of the three NMs differs significantly from the toxicity profiles based on the assumed influence of the PC-parameters. The differences in the PC-parameters are negligible, whereas based on nominal and analytical concentrations the three TiO₂ NMs have a significantly different ecotoxicity, with the Fe doped NMs having the lowest toxicity on algae. Doping resulted in a modified crystalline structure (rutile) compared to the undoped TiO₂-NM (anatase) which did not become obvious by the investigated PC-parameters but relates to ecotoxicity with the highest ecotoxicity of the undoped NM.

Table 30: TiO₂-NM - EC₅₀-values (basis: nominal concentrations) for algae, daphnids and fish embryos

TiO ₂ NMs	Algae (OECD TG 201): EC ₅₀ [mg/L]	Daphnids (OECD TG 202) EC ₅₀ [mg/L]	Fish embryo (OECD TG 236) EC ₅₀ [mg/L]
Eu doped (5 %)	0.91 (0.75-1.10)	> 100 mg/L	> 100 mg/L
Fe doped (5%)	3.6 (2.6-4.8)	> 100 mg/L	> 100 mg/L
Undoped	0.38 (0.33-0.43)	> 100 mg/L	> 100 mg/L
Fe doped, with illumination (≠ test guideline)	---	> 100 mg/L	---
undoped with illumination (≠ test guideline)	---	> 100 mg/L	---

Figure 10: Toxic effect of three TiO₂ NMs (basis: nominal concentrations) on algae, daphnids and fish embryos and results for selected PC-parameters in the three different test media (consider the additional reciprocal value of the EC-values to make the relationships more obvious.)
 n = zeta potential is negative in the test medium (due to the logarithmic scale negative values cannot be presented)



In Table 31 the EC50 values for algae based on the surface area are listed. The differences in ecotoxicity are still apparent indicating that surface area isn't the single driver of algae toxicity of the investigated nanoforms of TiO₂. The higher toxicity of the undoped NM is still obvious. This differs from the results obtained with CeO₂ as for CeO₂ doping did not affect the ecotoxicity uniformly resulting lower toxicity. The different toxicities of the three TiO₂ NMs on mass basis which cannot be repealed by consideration of the surface area indicate a specific reactivity of the undoped nanomaterial. However, this reactivity was not obvious by the applied reactivity measurements including the measurement on photocatalytic reactivity (see chapter 3.3).

Table 31: TiO₂-NMs - EC50 values for algae referring to surface area compared to EC50 values on mass

NM	Surface area [m ² /g]	EC50 - algae [mg/L]	EC50 - algae [m ² /L]
Eu doped (5%)	148	0.91	0.13
Fe doped (5%)	63	3.6	0.23
Undoped	78	0.38	0.03

4.5.1 Verification of the hypotheses

In Table 32 the expected and determined toxicity rankings are listed. None of the hypotheses was confirmed. Based on the PC-parameters, the significant ecotoxicity to algae as well as the differences between the NMs were not expected. The NMs can be activated by illumination in the test medium used for the test with daphnids (see chapter 3.3). However, this effect did not cause ecotoxicity. It is unknown whether the concentration of transformation products caused by photocatalytic activity is too low to induce ecotoxicological effects to daphnids or whether the test organism or excretion products inactivate the transformation products, so that they cannot result in toxic effects. The latter was shown for carbon containing NMs (Fenoglio et al., 2006; Hellack et al., 2015).

Table 32: Perspective „nanomaterial“ and “test organism” – expected and determined ecotoxicity of the selected NMs (basis: nominal concentrations)

Perspective	Expected	Determined
Nanomaterial	Eu doped ~ Fe doped ~ undoped	Test with algae: undoped > Eu doped > Fe doped No toxicity to daphnids and fish embryos.
Test organism	If the chemical composition results in toxic effects at all, due to the higher dispersion stability in the test medium used for the test with algae, following order based on nominal concentrations is expected: algae > daphnids ≥ fish embryo	The results of the NMs didn't differ: algae >> daphnids, fish embryos (no toxicity to daphnids and fish embryos).

4.5.2 Conclusion

The significant ecotoxicity to algae was not expected based on the PC-parameters. It is assumed that the high toxicity of the undoped NM is based on a surface dependent effect different from the measured reactivity with EPR.

4.6 Cu

Cu was included in the investigations as unusual material. Usually CuO is investigated. Information on Cu NMs is rare. Cu was added as additional spherical, ion-releasing NM with known ecotoxicity of the chemical substance.

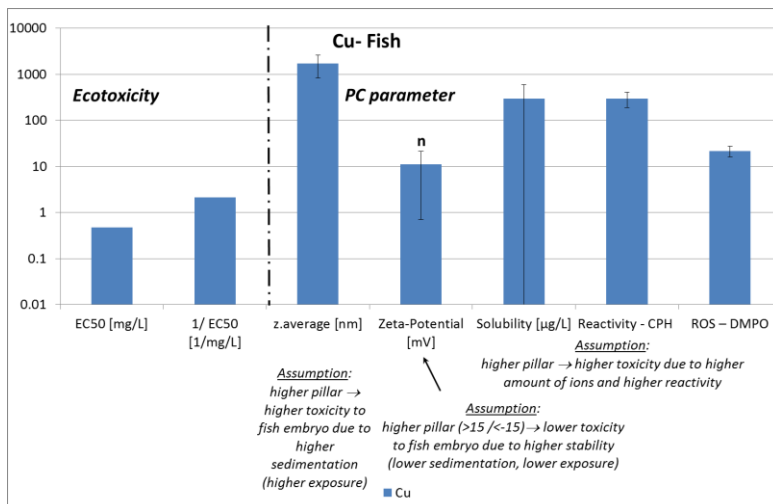
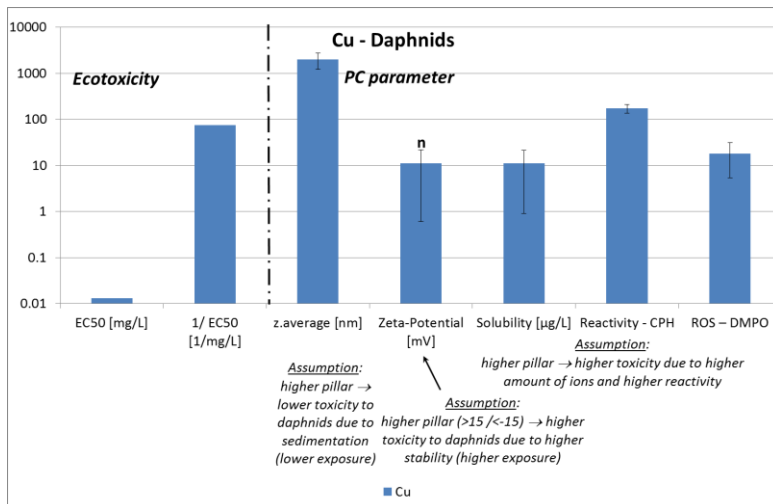
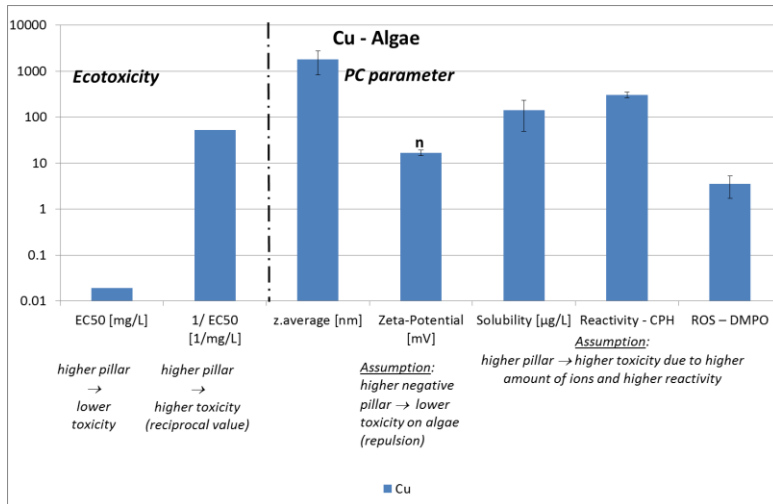
The EC50 values determined in the ecotoxicological tests with algae, daphnids and fish embryo are listed in Table 33. Additionally, the dissolution of the NMs in the test media, which is the most essential parameter according to the considerations described in chapter 3.2, is included. In contrast to the other NMs for Cu also obvious reactivity was determined. In Figure 11 besides the EC50-values and the solubility, also the PC-parameters z.average, zeta potential, and reactivity are presented.

Table 33: Cu-NMs - EC50-values (nominal concentrations) and solubility in the test media

Cu NMs	Algae (OECD TG 201):		Daphnids (OECD TG 202)		Fish embryo (OECD TG 236)	
	EC ₅₀ [mg/L]	Solubility 72h [µg/L]	EC ₅₀ [mg/L]	Solubility 72h [µg/L]	EC ₅₀ [mg/L]	Solubility 72h [µg/L]
Cu	0.0198 (0.0189 – 0.0208)	142 ± 93	0.0132 (0.0007 – 18.1792)	11 ± 10	0.4735 (0.3895 – 0.5574)	297 ± 29.8

Fish embryo test: delayed hatching

Figure 11: Toxic effect of Cu NM (basis: nominal concentrations) on algae, daphnids and fish embryos and results for selected PC-parameters in the three different test media (consider the additional reciprocal value of the EC-values to make the relationships more obvious.)
 n = zeta potential is negative in the test medium (due to the logarithmic scale negative values cannot be presented)



A calculation of the EC50 values based on surface was not performed. This value is only of interest if the toxicities of various NMs on the same organism shall be compared and if an assessment is required whether differences in the ecotoxicity of the NMs can be reduced if the surface area is taken into account.

In Table 34 the expected and determined toxicity rankings are listed. The hypotheses based on solubility were not confirmed. Also the reactivity cannot explain the observed differences. Comparable to Ag and ZnO the fish embryo test was rather insensitive.

Table 34: Perspective „test organism“ – expected and determined ecotoxicity of the selected NMs

Perspective	Expected	Determined
Test organism	fish embryos > algae > daphnids	Based on the nominal values: Algae ≥ daphnids ≥ fish embryos Based on surface area: Algae ~ daphnids ~ fish embryos Based on dissolved ions: daphnids > Algae > fish embryos

Conclusion

The expected and the measured ecotoxicity differed. However, if only algae and daphnids are considered, the prediction based on solubility corresponds to the toxicity based on dissolved Cu ions. Fish embryos are protected by the chorion. Once hatched, the situation might change. For Ag-NMs, there was no difference in toxicity observed between an onset of exposure at 2 hours post fertilization (hpf), 26 h hpf and 71 hpf, with the latter stage exposed without chorion (Böhme et al., 2015). On the contrary, a high sensitivity of young fish towards Ag-NMs in contrast to the fish embryos was already shown in another project funded by the UBA (FKZ 3709 65 418) and may also be expected for Cu-NMs due to its material specific and concentration dependent toxicity.

4.7 General conclusion

Most of the hypotheses, usually based on one parameter, were wrong. However, it seems that complex interactions have to be considered which make predictions between test organisms and between various test materials more difficult. At least the following parameters and their potential interactions have to be considered:

- ▶ Toxicity of the chemical composition (e.g. Ag is more toxic than ZnO).
- ▶ Dissolution in the test medium.
- ▶ Contact, uptake and internalization of NMs by test organisms (influenced by shape, stability and chemical composition of the NM). Sedimentation of the NMs, the behavior of the test organism, the test design (e.g. continuous shaking vs. no turbulence), place of the interaction of NM and test organism (e.g. on outer surface or only internal interaction sites; for the latter effects can only occur if there is uptake of the NM) have to be considered.
- ▶ Reactivity of the nanomaterials which can be influenced by the crystalline structure of the NMs; in our study the methods applied to determine the reactivity were not suitable to indicate differences. Other methods regarding reactivity allowing the prediction of ecotoxicity have to be used (Jackson et al., 2007; Warwar et al., 2011).

5 Grouping of the selected NMs and identification of relevant PC-parameters

Two fundamentally different approaches for the identification of PC-parameters relevant for the ecotoxicity of NMs and therefore also for the grouping regarding the hazard were applied.

One approach for a grouping of NMs was the arrangement regarding their PC data using various statistical approaches. Additionally, all information of the PC data and the toxicity of the NMs were evaluated together. If the grouping based on the PC-parameters and the grouping based on PC-data and ecotox-data are comparable, the selected PC-properties are suitable to predict the ecotoxicity and the groups seem to be meaningful regarding the environmental hazard.

In a second approach, only toxicity values and toxicity profiles were used to identify groups. The assessment was based on "expert knowledge" (no statistical approaches). Based on the groups the PC-parameters should be identified which could be responsible for the differentiation. It was assumed that with the ecotoxicological approach PC-parameters or combinations of them can be identified which are not obvious if the differentiation is just performed on the PC-parameters.

5.1 Grouping based mainly on PC-parameters

5.1.1 Volume of data and validity of the statistical results

Fourteen different NM types and subtypes had been tested and twelve different PC-parameters in four media (DI water and the three ecotoxicity test media (ADaM, ISO medium and OECD medium)) had been measured, all together building the data basis for the further evaluation. However, not for every toxicity endpoint or every tested PC-parameter data were available, for example if no effect on organisms had been observed or the material showed no dissolution or reactivity. Consequential, the data matrix for the statistical analysis was reduced and in turn the validity of the statistical results was affected. In the following figures the data availability for the statistical analysis for all media together and differentiated for each medium are presented in Figure 12 to Figure 15. The grey color indicates where data were available for the statistical test whereas the white color occurs where data were missing, for example if a NM is not soluble. For most NMs a minimum of two EC50 replicates were available for the statistical analyses for daphnids and fish embryos. For algae only one value was available for the statistical analysis as only one main test had been performed. For this test, the validity of the EC50 result was guaranteed as the range finder test, which had been conducted in advance, showed comparable toxicity for the NMs.

Figure 12: Available data for the statistical analysis, data shown were measured in all four media (water, ADaM, ISO water and OECD medium).

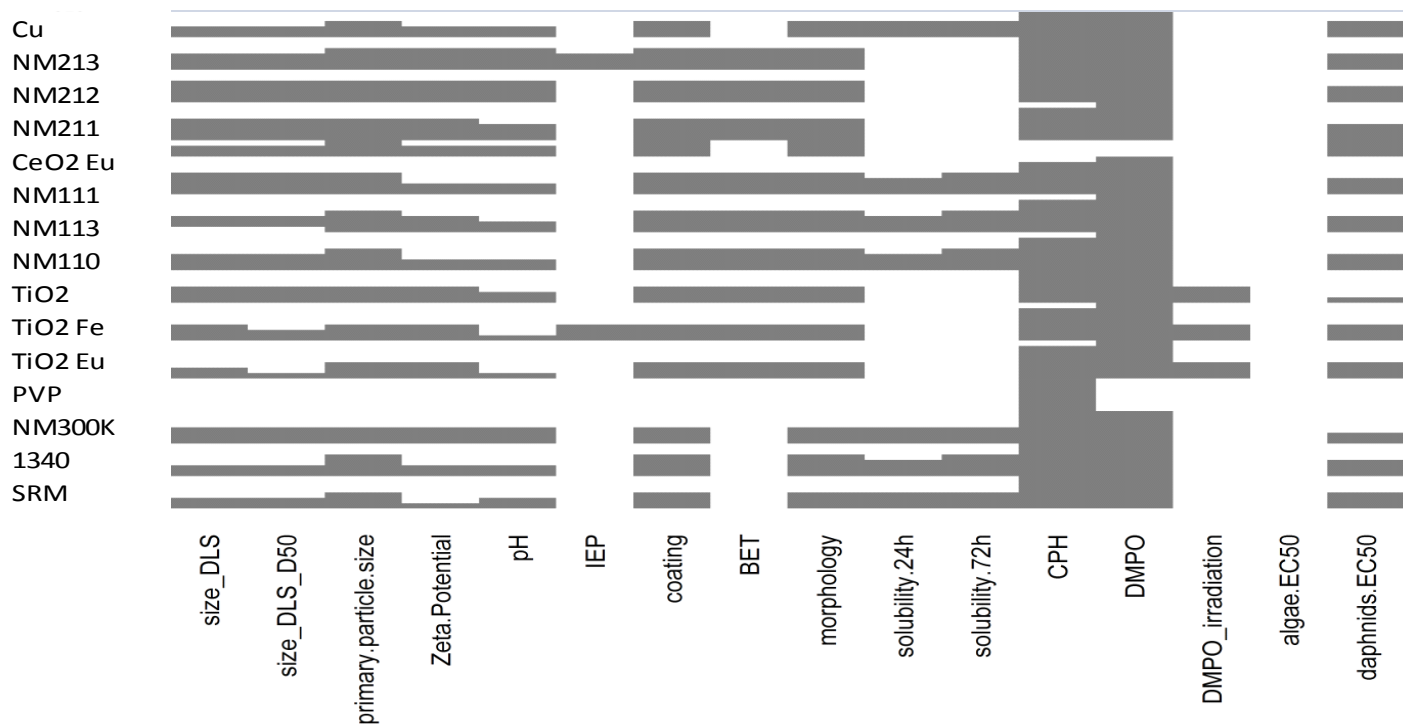


Figure 13: Data availability for the statistical analysis for daphnids (ADaM)

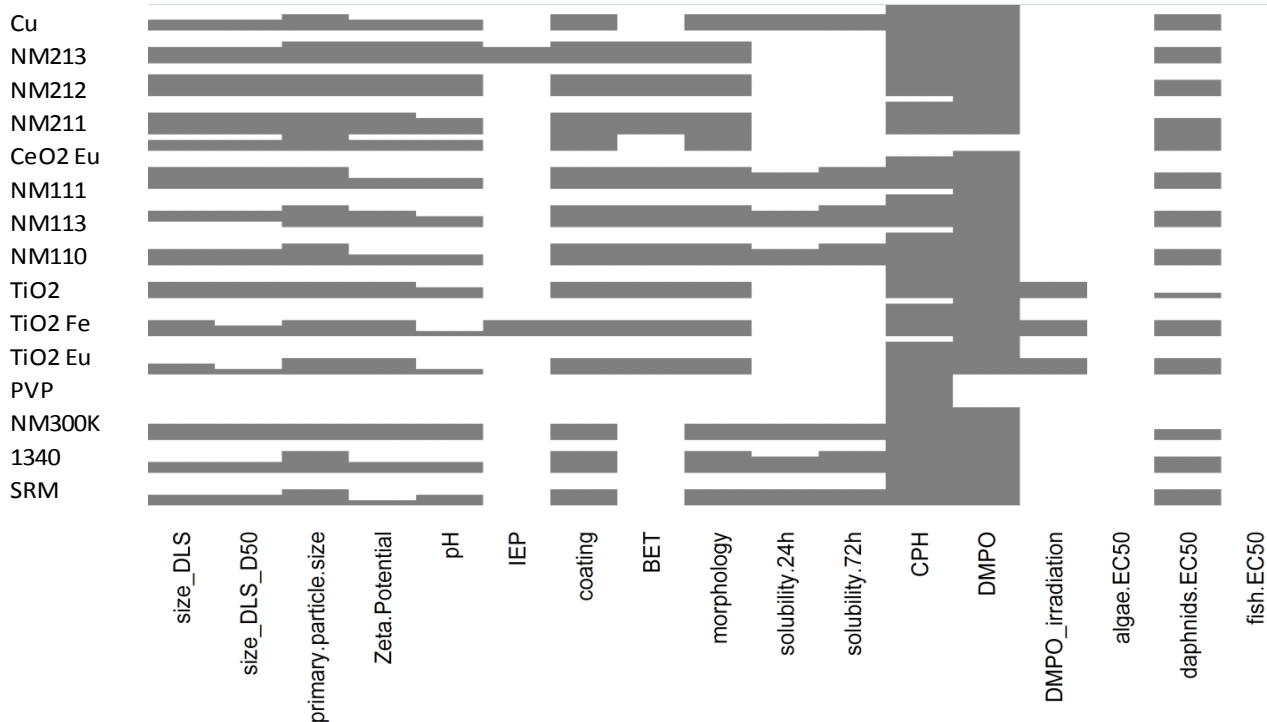


Figure 14: Data availability for the statistical analysis for fish embryo (ISO water)

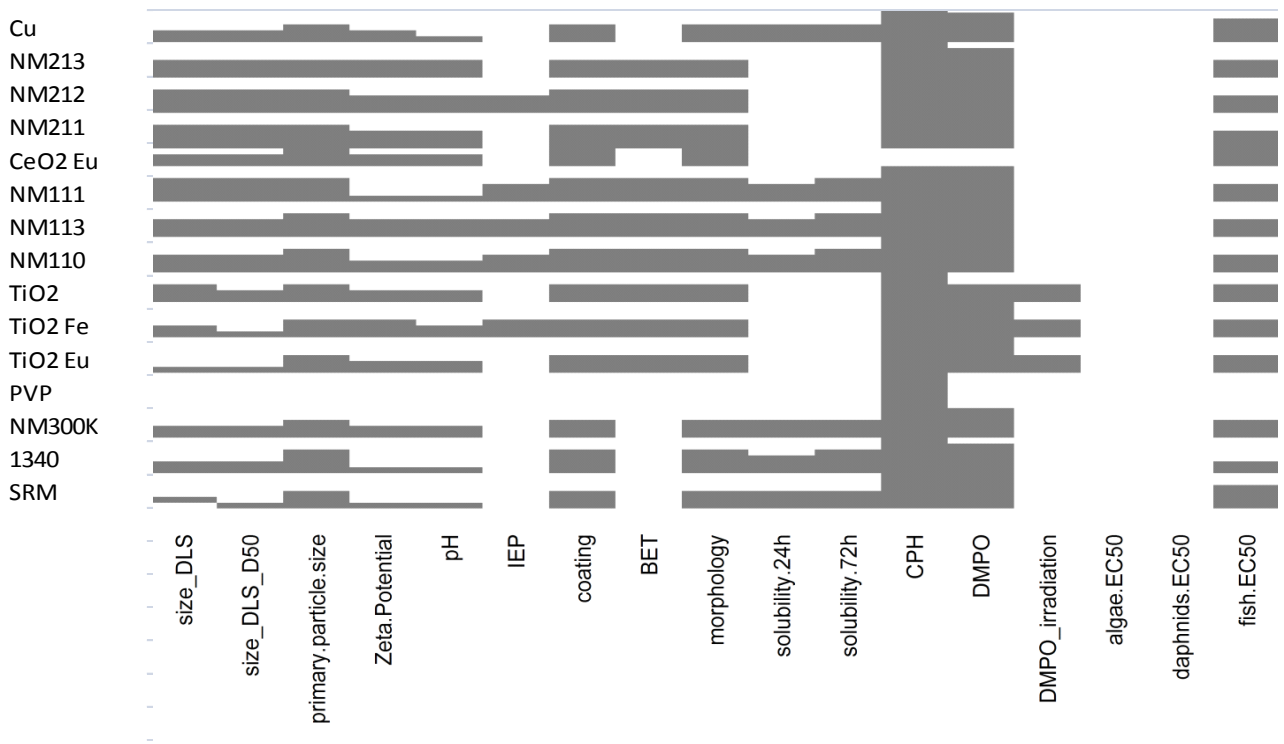
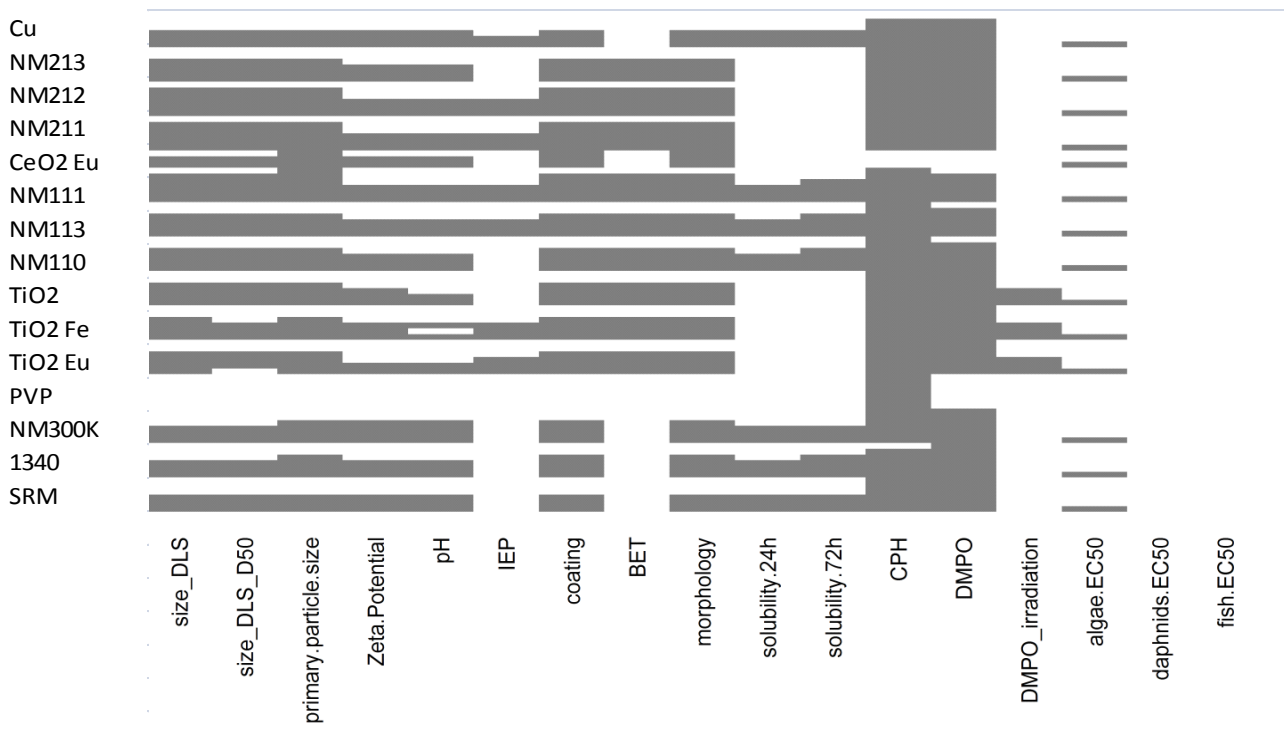


Figure 15: Data availability for the statistical analysis for algae (OECD Media)



Conclusion

From the figures it is obvious that any interpretation of the data with statistical methods has to be done with care due to limited set of data and lack of data for some endpoints.

5.1.2 Comparison of the NM suspension parameters in different media

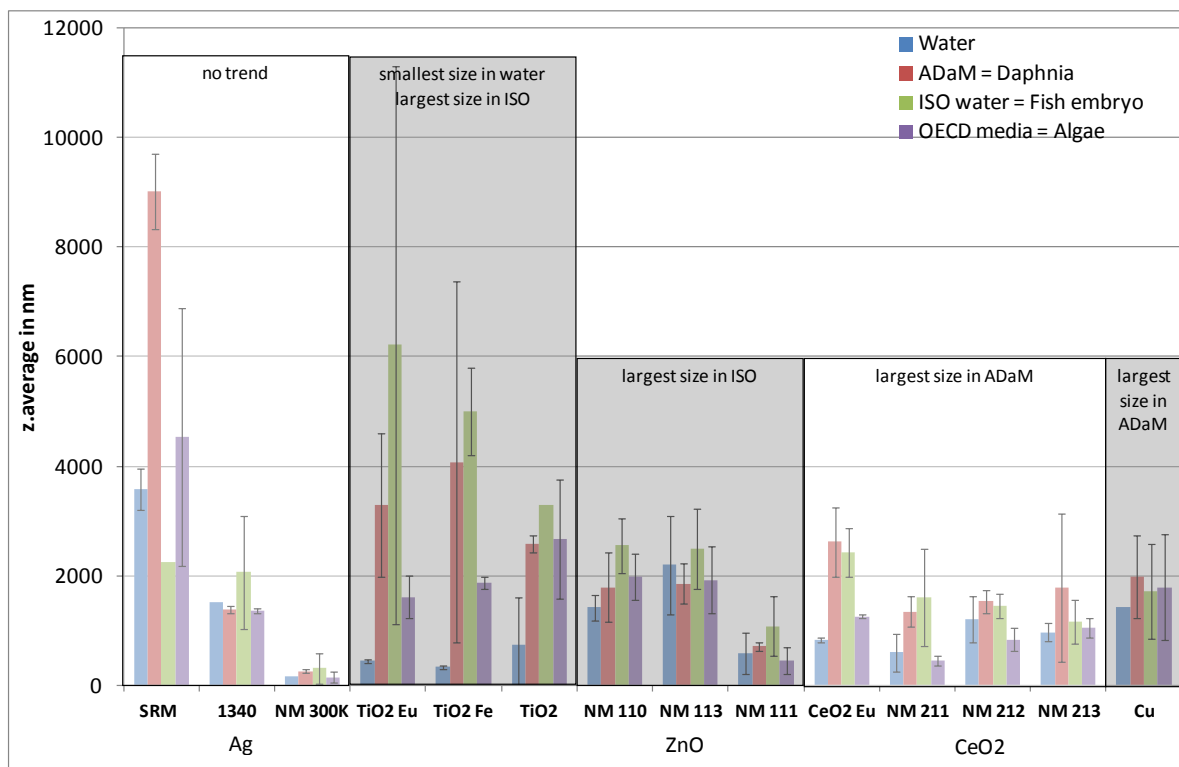
In this approach the similarities of the media dependent PC-parameters (hydrodynamic diameter, zeta potential, solubility and reactivity) were analyzed. This was done separately for every NM in every medium. Based on this analysis, similarities in the data or comparable trends of the different types of NMs or between the subtypes could be identified, which may also be used for a further classification / grouping of the NMs. Additionally the effect of the test media could be identified.

The results for the hydrodynamic diameter are shown in Figure 16.

Based on the measured hydrodynamic diameter for the nanomaterial types no clear trend was observed which occurs for all NM or media. However, for the TiO₂ and ZnO NMs the largest size was observed in ISO water and for CeO₂ NMs and Cu in ADaM. However, these differences were not significant (Tamhane, p = 0.05), only the differences between water and OECD medium for the doped TiO₂ NM showed significant differences.

For the different subtypes (except the two Ag wires) for all media the lowest hydrodynamic diameter was observed for NM-300K whereas no conformity was observed for the largest size. In ADaM the largest size was observed for TiO₂ Fe, in ISO water for TiO₂ Eu and in OECD medium for TiO₂ undoped.

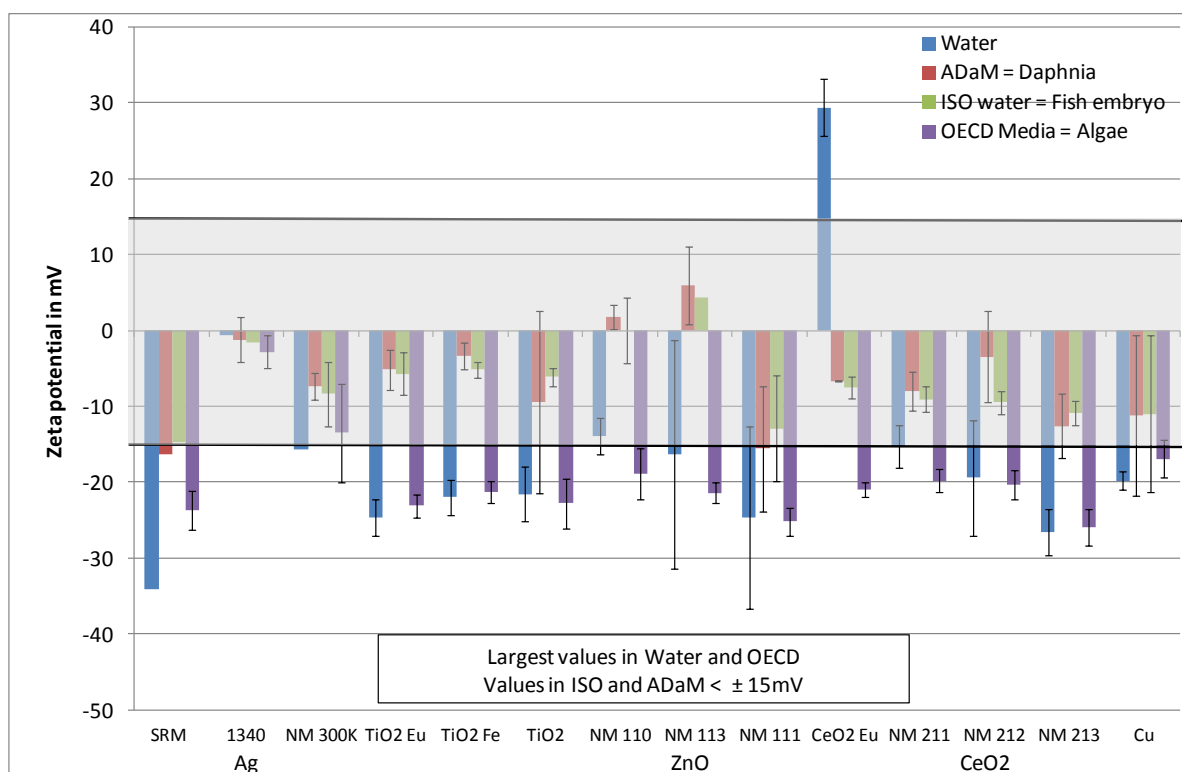
Figure 16: Hydrodynamic diameter of the NM (z.average) measured with DLS in the different media, NM concentration 100 mg/L, n = 3. ADaM = Daphnia medium, ISO water = Fish embryo medium, OECD medium = Algae medium.



In Figure 17 the results of the zeta potential measurements are presented. It is obvious that in the OECD medium the highest and always negative zeta potential values were measured. Only for the Ag-NMs Batch 1340 and NM-300K the values were below -15 mV. In ADaM and ISO water none of the NMs

showed values above ± 15 mV. Therefore, in these media a higher agglomeration and sedimentation rate of the NMs is expected resulting in a lower exposure concentration for daphnids and a high exposure concentration for fish embryos. Based on this parameter no grouping between the different NMs or within the subtypes was possible, but a clear media dependency was obvious.

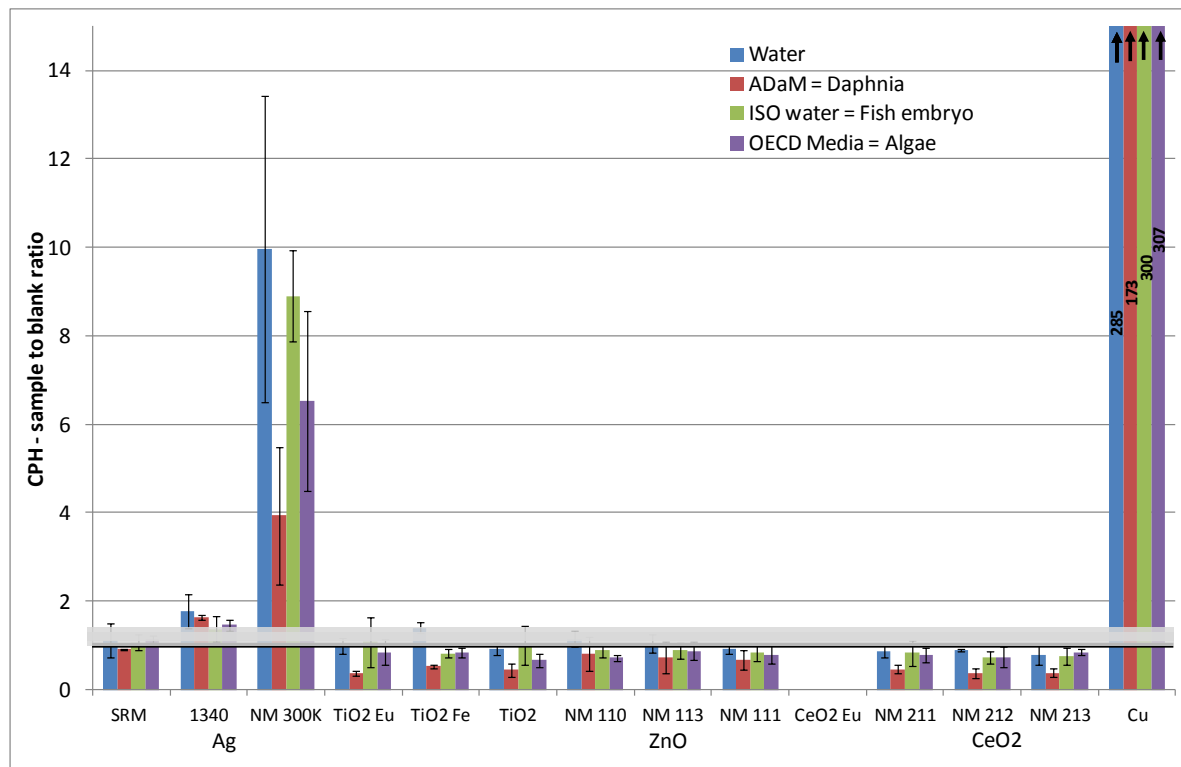
Figure 17: Zeta potential of the NM in the different media, measured with ELS. The black line indicates the value larger ± 15 mV. NM concentration 100 mg/L; n = 3; the area between -15 and + 15 mV indicates instability. ADaM = Daphnia medium, ISO water = Fish embryo medium, OECD medium = Algae medium.



In Figure 18 (CPH Method) and Figure 19 (DMPO Method) the reactivity of the NMs in the different media as sample to blank ratio are shown. Values ≥ 1.3 were used as indicator for a reactivity exceeding the blank values. The hypothesis was that forms of NMs which show reactivity may also show toxicity and can be classified in groups according to their reactivity. Additionally to this approach for the TiO_2 NM the photocatalytic activity was measured after UV irradiation (Figure 20). The TiO_2 NM were chosen as for these NM photocatalytic activity is described and these NM were used for this in technical application.

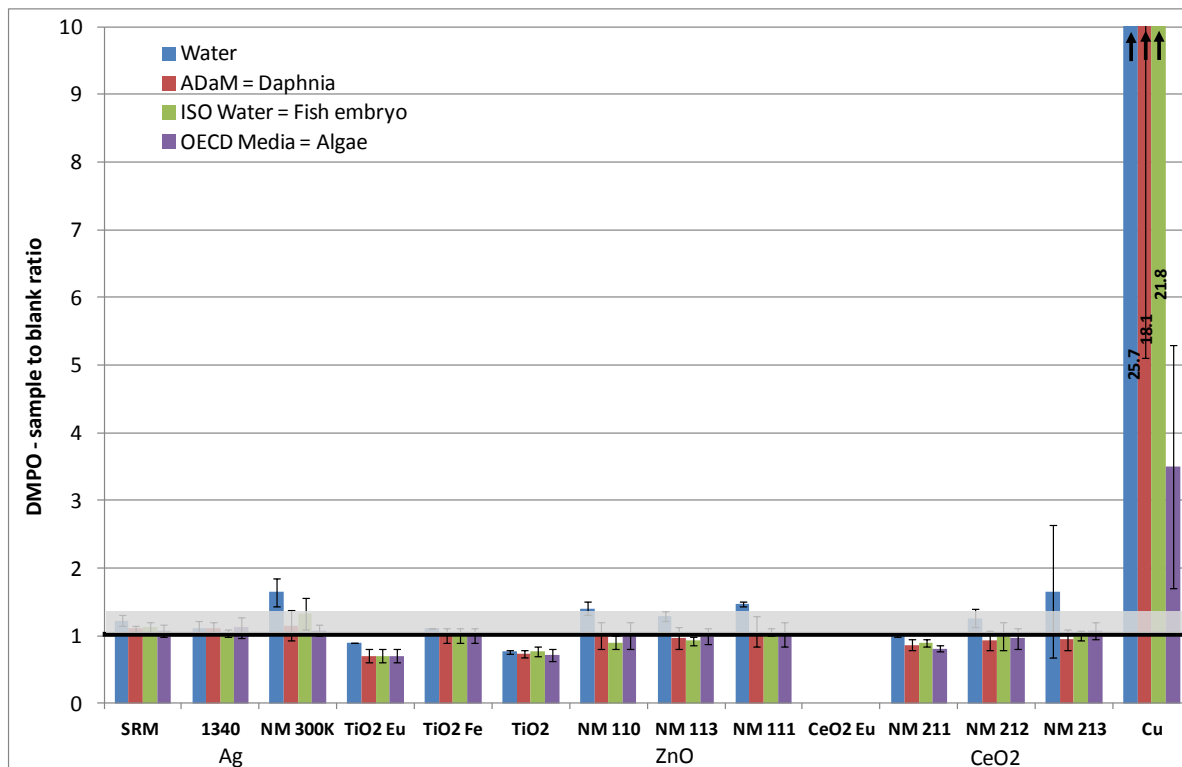
With the CPH method ("general" reactivity) only for Cu and NM-300K reactivity was observed in all media. For the silver NM 1340 reactivity was also observed in each media but the detected reactivity was in the range of the uncertainty of the measurements. For NM-300K the significant highest reactivity was detected in ISO water and the significant lowest in ADaM (Tamhane, $p = 0.05$). For Cu the highest reactivity over all were observed with no significant differences between the media (Tamhane, $p = 0.05$).

Figure 18: CPH sample to blank ratio of the NM in the different media measured with EPR. The black line shows the value 1.3, all values above this threshold indicate reactivity. The grey area 1-1.3 indicates the critical range of the measurements. Including the uncertainty of the measurements only values above 1.3 can be interpreted as reactive. NM concentration 100 mg/L; n ≥ 2. ADaM = Daphnia medium, ISO water = Fish embryo medium, OECD medium = Algae medium.



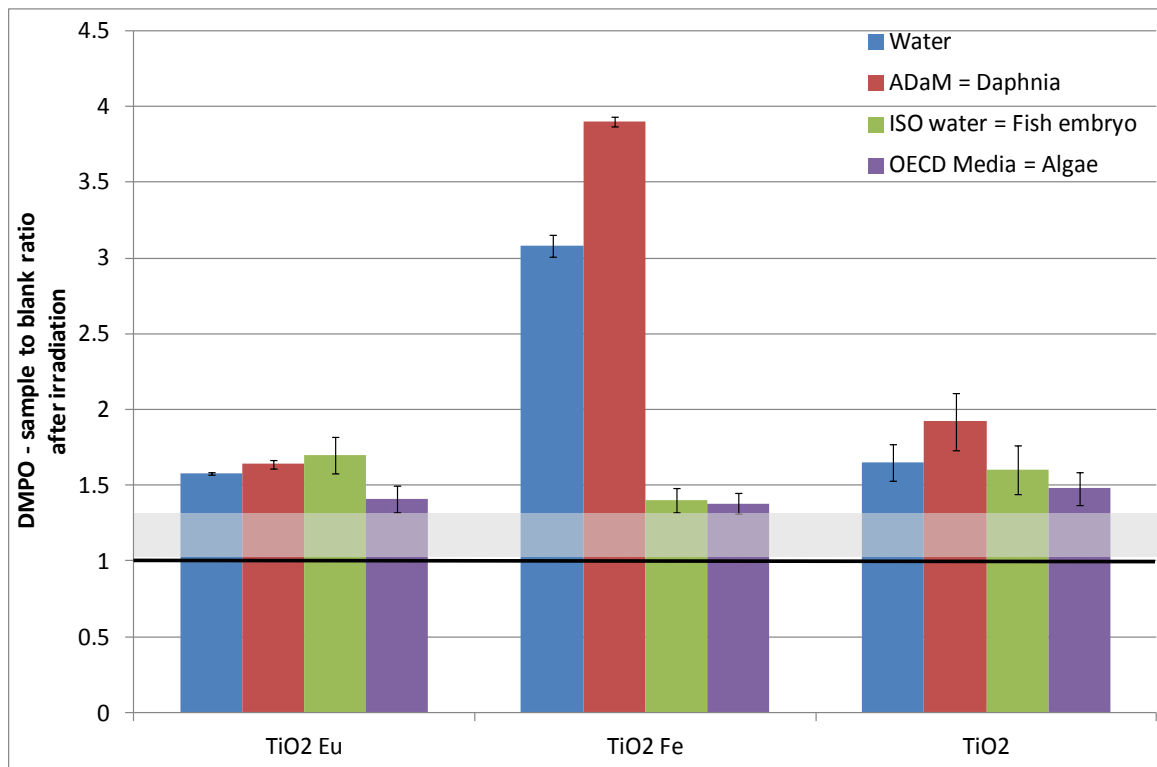
With the DMPO method (detection of ROS) only Cu showed a medium dependent significant reactivity, with the highest reactivity in ISO water and the lowest in OECD medium. For ZnO NM-110, -111, -113 as well as for the CeO₂ NM-213 and the silver NM-300K a higher reactivity was observed only in water. Based on the similarity for this PC parameter for water a group, with NM-110, 111, 113 and NM-213 and NM-300K can be derived. However, as the higher reactivity was only measured in water and not in the test media, no effect on the organisms is expected and therefore this grouping doesn't seem to be useful.

Figure 19: DMPO sample to blank ratio of the NM in the different media measured with EPR without irradiation. The black line shows the value 1.3, all values above this threshold indicate ROS production. The grey area 1-1.3 indicates the critical range of the measurements. Including the uncertainty of the measurements only values above 1.3 can be interpreted as reactive. NM concentration 100 mg/L; n ≥ 2. ADaM = Daphnia medium, ISO water = Fish embryo medium, OECD medium = Algae medium.



The measurements were conducted without irradiation, to test if the irradiation with UV light has an effect on the ROS generation the three photoactive TiO₂ NMs were activated with UV light and ROS was detected with the DMPO method afterwards (Figure 20). Thereupon for all TiO₂ NM in all media reactivity was detectable but with different characteristics. For all TiO₂ NMs in OECD medium the lowest reactivity (in the range of the uncertainty of the measurement) was observed. For TiO₂ Eu the highest values were detected in ISO water, whereas for TiO₂ and TiO₂ Fe the highest reactivity was observed in ADaM, with the overall significant highest reactivity for ADaM and TiO₂ Fe.

Figure 20: DMPO sample to blank ratio of the TiO₂ NMs in the different media measured with EPR after UV irradiation. The black line indicates data with value 1. The grey area 1-1.3 indicates the critical range of the measurements. Including the uncertainty of the measurements only values above 1.3 can be interpreted as reactive. NM concentration 100 mg/L; n ≥ 2. ADaM = Daphnia medium, ISO water = Fish embryo medium, OECD medium = Algae medium.



In Figure 21 to Figure 22 the solubility of the NMs in the four different media after 24 h and 72 h is presented as percent of the initial concentration.

The significant highest solubility after 24 h and 72 h was determined for NM-300K followed by the three ZnO NM, the Ag-wire 1340 and Cu. Furthermore a medium dependent but not significant (Tamhane p = 0.05) difference was observed. Additionally, there is no clear trend for the media and NMs. If the solubility is used as classification / grouping criteria for all NMs and media, NM-300K, NM-110, NM-111, NM-113, Ag-wire 1340 and Cu can be grouped together. For the different sub types no clear trend between the media is obvious for the silver NM, whereas for ZnO, the lowest solubility was determined in ADaM after 72h.

Figure 21: Dissolution of the NM in the different media after 24 h in %; the values for NM-300K are plotted on the second Y axis, please consider the different scale; n ≥ 2. Concentration of the stock suspension was 1 g/L, for NM300K 10 g/L.

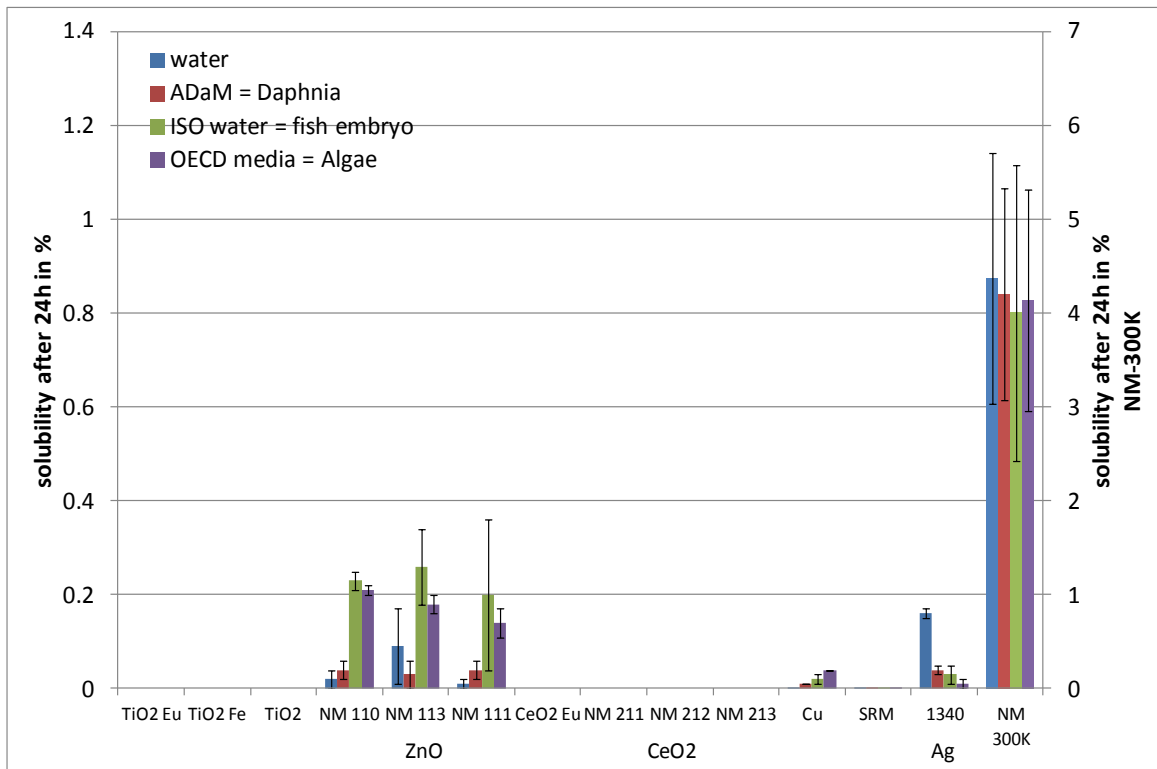
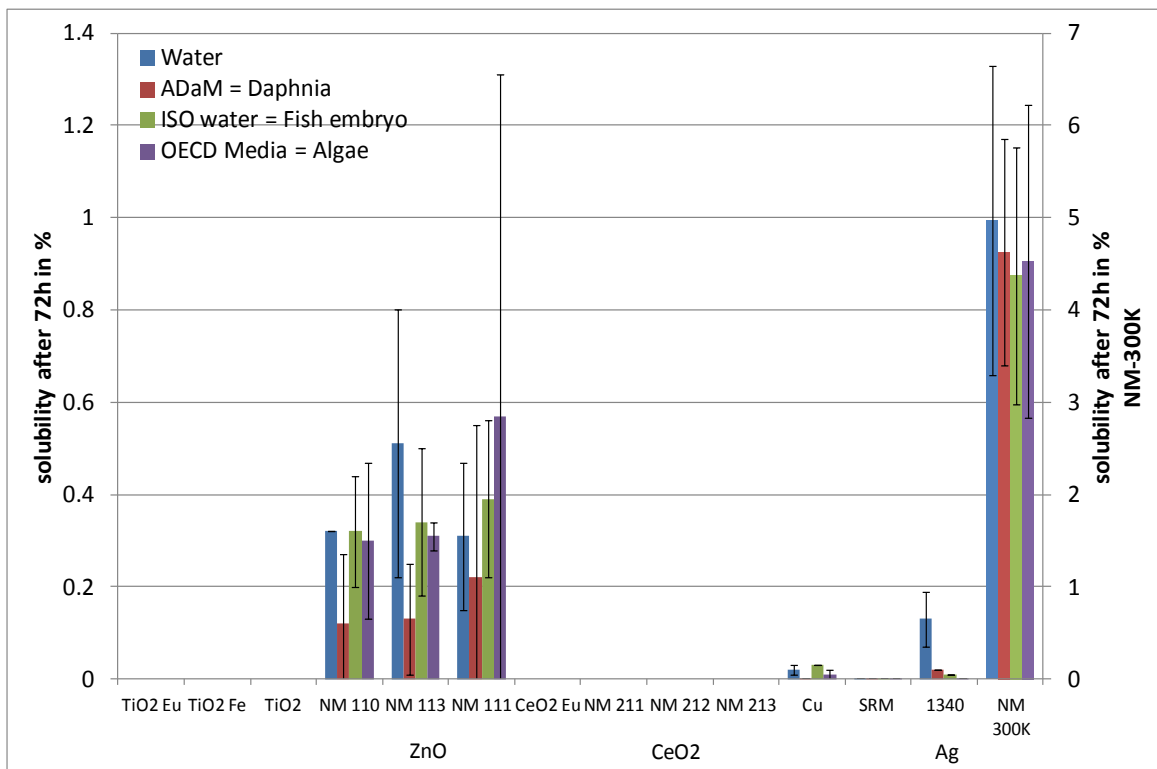


Figure 22: Dissolution of the NM in the different media after 72 h in %; the values for NM-300K are plotted on the second Y axis, please consider the different scale; n ≥ 2. Concentration of the stock suspension was 1 g/L, for NM300K 10 g/L.



5.1.3 Characterization of the PC-parameters in water and in test media

In a first step it was tested if the analysis of PC-parameters in water can be used as surrogate for other media. Therefore, the measured PC-parameters were classified in different categories (Table 35). The resulting categories in water and the test media were compared. Comparable categories would indicate that results in water can be used as surrogate for the other media. The results of the categories of the parameters which were affected by the media are shown in Table 36 to Table 38. In Table 36 the comparison for water and ADaM, in Table 37 for water and ISO water and in Table 38 for water and OECD medium are shown. Differences in categories of PC parameters between water and the presented media are marked in blue.

Table 35: Categories of the PC-parameters used for the comparison between water and the other media

categories	primary particle size nm	BET m ² /g	coating	morphology	z.average nm	Zeta potential mV	pH	IEP pH	solubility %	reactivity sample to blank ratio
1	≤10	≥ 100	Yes	Spheres	≤500	negative ≥15	≤ 6	≤ 6	Not soluble	≥ 1.3
2	10-50	50-100	No	Wire/Rods	500-1000	positive ≥15	6-8	6-8	≤0.1	≤ 1.3
3	50-100	10-50		Cubes	≥ 1000	negative ≤15	≥ 8	≥ 8	0.1-1	
4	≥ 100	≤10				positive ≤15			≥ 1	

Table 36: Comparison of the categories of the media affected PC data measured in water and Daphnia medium (ADaM). A different category is marked in blue.

Water/ADaM	SRM 110525	1340	NM 300K	TiO2 Eu doped (5 %)	TiO2 Fe doped (5 %)	TiO2	NM 110	NM 113	NM 111	CeO2 Eu doped (5 %)	NM 211	NM 212	NM 213	Cu
z.average	3	3	1	3	3	3	3	3	2	3	3	3	3	3
Zeta Potential	1	3	3	3	3	3	4	4	1	3	3	3	3	3
pH	2	2	2	2	3	3	2	2	2	2	2	2	2	2
IEP	4	4	4	4	4	4	4	4	4	4	4	4	4	4
solubility 24h	2	2	4	1	1	1	3	3	3	1	1	1	1	2
solubility 72h	2	2	4	1	1	1	3	3	3	1	1	1	1	2
CPH	2	1	1	2	2	2	2	2	2	2	2	2	2	1
DMPO	2	2	2	2	2	2	2	2	2	2	2	2	2	1
DMPO_irradiation	-	-	-	1	1	1	-	-	-	-	-	-	-	-

For ADaM in contrast to water a higher hydrodynamic diameter and a lower zeta potential, solubility after 72 h and reactivity (DMPO) was observed. Furthermore often no IEP was detectable.

Table 37: Comparison of the categories of the media affected PC data measured in water and fish embryo medium (ISO water). A different category is marked in blue.

Water/ISO	SRM 110525	1340	NM 300K	TiO2 Eu doped (5 %)	TiO2 Fe doped (5 %)	TiO2	NM 110	NM 113	NM 111	CeO2 Eu doped (5 %)	NM 211	NM 212	NM 213	Cu
z.average	3	3	1	3	3	3	3	3	3	3	3	3	3	3
Zeta Potential	3	3	3	3	3	3	3	4	3	3	3	3	3	3
pH	2	2	2	2	2	2	2	2	2	2	2	2	2	2
IEP	4	4	4	4	4	4	2	4	4	4	4	4	4	4
solubility 24h	2	2	4	1	1	1	3	3	3	1	1	1	1	2
solubility 72h	2	2	4	1	1	1	3	3	3	1	1	1	1	2
CPH	2	1	1	2	2	2	2	2	2	2	2	2	2	1
DMPO	2	2	1	2	2	2	2	2	2	2	2	2	2	1
DMPO_irradiation	-	-	-	1	1	1	-	-	-	-	-	-	-	-

The same observation was made for ISO water. In contrast to water a higher hydrodynamic diameter and a lower zeta potential, solubility and reactivity (DMPO) was observed. Furthermore often no IEP

was detectable. With exception of the two silver wires a higher solubility was observed in the ISO water.

Table 38: Comparison of the categories of the media affected PC data measured in water and algae medium (OECD medium). A different category is marked in blue.

Water / OECD	SRM 110525	1340	NM 300K	TiO ₂ Eu doped (5%)	TiO ₂ Fe doped (5%)	TiO ₂	NM 110	NM 113	NM 111	CeO ₂ Eu doped (5%)	NM 211	NM 212	NM 213	Cu
z.average	3	3	1	3	3	3	3	3	1	3	1	2	3	3
Zeta Potential	1	3	3	1	1	1	1	1	1	1	1	1	1	1
pH	2	2	2	2	2	2	2	2	2	2	2	2	2	2
IEP	4	4	4	1	4	2	4	2	2	4	1	2	4	2
solubility 24h	2	2	4	1	1	1	3	3	3	1	1	1	1	2
solubility 72h	2	2	4	1	1	1	3	3	3	1	1	1	1	2
CPH	2	1	1	2	2	2	2	2	2	2	2	2	2	1
DMPO	2	2	2	2	2	2	2	2	2	2	2	2	2	1
DMPO_irradiation	-	-	-	1	1	1	-	-	-	-	-	-	-	-

For the algae medium the differences to water are smaller as for the other two media. In contrast to ADaM and ISO water only slight differences for the hydrodynamic diameter were observed. If differences for the zeta potential were identified the values in the media were higher and always negative. For the reactivity and IEP the same observation as for the other two media were made. Whereas for the solubility after 72 h a lower ion release was detected for the silver NM 1340 but a higher solubility for ZnO in contrast to water.

Conclusion

For all NMs no clear trend was observed between the detected values in water and the media. Considering all PC-parameters, only CPH and DMPO irradiation resulted in comparable categories for water and the three media. Overall, for ADaM and ISO water no NM shows the same set of categories in the media and water. For OECD medium only the PC parameters of Cu were comparable to those in water. The categories for Cu in water were the same as in the OECD media.

A comparison between the different media demonstrated that for ADaM and ISO water half of the NMs (1340, TiO₂ Eu, NM-113, CeO₂ Eu, NM-211, NM-212, Cu) showed the same categories in both media. A lower conformity was observed between ADaM and OECD medium. In these media only for the silver NMs the same categories were detected. The lowest conformity was observed between ISO water and OECD medium. Here only for 1340 the same categories were observed. This observation clearly demonstrates that the media affected the different PC-parameters. This means that the results for the PC-parameters measured in water are not directly transferable to the other media. From this it follows that the PC characterization should be conducted in each test media for reliable results.

A comparison of the PC-parameter categories for the different NM for each media can be one approach for a grouping of the NMs.

For ADaM the same categories for TiO₂ Eu doped, CeO₂ Eu doped, NM-211 and NM-212 were demonstrated as well as another group with TiO₂ Fe doped and TiO₂ undoped and a third group with NM-110 and NM-113. For the other NMs different categories were demonstrated.

In ISO water the same categories were detected for the “stable” materials (all TiO₂ and CeO₂ NM) whereas all other NM showed different categories at least for one PC-parameter.

For OECD medium only CeO₂ Eu doped, TiO₂ Fe doped and NM-213 showed the same categories the other NM showed different ones.

5.1.4 Relationship between PC categories and ecotoxicity

Based on the categories of Table 35 and the information on toxicity we analyzed if any PC-parameter can be identified which causes the toxicity.

The results for the daphnids and the PC parameters measured in ADaM are presented in Table 39. From the results it can be concluded that based on the PC-parameters BET, z.average/size_DLS, pH, IEP, DMPO and DMPO irradiation no relationship to the toxicity was observed. For the primary particle size it can be concluded that the smallest particle size shows no toxicity, the toxicity was observed for particles > 10 nm. Additionally, for daphnids no effect of the morphology can be identified if all NM types were compared. Toxic effects in the same range were observed independently of the morphology. However, if only the silver subtypes were compared, then an effect of the morphology is obvious, with a higher toxicity for the Ag wires compared to the spherical Ag NM. In this case the effect of the morphology was higher than the differences in the solubility, as the spherical Ag NM NM-300K shows the highest solubility but not the highest EC50.

For the zeta potential the highest toxicity was observed for NMs with a negative ZP independently if the values were larger or smaller 15 mV. Therefore the charge of the ZP can be one important parameter related to the toxicity. For the solubility we can conclude that the insoluble NMs showed no toxic effect. Furthermore we observed that the NMs with the highest solubility did not cause the highest toxic effect, this means that the solubility can be the driver for the observed results, but not the only one. For the reactivity measured with CPH we can conclude that NMs which show reactivity are also toxic. However, reactivity is not prerequisite for ecotoxicity. Therefore, reactivity is a relevant parameter but not the only one.

Anyway, the identified categories for the daphnia medium (ADaM) shown in Table 39, based on the same PC-parameter categories as presented in Table 35, do not correspond to the observed toxic effects measured for the organisms. Transparency on the correlation of the established categories and toxicity might be improved when considering nanoforms of only 1 chemical identity. However for such an exercise a bigger data set is needed.

Table 39: Categories of the measured PC-parameters for the different NMs in ADaM and their corresponding toxicity for daphnids. The green color in the table head stands for the lowest toxicity and the dark red color for the highest toxicity, with increasing toxicity from yellow to red.

ADaM	SRM 110525	1340	NM 300K	TiO2 Eu doped (5%)	TiO2 Fe doped (5%)	TiO2 undotiert	NM 110	NM 113	NM 111	CeO2 Eu doped (5%)	NM 211	NM 212	NM 213	Cu
primary particle size	4	4	2	2	2	2	2	2	2	2	1	2	2	3
BET	-	-	-	1	2	2	3	4	3	2	2	3	4	-
coating	2	2	2	2	2	2	2	2	1	2	2	2	2	2
morphology	2	2	1	1	1	1	3	3	3	1	1	1	3	1
size_DLS	3	3	1	3	3	3	3	3	2	3	3	3	3	3
Zeta Potential	1	3	3	3	3	3	4	4	1	3	3	3	3	3
pH	2	2	2	2	3	3	2	2	2	2	2	2	2	2
IEP	4	4	4	4	4	4	4	4	4	4	4	4	4	4
solubility 24h	2	2	4	1	1	1	3	3	3	1	1	1	1	2
solubility 72h	2	2	4	1	1	1	3	3	3	1	1	1	1	2
CPH	2	1	1	2	2	2	2	2	2	2	2	2	2	1
DMPO	2	2	2	2	2	2	2	2	2	2	2	2	2	1
DMPO_irradiation	-	-	-	1	1	1	-	-	-	-	-	-	-	-

colour code	EC 50
Green	> 100 mgL
Yellow	100-10 mgL
Orange	1-10 mgL
Red	1-0.1 mgL
Dark Red	< 0.1 mgL
Black	< 0.01 mgL

In Table 40 the results for the fish embryos and the PC parameters measured in ISO water are presented. From the results it can be concluded that based on the PC-parameters BET, z.average, zeta potential, pH, IEP, DMPO and DMPO irradiation no relationship between the categories and the toxicity was observed. Regarding the primary particle size, the smallest particle size shows no toxicity, but that

toxicity was observed for particles > 10 nm which corresponds to the observations for daphnids. For the fish embryos only wires and spherical particles shows an effect, whereas no effect was observed for the cubes (ZnO-NM). For the solubility we can deduce that insoluble NMs showed no toxic effect. Furthermore we observed that NMs with a low solubility show lower toxic effects. Comparable to daphnids, NMs which are CPH reactive are also ecotoxic, but reactivity is not a general and solely prerequisite for ecotoxicity. Therefore, it was concluded that reactivity is a relevant parameter but not the only one.

Regarding the categories based on the PC-parameter measured in the media the same observation as for Daphnia medium ADaM was made. The identified categories based on the value of the different PC parameters in the Fish embryo media ISO do not correspond to the observed toxic effects, this means that a comparable PC category do not lead to a corresponding toxicity and no clear trend can be observed.

Table 40: Categories of the measured PC-parameters for the different NMs in ISO water and their corresponding toxicity for fish embryos. The green color in the table stands for the lowest toxicity and the dark red color for the highest toxicity, with increasing toxicity from yellow to red.

ISO	SRM 110525	1340	NM 300K	TiO2 Eu doped (5%)	TiO2 Fe doped (5%)	TiO2 undotiert	NM 110	NM 113	NM 111	CeO2 Eu doped (5%)	NM 211	NM 212	NM 213	Cu
primary particle size	4	4	2	2	2	2	2	2	2	2	1	2	2	3
BET	-	-	-	1	2	2	3	4	3	2	2	3	4	-
coating	2	2	2	2	2	2	2	2	1	2	2	2	2	2
morphology	2	2	1	1	1	1	3	3	3	1	1	1	3	1
size_DLS	3	3	1	3	3	3	3	3	3	3	3	3	3	3
Zeta Potential	3	3	3	3	3	3	3	4	3	3	3	3	3	3
pH	2	2	2	2	2	2	2	2	2	2	2	2	2	2
IEP	4	4	4	4	4	4	2	4	4	4	4	4	4	4
solubility 24h	2	2	4	1	1	1	3	3	3	1	1	1	1	2
solubility 72h	2	2	4	1	1	1	3	3	3	1	1	1	1	2
CPH	2	1	1	2	2	2	2	2	2	2	2	2	2	1
DMPO	2	2	1	2	2	2	2	2	2	2	2	2	2	1
DMPO_irradiation	-	-	-	1	1	1	-	-	-	-	-	-	-	-







colour code	EC 50
	> 100 mg/L
	100-10 mg/L
	1-10 mg/L
	1-0.1 mg/L
	< 0.1 mg/L
	< 0.01 mg/L

In Table 41 the results for the algae and the PC Parameter measured in OECD media are presented. From the results it can be concluded that based on the PC-parameters primary particle size, BET, morphology, z.average, pH, IEP, DMPO and DMPO irradiation no relationship between the categories and the toxicity was observed. For the zeta potential we can identify that the highest toxicity was observed if the NMs shows a negative ZP with values increasing -15 mV. Therefore the ZP was identified as one important parameter which can be correlated with the toxicity. For the solubility we can conclude that the insoluble NMs show a lower toxic effect compared to soluble ones. If no solubility was detected after 24 h EC50 values > 0.1 mg/L were observed and if no solubility was observed after 72 h the EC increased to a value of > 1 mg/L. For the reactivity measured with CPH the same observation as for the daphnia medium ADaM and fish embryo ISO water was deduced. If reactivity was observed, we also observed toxicity, but we also observed toxicity if no reactivity was observed. An indication, that the reactivity can be a relevant parameter but not the only one explaining the detected toxicity.

Regarding the PC-parameter categories the same observation as for the other two organisms and media was observed. The value of the different PC parameters do not correspond to the observed toxic effects, this means that a comparable PC category do not lead to a corresponding toxicity.

Table 41: Categories of the measured PC-parameters for the different NMs in OECD water and their corresponding toxicity for algae. The color in the table stands for the measured toxicity, with increasing toxicity from yellow to red.

OECD	SRM 110525	1340	NM 300K	TiO2 Eu doped (5%)	TiO2 Fe doped (5%)	TiO2 undotiert	NM 110	NM 113	NM 111	CeO2 Eu doped (5%)	NM 211	NM 212	NM 213	Cu
primary particle size	4	4	2	2	2	2	2	2	2	2	1	2	2	3
BET	-	-	-	1	2	2	3	4	3	2	2	3	4	-
coating	2	2	2	2	2	2	2	2	1	2	2	2	2	2
morphology	2	2	1	1	1	1	3	3	3	1	1	1	3	1
size_DLS	3	3	1	3	3	3	3	3	1	3	1	2	3	3
Zeta Potential	1	3	3	1	1	1	1	1	1	1	1	1	1	1
pH	2	2	2	2	2	2	2	2	2	2	2	2	2	2
IEP	4	4	4	1	4	2	4	2	2	4	1	2	4	2
solubility 24h	2	2	4	1	1	1	3	3	3	1	1	1	1	2
solubility 72h	2	2	4	1	1	1	3	3	3	1	1	1	1	2
CPH	2	1	1	2	2	2	2	2	2	2	2	2	2	1
DMPO	2	2	2	2	2	2	2	2	2	2	2	2	2	1
DMPO_irradiation	-	-	-	1	1	1	-	-	-	-	-	-	-	-

colour code	EC 50
	> 100 mg/L
	100-10 mg/L
	1-10 mg/L
	1-0.1 mg/L
	< 0.1 mg/L
	< 0.01 mg/L

Conclusion

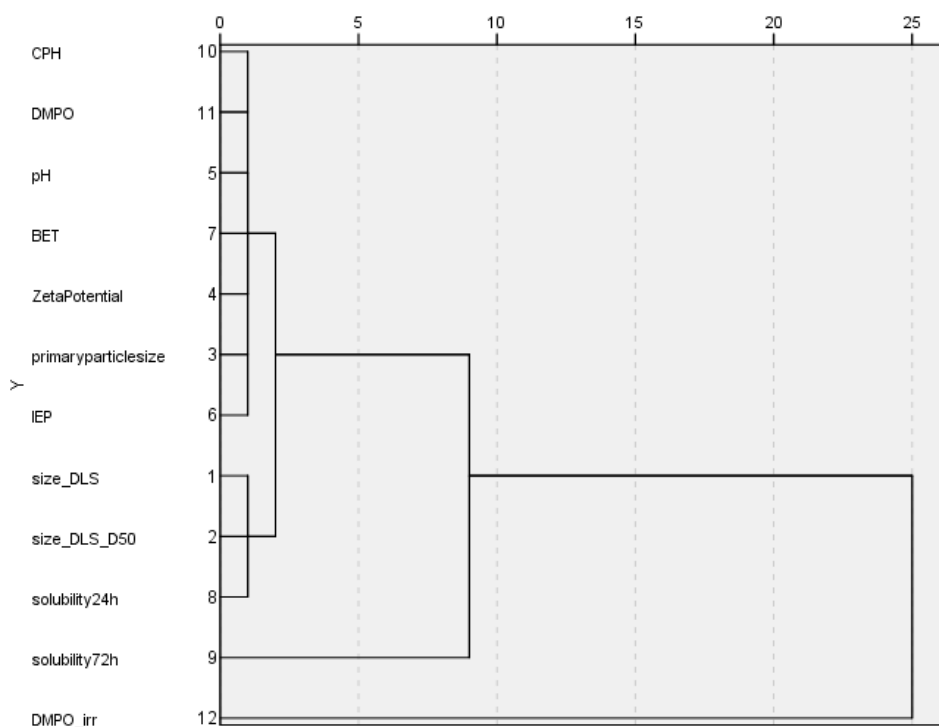
From these observations we can conclude that (i) not just one PC-parameter is the driver for the observed toxicity and (ii) for the different organisms different PC parameter may be relevant. However, the solubility and reactivity measured with CPH and morphology were identified as relevant but not the only parameter driving toxicity for all tested organisms. The zeta potential was identified as relevant for toxicity in daphnids and algae and the primary particle size for toxicity in daphnids and fish embryos.

5.1.5 Cluster analysis of the PC-parameters

In the following sections different statistical methods were used to identify if, based on our data, different groups can be identified based on statistical similarity. One statistical method for the categorization of data is a hierarchical Cluster analysis (linkage between the groups, squared Euclidian distance). The results of the analysis are shown in Figure 23. In this step it was analysed if different PC-parameters can be grouped together, this means that they would show the same pattern e.g. combination of small agglomerates and high zeta potential. The analysis was done for all NM for all media. Based on this analysis four clusters were identified:

- ▶ Cluster one: CPH, DMPO, pH, BET, ZP, IEP and primary particle size
- ▶ Cluster two: z.average and solubility (24 h)
- ▶ Cluster three: solubility (72 h)
- ▶ Cluster four: reactivity after irradiation

Figure 23: Dendrogram of the PC-parameters of all NMs in all media based on the linkage between the groups, squared Euclidian distance



It has to be noted that interpretation of the cluster analysis has to be taken with care due to the small data set. For example less than the half of the tested NM showed a relevant solubility and only three NM values for the measurements with DMPO after irradiation. This means that data for this parameter were available only in a limited number. However, all parameter were included in the analysis with the same weighting which could cause a wrong interpretation of the results exemplarily due to a spurious correlation. For example, it is not explainable that the solubility after 24h are closely linked to the hydrodynamic particle size, whereas the solubility data after 72h stands separately.

It is conceivable that some parameters show a different pattern in the different media (e.g. zeta potential or agglomerate size), which may affect the cluster results. Correlations between parameters which occur in one media can be missed if all media are examined together. Therefore in the following cluster analyses (linkage between the groups, squared Euclidian distance) the different media were analyzed separately.

In the following cluster analysis possible groups/clusters for the tested nanomaterials based on their PC data in the corresponding media were identified.

Water:

- ▶ Cluster one: ZnO NM-111, CeO₂ NM-212, Silver NM-300K
- ▶ Cluster two: undoped TiO₂, Fe doped and Eu doped and the CeO₂ NM-211
- ▶ Cluster three: ZnO NM-110 and NM-113, CeO₂ NM212, Cu and CeO₂ doped with Eu and the silver NM SRM and 1340

ADaM:

- ▶ Cluster one: CeO₂ NM-211, Silver SRM, TiO₂ undoped, TiO₂ Fe and Eu doped
- ▶ Cluster two: ZnO NM-110, NM-113, CeO₂ NM-212 and doped with Eu, Silver NM 1340 and NM-300K
- ▶ Cluster three: ZnO NM-111, CeO₂ NM-213, Cu

ISO water:

- ▶ Cluster one: ZnO NM-110, CeO₂ NM-211, NM-212, NM-213 and Eu doped, Cu and the silver NM NM-300K, 1340 and SRM
- ▶ Cluster two: ZnO NM-111, NM-113
- ▶ Cluster three: TiO₂ undoped, TiO₂ Fe and Eu doped

OECD medium:

- ▶ Cluster one: ZnO NM-110, NM-113, CeO₂ NM-211, NM-212, Eu doped, Cu and Silver NM SRM, 1340 and NM-300K
- ▶ Cluster two: ZnO NM-111, CeO₂ NM-213
- ▶ Cluster three: TiO₂ undoped, TiO₂ Fe and Eu doped

For each medium different clusters of the NMs were identified. Furthermore, the different subtypes of the NMs are clustered in different groups, excluded the TiO₂ NMs (all media) and additionally the CeO₂ NMs and Ag NMs in the fish embryo media, which were clustered together. This result for TiO₂ indicates that the PC parameters of the different TiO₂ NMs show the highest conformity of the tested NMs in all media.

Conclusion

The results of the cluster analysis show (i) four cluster for the PC-parameters over all media and (ii) different NM clusters for each medium. The TiO₂ NMs show the highest conformity in all tested media, as they were clustered together.

5.1.6 Correlation analysis of the PC-parameters

Another possibility for a grouping of the data is the correlation analysis. Here the statistical relationship between two or more data/parameters can be identified.

In a first step it was analyzed if a correlation of the PC parameter over all media could be identified (Spearman Rho rank correlation analysis with $p < 0.05$). The hydrodynamic diameter of the NMs in suspension (size_DLS, z.average), the primary particle size, the zeta potential, BET surface, solubility after 24 h and 72 h, the reactivity based on CPH and DMPO as well as after irradiation (DMPO_irradiation) were used as PC-parameters. However, no reasonable correlations were detected over all media and therefore no correlation analyses for the individual media were performed.

5.1.7 Correlation and cluster analysis of PC-parameters and ecotoxicity data

In a second step it was analyzed if a correlation of the PC parameter and ecotoxicity data can be identified. Therefore, correlation analyses (Spearman Rho rank correlation analysis with $p < 0.05$) for each medium including the toxicity data were conducted. The agglomerate size (size_DLS – z.average), primary particle size, zeta potential, BET surface area, solubility after 24 h and 72 h, reactivity based on CPH and DMPO, and DMPO after irradiation (DMPO_irradiation) as well as the EC50 values for algae, daphnids and fish embryos were used.

For Algae a negative significant correlation for the EC50 and the PC-parameter zeta potential was detected. For the EC50 for daphnids a negative significant correlation for primary particle size, CPH and DMPO was observed and a positive correlation for BET. For the EC50 for fish embryos a negative significant correlation was identified for primary particle size, CPH and DMPO.

An additional correlation analysis based on the Pearson correlation coefficient ($p < 0.05$) showed different correlation for any organism and the PC-parameters. For daphnids a negative correlation of the EC50 was observed with the morphology and a positive with BET, whereas the correlation with the reactivity was weak (Figure 24). For the EC50 found for fish embryos the same correlation as with

the Spearman correlation was observed (Figure 25) and for the EC50 for algae a strong negative correlation was observed for the DMPO irradiation results and the IEP (Figure 26).

A cluster analysis with the Pearson coefficient as distance mass was also conducted. For daphnids four main clusters were observed. The EC50 values were clustered with BET, coating/surface modification and IEP in the corresponding test media.

For fish embryo five main clusters were identified. The EC50 values were clustered with coating/surface modification in the corresponding test media.

For algae six main clusters were identified. The EC50 value was clustered with coating/surface modification and BET in the corresponding test media.

The cluster analysis based on the Pearson coefficient as distance mass shows for all organisms a relation with the EC50 values and the coating/surface modification. However, as only one NM shows a coating/surface modification (NM-111), we conclude that the cluster with the EC50 values and coating/surface modification does not correspond to the real observation.

Figure 24: Pearson correlation analysis plot for the PC-parameters in ADaM (100 mg/L, except solubility values concentration 1g/L and 10g/L for NM-300K) and the EC50 values for daphnids. A reddish circle around the values stands for a positive correlation and a bluish circle a negative correlation. With increasing intensity of the color and smaller circles a higher correlation is indicated. On the right site a hierarchical cluster analysis with the Pearson coefficient as distance mass is shown.

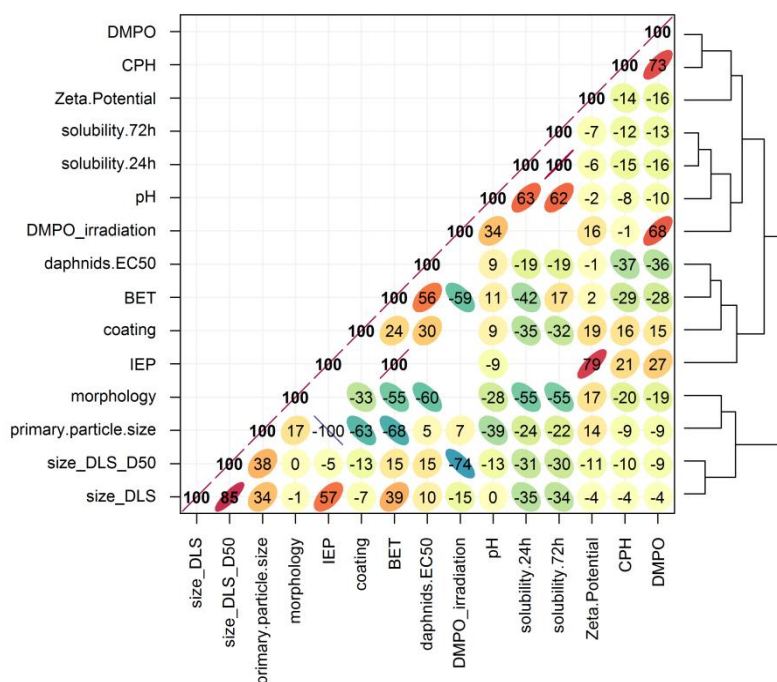


Figure 25: Pearson correlation analysis plot for the PC-parameters in ISO water (100 mg/L, except solubility values concentration 1g/L and 10g/L for NM-300K) and the EC50 values for fish embryos. A reddish circle around the values stands for a positive correlation and a bluish circle a negative correlation. With increasing intensity of the color and smaller circles a higher correlation is indicated. On the right site a hierarchical cluster analysis with the Pearson coefficient as distance mass is shown.

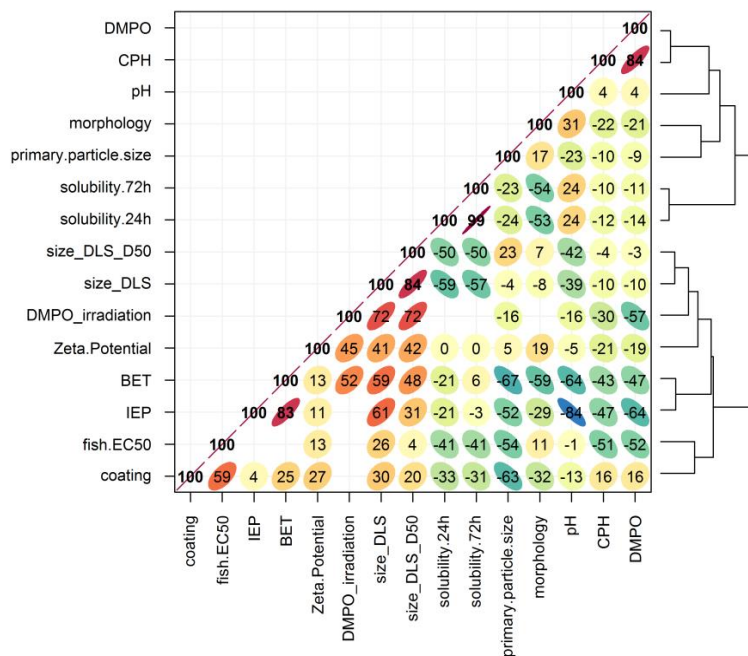
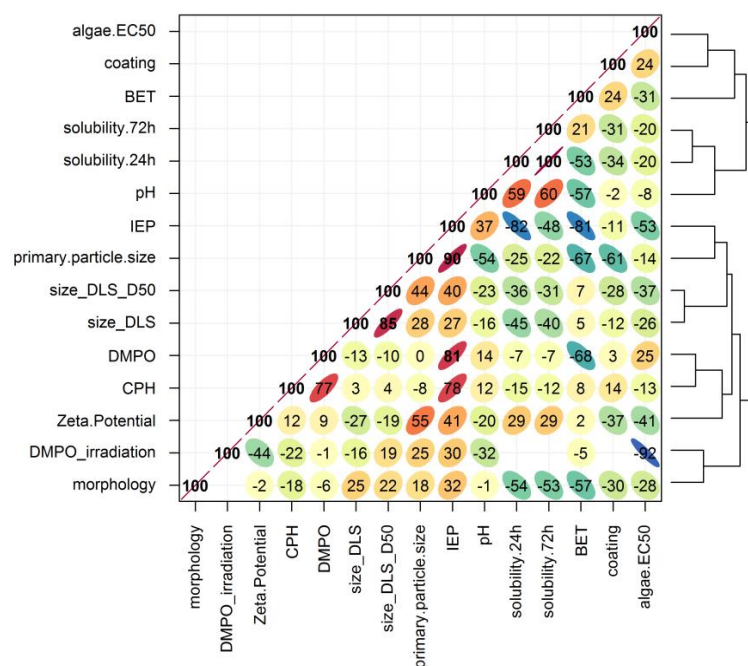


Figure 26: Pearson correlation analysis plot for the PC-parameters in OECD media (100 mg/L, except solubility values concentration 1g/L and 10g/L for NM-300K) and the EC50 values for algae. A reddish circle around the values stands for a positive correlation and a bluish circle a negative correlation. With increasing intensity of the color and smaller circles a higher correlation is indicated. On the right site a hierarchical cluster analysis with the Pearson coefficient as distance mass is shown.



Conclusion

Based on the results of the Spearman Rho rank correlation for daphnids and fish embryo the reactivity and primary particle size were identified as relevant for the EC50 values in these test organisms and additionally the BET for daphnids and for algae the zeta potential.

Based on the results of the Pearson correlation analysis for the different organisms and PC-parameters no parameter can be identified which solely can be used for a grouping of the NMs.

5.1.8 Mean comparison (Whitney-U) test of the PC-parameters

Statistical differences for the PC-parameters in the different media were analyzed using the mean comparison test. It was observed that BET, solubility (24 h) and DMPO were not significant different between the four media, IEP and solubility (72 h) were less clear but also not significant different, whereas agglomerate size, zeta potential, DMPO irradiation and CPH were affected by the media ($p < 0.05$).

5.1.9 Mean comparison (Whitney U test) of the NM, based on values of PC-parameters and the ecotoxicity data (EC50 values)

The mean comparison test of the different NMs showed for daphnids and fish embryos significant differences.

For daphnids following ranking was observed:

$\text{Cu} < \text{NM-300K} < \text{NM-110} < \text{NM-111} < \text{NM-113} < 1340 \leq \text{SRM} < \text{TiO}_2 \text{ undoped, TiO}_2 \text{ Fe doped and TiO}_2 \text{ Eu doped, CeO}_2 \text{ Eu doped, NM-211, NM-212, NM-213}$

For Fish embryos following ranking was observed:

$1340 < \text{Cu} < \text{NM-300K} < \text{SRM} < \text{NM-110, NM-111, NM-113, NM-211, NM-212, NM-213, CeO}_2 \text{ Eu doped, TiO}_2 \text{ undoped, TiO}_2 \text{ Fe doped and TiO}_2 \text{ Eu}$

For algae no significant differences based on the mean comparison test were observed.

Further mean comparison tests (Kruskal Wallis) showed no significant differences between NMs with different shape, or reactivity as measured in the algae OECD media. For the daphnia medium no significant differences were observed for reactive NMs whereas a significant difference was observed for NMs with different shape. For the fish embryo medium – ISO water, coating/surface modification, shape and morphology showed significant differences.

Conclusion

The mean comparison test of the PC-parameters identified a medium dependency of the PC-parameters agglomerate size, zeta potential, DMPO irradiation and CPH.

The mean comparison test of the NMs and the organisms shows no significant differences for algae, but significant differences for daphnia and fish embryo media. For the daphnia medium significant differences for each soluble NM and the soluble NM and the “stable” NM were observed and significant differences between the silver NM and Cu and the rest of the NMs for fish embryo media.

5.1.10 Grouping based on the ECETOC system

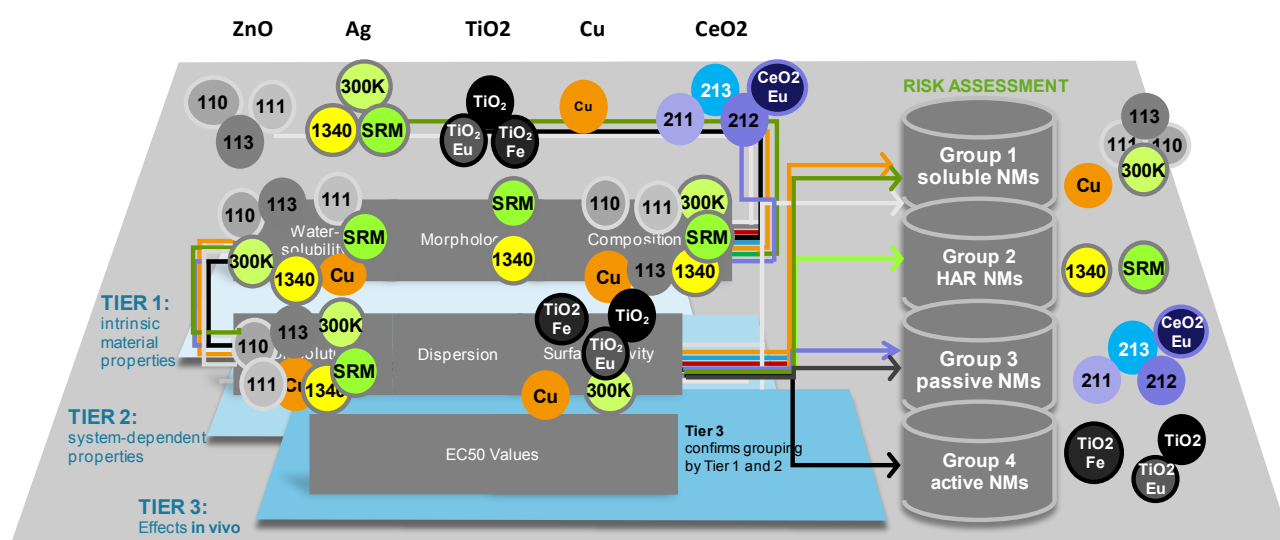
In the DF4nano group a tiered approach was developed which classified NMs due to their PC data and their human toxicity (inhalation toxicity) in four groups: soluble NMs, high aspect ratio (HAR) NMs, passive NMs and active NMs (Arts et al., 2016). With increasing tier the complexity of information which is needed increases. This means that in tier 1 intrinsic material properties are used for the grouping. The relevant PC-parameters are the composition, morphology and water solubility of the NMs. If these data are not sufficient for a grouping, in tier 2 system dependent properties are measured like the dissolution in relevant media, dispersion and surface activity. Additionally, in tier 3

toxicity data are included. This approach was introduced for the grouping/ranking of NM regarding their human toxicity, mainly inhalation effects of NMs. Therefore, some thresholds and suggested *in vitro* methods are specific for this scope. As existing and accepted concept, it was tested if this concept can also be used for environmental issues. As some thresholds and methods are specific for human toxicology some adaptations were made.

- i) The threshold value for solubility (100 mg/L) was not used, for the environmental approach no lower solubility limit was defined.
- ii) The aspect ratio (WHO definition) for HAR NM was not used. For the environmental approach a NM was defined as fiber/wire only on the obvious shape.
- iii) The categorization in Group 1 “soluble NMs” does not mean that no NM specific properties have to be considered. However, it means that beside the particle effect also ions will be released from the NM which may cause additional effects.

The results of this slightly adapted grouping approach using data of the NMs investigated in this project are shown in Figure 27.

Figure 27: Grouping of the used NM based on the grouping approach of (Arts et al., 2016) under consideration of the PC data.



Based on this approach the NMs used in this study were grouped as follows:

1. Soluble/ion releasing NM: Ag NM-300K; ZnO NM-110, NM-111, NM-113; Cu
2. HAR NM: Ag wire 1340, Ag wire SRM
3. Passive NM: TiO₂ undoped, TiO₂ Eu doped, CeO₂ Eu doped, NM-211, NM-212, NM-213
4. Active NM: TiO₂ Fe doped

It has to be noted that the sequence of these groups 1-4 do not feature a general hierarchy of toxic relevance.

5.1.11 Grouping based on the adapted ECETOC system for PC-parameters and ecotoxicity data

In a next step, in the adapted ECETOC approach information using EC50 values was introduced as final tier to confirm the grouping decisions of the previous tiers. Using this approach NM which were grouped as “passive” switched to the “active” group in case toxicity was found and vice versa. Again, it has to be noted that the sequence of these groups 1-4 do not feature a general hierarchy of toxic relevance.

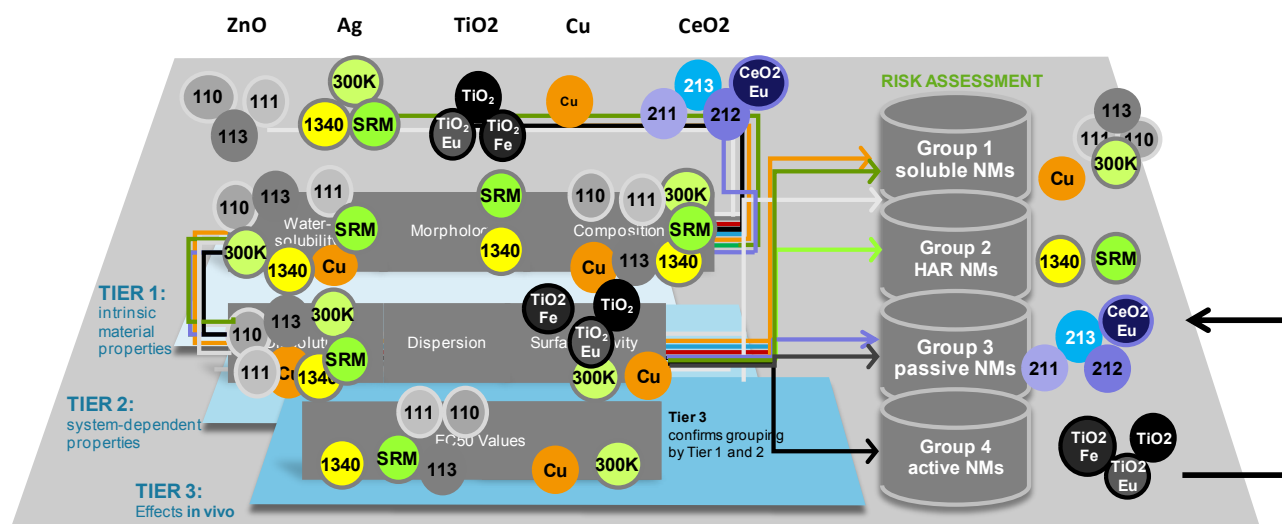
Following results can be conducted using the data generated in this project for each of the investigated toxicity tests:

For the **daphnids** based on this approach the NMs used in this study were grouped as follows:

1. Soluble NM: Ag NM-300K, ZnO NM-110, NM-111, NM-113, Cu
2. HAR NM: Ag wire 1340, Ag wire SRM
3. Passive NM: TiO₂ undoped, TiO₂ Eu doped, TiO₂ Fe doped CeO₂ Eu doped, NM-211, NM-212, NM-213
4. Active NM: none

In contrast to the grouping based on PC data only the TiO₂ Fe doped NM was shifted from 4 - active NM to 3 - passive NM as the observed reactivity after illumination did not lead to a toxic response.

Figure 28: Grouping of the used NM based on the grouping approach of (Arts et al., 2016) under consideration of the PC data and EC50 values for daphnids.

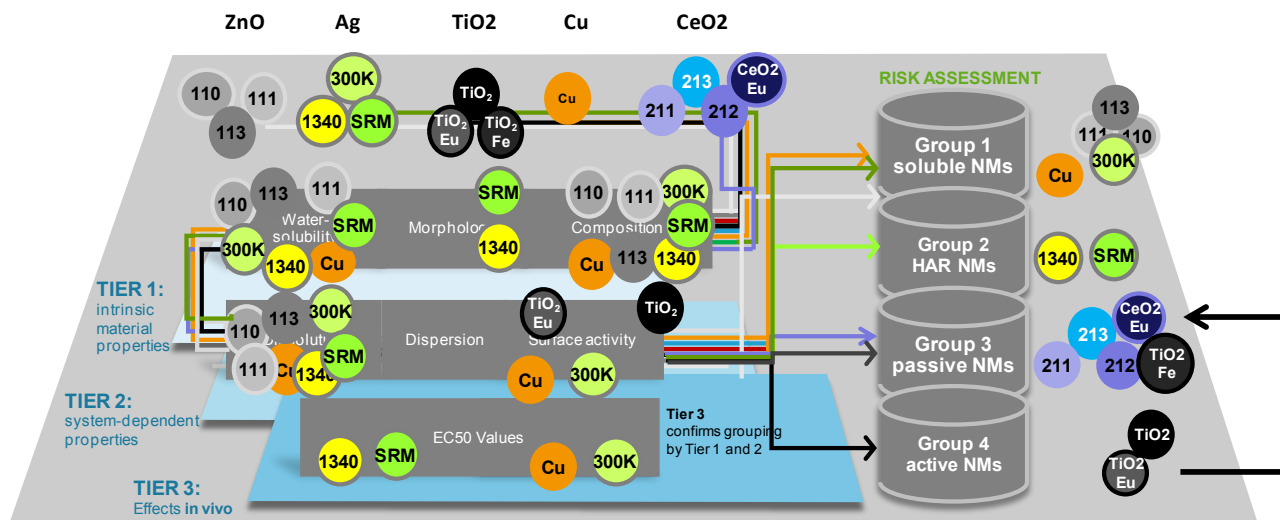


For the **fish embryos** based on this approach the NMs used in this study were grouped as follows:

1. Soluble NM: Ag NM-300K, ZnO NM-110, NM-111, NM-113, Cu
2. HAR NM: Ag wire 1340, Ag wire SRM
3. Passive NM: TiO₂ undoped, TiO₂ Eu doped, TiO₂ Fe doped CeO₂ Eu doped, NM-211, NM-212, NM-213
4. Active NM: none

In contrast to the grouping based only on the PC data the TiO₂ Fe doped NM was shifted from 4 - active NM to 3 - passive NM as the observed reactivity after illumination did not lead to a toxic response (EC50).

Figure 29: Grouping of the used NM based on the grouping approach of (Arts et al., 2016) under consideration of the PC data and EC50 values for fish embryo.

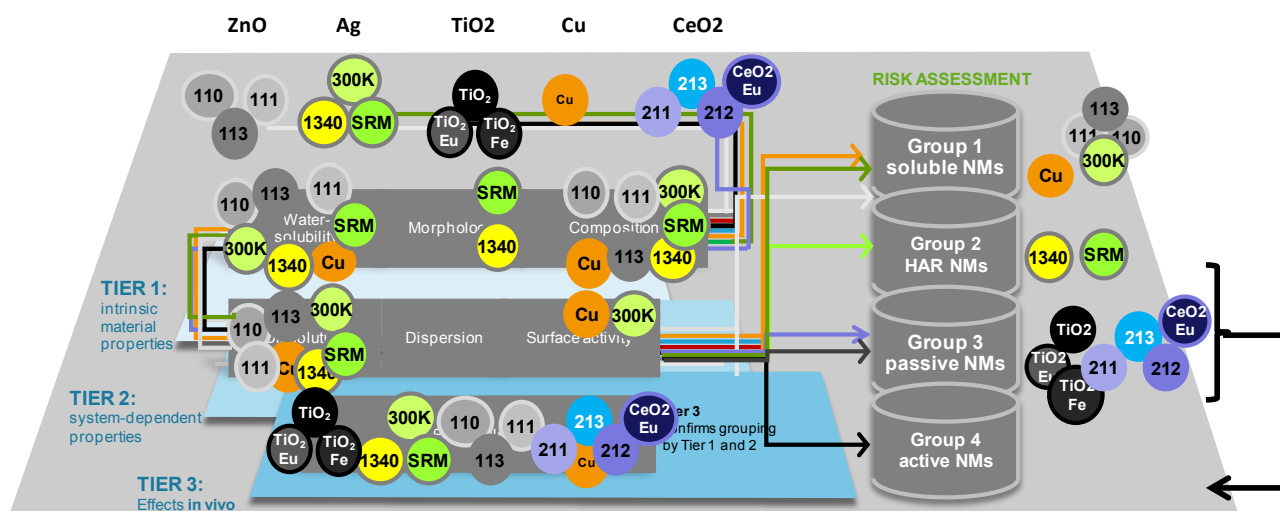


For the **algae** based on this approach the NMs used in this study were grouped as follows:

1. Soluble NM: Ag NM-300K, ZnO NM-110, NM-111, NM-113, Cu
2. HAR NM: Ag wire 1340, Ag wire SRM
3. Passive NM: none
4. Active NM: TiO₂ undoped, TiO₂ Eu doped, TiO₂ Fe doped CeO₂ Eu doped, NM-211, NM-212, NM-213

In contrast to the grouping based only on the PC data all materials further grouped in 3 – passive NM were shifted to 4 – active NM as for all materials EC50 values were detected.

Figure 30: Grouping of the used NM based on the grouping approach of (Arts et al., 2016) under consideration of the PC data and EC50 values for algae.



Conclusion

It was tested if the already existing and accepted grouping approach of the DF4nanoGroup, which was established for human (inhalation) toxicity effects, can also be used with some adaptations for environmental issues. Therefore the results for the tested NMs in the three different media were used and classified based on this approach. If the grouping based on PC-parameters represented the

ecotoxicity, no modifications by the consideration of the ecotoxicological results in the grouping approach should be the case.

It was shown that this approach was, also with the adaptations, not suitable for environmental issues. Some NMs were classified in wrong groups, for fish embryo (ISO water) and daphnids (ADaM) false positive groups were identified. An undesired mistake but which has no negative consequences. However, for algae (OECD medium) tier 1 and tier 2 lead to wrong / false negative classification. This mistake is relevant as the toxicity of NMs was underestimated which can lead to negative effects in the environment if an exposure occurs.

Therefore, we developed a new grouping/ranking approach which can also be used for environmental relevant questions (chapter 5.2.3).

5.2 Grouping based on ecotoxicological test results and PC-parameters identification based on „expert knowledge“

In this approach the EC50-values determined in the present project were used as the basis for the grouping. Toxicity values but also toxicity profiles have to be considered in this context. The term „toxicity value“ is defined as EC-value indicating a specific toxicity. The term „toxicity profile“ is used to describe the ranking of the sensitivity of the three applied test organisms for a specific NM.

Various levels of complexity are presented in the following.

5.2.1 Basis: Toxicity yes / no

In the first approach it was just considered whether toxicity was determined. Toxicity was defined as “calculation of EC50 values possible”. The results are presented in Table 42.

Table 42: Characterization of the NMs according to their ecotoxicity (calculation of EC50 values; basis: nominal values) - distinction between “toxic” (+) and non-toxic (-)

Nanomaterial	Algae	Daphnids	Fish embryo	Group
Ag: SRM 110525	+	+	+	1
Ag: Batch 1340	+	+	+	1
Ag: NM-300K	+	+	+	1
ZnO: NM-110	+	+	-	2
ZnO: NM-113	+	+	-	2
ZnO: NM-111	+	+	-	2
CeO ₂ : Eu-doped	+	-	-	3
CeO ₂ : NM-211	+	-	-	3
CeO ₂ : NM-212	+	-	-	3
CeO ₂ : NM-213	+	-	-	3
TiO ₂ : Eu-doped	+	-	-	3
TiO ₂ : Fe-doped	+	-	-	3
TiO ₂ :	+	-	-	3
Cu	+	+	+	1

With this approach three groups can be distinguished:

- ▶ Group 1
Effects on all three test organisms observed
⇒ ion releasing NMs (Ag, Cu) with high known toxicity
- ▶ Group 2
Effects only on algae and daphnids
⇒ ion releasing NMs (ZnO) with lower known toxicity
- ▶ Group 3
Effects only on algae
⇒ “stable“ NMs (TiO₂, CeO₂)

Based on this approach, NMs with the same chemical identity cannot be distinguished. Group 2, which is represented by the ion-releasing ZnO, could be based on the lower ecotoxicity of the chemical composition. Hence, based on this very roughly determined groups hardly any predictions on toxicity of a given NM can be made, and read-across approaches can't be carried out.

5.2.2 Basis: differentiated toxicity

In a second approach the ecotoxicity was divided in high, medium, low and negligible. By this approach, a stronger differentiation in grouping compared to the basic toxicity approach (presented in 5.2.1) was obtained, allowing the allocation of some sub-groups of the same chemical identity to different groups.

For this differentiation following thresholds regarding the EC50-values on nominal mass basis were applied:

- ▶ < 1 mg/L: high toxicity (toxicity level 4)
- ▶ 1 - < 10 mg/L: medium toxicity (toxicity level 3)
- ▶ 10 - < 100 mg/L: low toxicity (toxicity level 2)
- ▶ ≥ 100 mg/L: negligible toxicity (toxicity level 1)

The result is presented in Table 43.

Table 43: Grouping of the NMs according to their ecotoxicity (EC50 values – basis mass, nominal values) - distinction between high, medium, low and negligible, a, b, c = subgroups

Nanomaterial	Algae	Daphnids	Fish embryo	Group
Ag: SRM 110525	3	4	2	1b
Ag: Batch 1340	4	4	4	1a
Ag: NM-300K	4	4	4	1a
ZnO: NM-110	4	3	1	2
ZnO: NM-113	4	3	1	2
ZnO: NM-111	4	3	1	2
CeO ₂ : Eu-doped	3	1	1	3b
CeO ₂ : NM-211	3	1	1	3b
CeO ₂ : NM-212	3	1	1	3b
CeO ₂ : NM-213	2	1	1	3c
TiO ₂ : Eu-doped	4	1	1	3a
TiO ₂ : Fe-doped	3	1	1	3b
TiO ₂ :	4	1	1	3a
Cu	4	4	4	1a

The three main groups can be differentiated in sub-groups using the differentiated approach.

- ▶ Group 1:
 - high and medium effects with the three test organisms
 - ⇒ Ion releasing NMs (Ag, Cu)
 - Group 1a: high effects on the three organisms (Ag: Batch 1340, NM-300K; spherical NM and long wire with small diameter; Cu)
 - Group 1b: high effects on daphnids; medium on algae and low on fish embryo (Ag: SRM 110525; long wire; due to the diameter no NM according to EU-definition)
- ▶ Group 2:
 - Effects only on algae and daphnids
 - ⇒ Ion releasing NMs (ZnO)
 - No further differentiation (high and medium effects on algae and daphnids; no effects on fish embryos)

To understand the separation between Ag, Cu (group 1) and ZnO (group 2) only the spherical NMs were considered. By this, misleading conclusions based on the different shapes (spherical NMs, wires) were avoided. Additionally, the ecotoxicity values were compared in more detail. For the spherical Ag (Nm-300K) and Cu, the toxicity in the tests with algae and daphnids is comparable (EC50 in the range of 0.01 – 0.1 mg/L). The sensitivity of the fish embryo test is about a factor of 10 lower (EC50 in the range of 0.1 – 1 mg/L). For ZnO the difference in the sensitivity of the test organisms differs compared to the results of Ag and Cu. The EC50 of ZnO on algae is in the range of 0.1 – 1, on daphnids in the range of 1 – 10 and no effect on fish embryos at all. These differences between the NMs indicate that two groups separating ZnO and Ag, Cu are justified. Although the difference could be based on metal oxides (ZnO) on the one hand, and pure metals (Ag, Cu) on the other hand, it is assumed that the main reason for the two groups is the different toxicity of the metals. Zn⁺ is less toxic than Ag⁺ and Cu⁺ (Okamoto et al., 2015).

► Group 3

Effects only on algae

⇒ “stable“ NMs (TiO₂, CeO₂)

Group 3a: high effects on algae (doped, non-doped NMs: TiO₂)

Group 3b: medium effects on algae (doped, non-doped NMs: TiO₂, CeO₂)

Group 3c: low effects on algae (non-doped NMs: CeO₂)

Following conclusions can be drawn:

- Based on the results there is a separation between metals and metal oxides.
- Doping does not affect ecotoxicity in general (doped NMs are always less or higher toxic than non-doped). Additionally, it is obvious that the results cannot be transferred from doped to undoped NMs and vice versa without further considerations.
An additional experiment using an undoped CeO₂ with PC-properties comparable to the doped CeO₂ had been performed. The undoped CeO₂ had been provided by IUTA and was considered as control or reference NM to the doped material. In this additional experiment the toxicities of both NMs on algae were determined. The EC50-value of the doped CeO₂ was verified (1.51 mg/L (confidence interval 1.06 – 2.14 mg/L) in the second experiment compared to 3.2 (2.6 – 3.8) mg/L in the first experiment. With 1.03 mg/L (confidence interval: 0.45 – 2.33 mg/L) the EC50-value of the undoped CeO₂ NM was comparable to the doped one supporting the conclusion that in the case of CeO₂- in contrast to TiO₂ - the doping does not affect the ecotoxicity.
- The crystalline structure of doped and undoped TiO₂ differed with anatase/rutile for the undoped TiO₂ and rutile for both doped TiO₂ NMs. The anatase/rutile TiO₂ was more toxic compared to the doped rutile TiO₂ indicating that the crystalline structure could be the reason for the observed toxicity. However, a phototoxic reactivity could not be shown with the applied procedure in the algae test medium.
There were no indications that the crystalline structure of the doped and undoped CeO₂ differed (Tim Hülser, IUTA e.V., personal communication).
Comparison of the individual PC-properties of the CeO₂ NMs indicates that the surface area of the CeO₂ with the lowest toxicity is about a factor of 10 smaller than the BET surface area of the more toxic CeO₂ NMs (4 m²/g compared to 28 – 66 m²/g). We assume that in the case of reactive surfaces, the surface area has an impact on the ecotoxicity with larger surface areas resulting in higher ecotoxicity. However, in the present project the applied procedures on reactivity did not indicate differences between the various CeO₂ NMs. Therefore, it remains unknown if a surface reactivity which was not determined by the applied methods is the reason for the low toxicity of the NM with the smallest surface area.

As it is often discussed to use the surface area as metric instead of the mass, the approach using high, medium, low, negligible toxicity to distinguish the NMs into groups was also based on the surface area. To address this approach the EC50-values on mass basis were converted to surface area and following threshold values regarding the EC50-values based surface area were applied:

- ▶ high toxicity (toxicity level 4)
Alternative 1 (A1): $< 10^{-4} \text{ m}^2/\text{L}$
Alternative 2 (A2) : $< 10^{-5} \text{ m}^2/\text{L}$
- ▶ medium toxicity (toxicity level 3)
A1: $10^{-4} - <10^{-2} \text{ m}^2/\text{L}$
A2: $10^{-5} - <10^{-3} \text{ m}^2/\text{L}$
- ▶ low toxicity (toxicity level 2)
A1: $10^{-2} - <1 \text{ m}^2/\text{L}$
A2: $10^{-3} - <10^{-1} \text{ m}^2/\text{L}$
- ▶ negligible toxicity (level 1)
A1: $\geq 1 \text{ m}^2/\text{L}$
A2: $\geq 10^{-1} \text{ m}^2/\text{L}$

No threshold values for classifying ECx values into high, medium and negligible based on surface area of NMs exist. From the available data suitable threshold values were not obvious. Therefore, two different alternatives A1 and A2 per toxicity level were applied. The result is presented in Table 44.

Table 44: Grouping of the NMs according to their ecotoxicity (EC50 values – basis surface area, nominal values) - distinction between high, medium and low ecotoxicity, a, b, c = subgroups.

Nanomaterial	Algae		Daphnids		Fish embryo		Group	
Ag: SRM 110525	A1: 3	A2: 2	A1: 4	A2: 3	A1: 2	A2: 2	A1: 1a	A2: 2a
Ag: Batch 1340	A1: 3	A2: 3	A1: 4	A2: 3	A1: 3	A2: 2	A1: 1a	A2: 2a
Ag: NM-300K	A1: 3	A2: 2	A1: 3	A2: 2	A1: 2	A2: 2	A1: 2a	A2: 3a
ZnO: NM-110	A1: 3	A2: 2	A1: 2	A2: 2	No toxicity		A1: 2b	A2: 3b
ZnO: NM-113	A1: 3	A2: 3	A1: 2	A2: 2	No toxicity		A1: 2b	A2: 2b
ZnO: NM-111	A1: 3	A2: 2	A1: 2	A2: 2	No toxicity		A1: 2b	A2: 3b
CeO ₂ : Eu-doped	A1: 2	A2: 1	No toxicity		No toxicity			
CeO ₂ : NM-211	A1: 2	A2: 1	No toxicity		No toxicity		A1: 3c	A2: 4c
CeO ₂ : NM-212	A1: 2	A2: 1	No toxicity		No toxicity		A1: 3c	A2: 4c
CeO ₂ : NM-213	A1: 2	A2: 1	No toxicity		No toxicity		A1: 3c	A2: 4c
TiO ₂ : Eu-doped	A1: 2	A2: 1	No toxicity		No toxicity		A1: 3c	A2: 4c
TiO ₂ : Fe-doped	A1: 1	A2: 1	No toxicity		No toxicity		A1: 4c	A2: 4c
TiO ₂ :	A1: 2	A2: 2	No toxicity		No toxicity		A1: 3c	A2: 3c
Cu	A1: 2	A2: 2	A1: 2	A2: 2	A1: 2	A2: 1	A1: 3a	A2: 3a

Four main groups can be differentiated based on this approach:

- ▶ Group 1: at least one organism is highly affected
- ▶ Group 2: at least one organism is medium affected
- ▶ Group 3: at least one organism is slightly affected
- ▶ Group 4: at least one organism is negligibly affected

The three selected organisms represent three trophic levels, algae = primary producer, daphnia = primary consumer, fish embryo = secondary consumer. Regarding the trophic level of the organisms the sub-groups in this grouping approach named with a, b and c can be formed according to the number of trophic levels which indicate toxicity (a: three trophic levels indicate toxicity; b: two trophic levels; c: 1 trophic level).

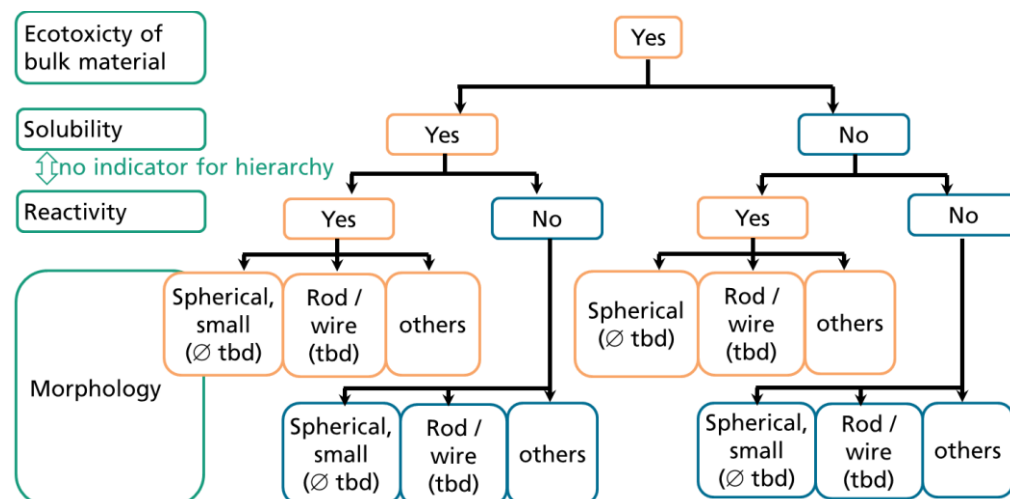
Based on the surface, a differentiation between ion-releasing and “stable” NMs was only possible by the sub-groups. Exemplarily, group 3 (A1) contains forms of TiO₂, CeO₂ and Cu NMs. Therefore, a grouping within these NMs is only possible if results for three trophic levels were available and the sensitivity of the species applied for the trophic levels is comparable between the NMs to be compared. Even if the limits of the groups are modified (A2), the classification was difficult to understand. Therefore, just on the surface area, a grouping regarding ecotoxicity does not seem to be targeted. It can be concluded that the surface area is no basis for the identification of PC-parameters relevant for grouping.

5.2.3 Grouping based on a proposed flow chart

In order to combine the tiered approach applying data on intrinsic and extrinsic PC properties as suggested in the ECETOC scheme with the specific requirements of grouping according to ecotoxicity, a flow chart is proposed. The basic idea is that NMs sorted to the same position in the scheme belong to the same group. The proposal is based on the PC-parameters considered during this project, but the scheme is flexible and hence consideration of further PC-parameters (e.g. coatings or crystal structure) is possible.

The scheme is presented in Figure 31.

Figure 31: Ecotox-scheme for the grouping of NMs (tbd = has still to be defined). The same scheme applies for materials with no toxicity of the bulk material.



In the project it became obvious that the toxicity of the chemical composition is an essential parameter if the grouping approach shall cover various compositions. Thus, the scheme starts with the question on known ecotoxicity data of the bulk material which is usually available (with some exemptions as carbon based have no direct bulk counterpart). This initial question will lead to a rough separation of NMs under investigation, e.g. in the project the ion-releasing NMs had a higher toxicity than the “stable” NMs such as TiO₂ and CeO₂. For a pragmatic approach just a rough differentiation in “yes” and “no” is proposed. As threshold values the highest test concentration recommended for the tests is proposed as EC50. This means 100 mg/L for the most sensitive aquatic species (and 1000 mg/kg for terrestrial species). In the following steps just PC-parameters are considered. If no information on ecotoxicity is available, the grouping approach starts on the level of the PC-parameter.

The PC-parameters solubility and reactivity were considered to be of major relevance. Although, solubility is above reactivity in the scheme, this indicates no hierarchy. For reactivity, any positive result (result indicating reactivity) measured by DMPO, CPH, with or without illumination was considered. As further parameter morphology is included. Three categories were considered. In the project the toxicity of wires became obvious. However, as only two wires were considered dimensions indicating increased ecotoxicity cannot be deduced. Ivask et al. (2014) observed that the toxicity of Ag NMs with a size range of 20 to 80 nm differed significantly from the toxicity of Ag NMs with a size of about 10nm. Additionally, they demonstrated the different bioavailability. Therefore, we included a category for small spherical NMs in the morphology. Whether a threshold value of 10 nm is suitable which corresponds to the size of the NMs studied by Ivask et al. (2014) or a different one (e.g. 15 nm) has to be discussed. NMs which were neither wires nor small spheres were grouped in a third category named "other".

The ecotox-scheme was applied to the NMs selected for the project. In Figure 32 the ion releasing NMs were categorized. As threshold value for small spherical NMs 10 nm is applied. Four groups were formed with (i) Cu and Ag NM-300K, (ii) and (iii) the two Ag wires separated due to their reactivity and (iii) the ZnO NMs. It is assumed that neither TiO₂ nor CeO₂ is toxic as bulk material. The grouping of these NMs is presented in Figure 33. Two groups separating the two chemical compositions TiO₂ and CeO₂ were formed. Based on the DMPO method the TiO₂ NMs were reactive while the CeO₂ NMs were non-reactive.

Figure 32: Ecotox-scheme regarding the grouping of NMs applied to the selected NMs with known toxicity of the bulk material (Ag, Cu, ZnO).

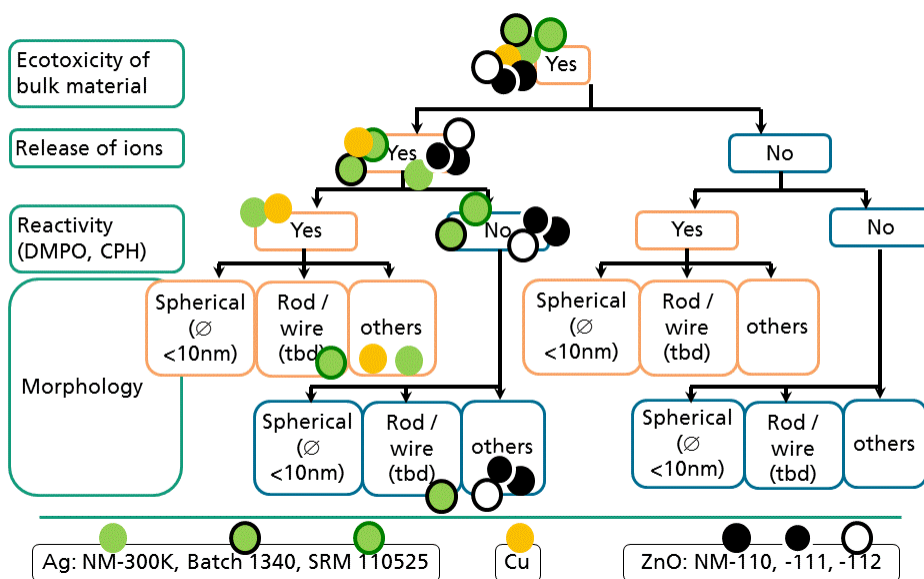
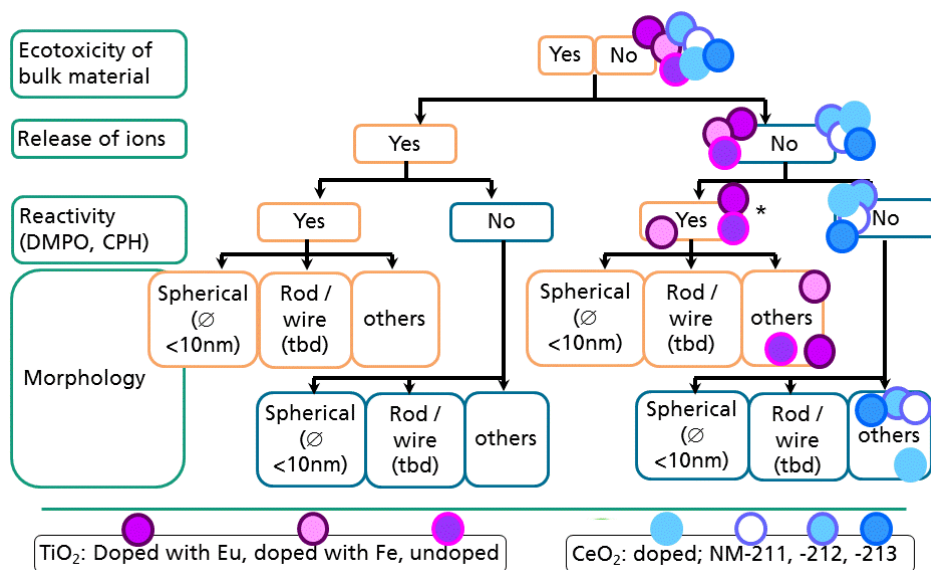


Figure 33: Ecotox-scheme regarding the grouping of NMs applied to the selected NMs for which it is assumed that the bulk material is non-toxic (TiO₂, CeO₂).



* Reactivity after illumination

To check the reasonability of the groups in Table 45 some characteristics were listed. The groups differ in their ecotoxicity (exception: Ag wires), in the affected organisms and in the rough characterization of the NMs. Therefore, the approach is considered to be more reliable compared to the ECETOC approach which was developed for the characterization of inhalation toxicity.

However, NMs with the same chemical identity cannot be conclusively distinguished (exception: Ag NMs). Therefore, read-across between chemicals of the same chemical identity cannot be carried out. It is unknown whether further PC-properties have to be considered or whether the impact of the selected modifications on ecotoxicity is too minor to result in significant ecotoxicological differences.

In the Ecotox grouping approach the two Ag wires are of special interest. Based on the reactivity they are separated in two groups although the toxicity for the most sensitive organism (daphnids) is similar. For the other chemical compositions the algae proved to be the most sensitive organisms. For nAg particle NM300K the EC₅₀ values regarding algae and daphnids have the same order of magnitude. If the toxicity of the wires on algae is considered, a clear difference is obvious: Ag 1340 resulted in an EC₅₀_{algae} of 0.022 mg/L, whereas the EC₅₀_{algae} for Ag 110525 was 2.4 mg/L. Therefore, for grouping the organisms have to be treated separately. The special features associated with wires have to be considered.

Table 45: Characterization of the groups formed by the ecotox-scheme

Groups of NMs	EC50 [mg/L] (value of the most sensitive organism)	Affected organisms	NM-characterization
1. Ag 1340	0.0016 (daphnids)	Algae, daphnids, fish	Wires, ion releasing NMs
2. Ag 110525	0.0085 (daphnids)	Algae, daphnids, fish	Wires, ion releasing NMs
3. Cu, Ag NM300K	0.01 – 0.043 (daphnids) ¹	Algae, daphnids, fish	Spherical, ion releasing NMs
4. ZnO	0.09 – 0.55 (algae)	Algae, daphnids	Spherical, ion releasing NMs
5. TiO ₂	0.38 – 3.6 (algae)	Algae	“stable” NMs
6. CeO ₂	3.2 – 43.8 (algae)	Algae	“stable” NMs

¹ EC50 for algae and daphnids have the same order of magnitude

6 Conclusion

For grouping of NMs for the ecotoxicological assessment for regulatory purposes, an approach is needed that allows the prediction of effects based on material parameters. The systematic and comprehensive testing of ecotoxicity and PC-parameters performed in the present project provided a multitude of new information useful for grouping as well as identifiable relationships and limitations. In the following the findings and conclusions were summarized:

Grouping of the selected NMs

- ▶ Based on the developed ecotox-scheme, six groups could be formed:
 - Ion releasing nanomaterials with DMPO/CPH reactivity and other morphology: Ag NM-300K, Cu.
 - Ion releasing nanomaterials with DMPO/CPH reactivity, wire: Ag, Batch 1340.
 - Ion releasing nanomaterials without DMPO/CPH reactivity, wire: Ag, SRM 110525.
 - Ion releasing nanomaterials without DMPO/CPH reactivity, other morphology: the three investigated ZnO nanomaterials.
 - Non-ion releasing nanomaterials, without DMPO/CPH reactivity and other morphology: the investigated four CeO₂ nanomaterials.
 - Non-ion releasing nanomaterials, with DMPO/CPH reactivity and other morphology: the investigated three TiO₂ nanomaterials.
- ▶ With exception of the Ag NMs, the differences in ecotoxicity of the sub-types of the selected NM-types were too small, to group differently. To affect ecotoxicity significantly, it seems that the variability in the PC-properties has to be much more pronounced than the variability included in this project.

PC-parameters

- ▶ The results for the PC-parameters of the tested NMs differed between the various test media (water, ISO water for fish embryo test, OECD-medium for algae, ADaM for daphnids). Thus, for predictions of the ecotoxicity only values determined in the relevant test medium are suitable (see chapter 0).
- ▶ Due to the low evidence regarding the relationships between PC-parameters and ecotoxicity we conclude that the usually discussed parameters are not sufficient to explain or even predict the ecotoxicity. For example, in the case of this study the solubility of the NMs as sole indicator is not suitable. ZnO was completely dissolved within a period of 72 h, whereas the dissolved ratio of Ag was much smaller. Nevertheless the toxicity of ZnO was as expected to be lower compared to that of Ag, due to the different toxicity of the chemical composition (Okamoto et al., 2015). Additionally, a comparison within one chemical group like the silver NM indicates that the NM with the highest solubility did not show the highest effects. Therefore, the effect data of the NM with the highest solubility cannot be used as worst case approach. The toxicity of the chemical substance and further parameters such as shape have to be considered.
- ▶ The influence of the *zeta potential* in an ecotoxicological test seems to be comparably small. Other indicators seem to be of higher relevance. Basis for this statement were the results with ZnO. Negative and positive zeta potentials were determined in the test with fish embryos and daphnids but a relationship to ecotoxicity was not obvious. However, in the algae medium (OECD medium) always high negative zeta potentials were measured and always toxic effects were observed. But especially in this test stability of the NMs seems to be of lower relevance as NMs and algae are in contact throughout the test due to extensive shaking. Currently it is unknown whether this statement can be generalized to further NMs.

- ▶ We assume *surface-reactivity* to be one reason for the observed ecotoxicity of the NMs TiO₂ and CeO₂. There is a need to identify or modify an existing method that the results correlate with the measured ecotoxicity which is assumed to be based on surface reactivity.
- ▶ The *morphology* of the NMs seems to be relevant. However, the differentiation in spherical and rods / wires is not sufficient (see also below: test species – daphnids). Threshold values have to be defined to indicate spheres and wires with a high toxic potential. A third group which comprises the remaining NMs is required.
- ▶ Several nanomaterials carried a *doping*. It is obvious that doping as sole information is not sufficient to indicate ecotoxicity. For TiO₂, doping modifies the crystalline structure followed by a modified ecotoxicity. For CeO₂ such a relationship is not obvious. The ecotoxicity of the Eu doped CeO₂ was comparable to the ecotoxicity of two non-doped CeO₂, but a third non-doped CeO₂ behaved differently. The existence of doping doesn't necessary lead to different groups in terms of toxicity.
- ▶ For NMs, obviously a set of PC-parameters need to be considered for grouping regarding similar ecotoxicity. The individual parameter has to be weighted taken the various test organisms and their behavior in the test system and the corresponding test media into account.
- ▶ Statistical analyses can be a useful tool to identify PC-parameters relevant for ecotoxicological effects. Statistical analyses provide only useful relationships if they are based on a large data set. The data set of this project is limited and has to be extended for robust conclusions (see outlook).

Test species

- ▶ The test species behave differently regarding the ranking of the NMs. Therefore, they have to be treated separately and need to be considered separately in terms of reasoning for a certain grouping and read-across.
- ▶ The bioavailability of NM is linked to their behavior in the different test media. This actually means that data on toxicity of one NM in one test species cannot be used to forecast the toxicity of another NM of the same chemical substance for another test species.
- ▶ The *fish embryo test* turned out to be rather insensitive and for the assessment of NMs modifications such as dechoriation may be considered (as shown in e.g. Henn and Braunbeck (2011); Bodewein et al. (2016)). Otherwise, chronic fish tests have to be performed although animal testing should be minimized. However, it has to be considered that a test period of 72 h was used in the project (instead of 96 h).
- ▶ *Algae* turned out to be most sensitive test species. We do not assume that shading due to turbid test dispersions is the reason. The effects were observed even in low test concentrations without significant turbidity.
- ▶ *Daphnids* are sensitive to thin and long Ag wire NMs. The range of critical dimensions still has to be investigated.

Ranking / Grouping based on statistical analyses

- ▶ Although various correlations between the selected PC-parameters (agglomerate size, primary particle size, zeta potential, BET surface, solubility, reactivity based on CPH and DMPO) could be calculated using various statistical approaches, the cluster analyses resulted in inconsistent results even though they were performed independently for every test medium. The groups are not comprehensible regarding the ecotoxicity found as ion releasing and "stable" NMs are mixed in the groups although obvious differences in toxicity were observed. Besides a larger volume of data (robust data set) and an improved selection of PC-parameters, a reason might be the differing conditions of investigated PC-parameters in the ecotoxicological test dispersions and the determination of these PC-parameters in the stock dispersions. Influencing

factors such as test organisms themselves, their movement and exudates, illumination as well as turbulence such as different levels of shaking or stirring need to be considered when interpreting PC-parameters.

Up to now it is not possible to predict the ecotoxicity based on a routine statistical analysis of PC-properties. Expert-knowledge regarding the interaction of the various parameters is still required for grouping of NMs based on their PC properties.

Ranking / Grouping based on ecotoxicological profiles

- ▶ Grouping / ranking based only on ecotoxicological data and profiles resulted in only rough groups. However, ecotoxicological profiles support the identification and weighting of PC-parameters relevant for ecotoxicity. Ion-releasing and “stable” NMs can be differentiated. For ion-releasing NMs of different chemical nature the differences in ecotoxicity can mainly be related to the toxicity of the chemical substance.
- ▶ However; grouping solely based on ecotoxicological data will not support the rationale for *read across*. For this aim, data on PC properties for comparison are needed to waive data acquisition on ecotoxicity of every member of one group.

Ecotox-scheme for grouping

- ▶ Grouping based on one PC-parameter is not possible. Several parameters have to be considered. An approach similar to the approach developed regarding human inhalation toxicity (ECETOC scheme) was developed regarding ecotoxicity.
- ▶ The flow chart developed for an ecotoxicological grouping within this project needs further specification with regard to some parameters, e.g. solubility and reactivity levels. Scientifically based but also pragmatic threshold values have to be defined. In any case, next to correlations between PC parameters and ecotoxicity, always expert judgment will be an essential element to judge on the grouping on a case by case basis.

7 Recommendations and outlook

Several recommendations shall support the further identification of relevant correlations between PC properties and ecotoxicity of NMs and thus, the development of the grouping / read across approach regarding ecotoxicity:

- ▶ Verification of the DMPO method as indicator for reactivity inducing ecotoxicological effects or development of methods characterizing reactivity which relates to ecotoxicity of surface reactive NMs.
- ▶ For identification of general correlations using statistical analyses: Increased number of robust dataset are required (additional EC50 values for the same test systems; PC-parameters for the NMs in each medium)
- ▶ Improved measurement of the exposure concentration (better recovery).
- ▶ A detailed investigation of the effect of the morphology by using different NM types with the same morphology like ZnO or TiO₂ rods / wires.
- ▶ A detailed characterization of the kinetics of solubility, zeta potential, reactivity and agglomeration is necessary, as no clear relationship between the measured PC-parameters and toxicity was observed. It seems possible that (i) the toxic response is the result of a combination of parameters (ii) not all relevant parameters important for the ecotoxicity were measured and (iii) measurements of PC-parameters without organisms and at one time point (as performed in this study) can be misleading, as indicated by the results of the soluble fraction in the tests with algae.
- ▶ In this project the zeta potential was identified by the statistical analyses as a potential parameter which can be related to the observed effects. However, in the test media most of the NMs had a zeta potential in the range indicating instable dispersions (-15 to +15 mV) with focus on negative values. Therefore, no clear correlation between the zeta potential and the effect could be identified. To determine if the zeta potential has an effect on the toxicity it would be necessary to test further NMs with a negative and positive zeta potential outside of this range.
- ▶ Verification of the presented ecotox-scheme with further NMs.

8 List of Annexes

- ▶ Study Report Algae test (OECD Guideline 201, 2011).
- ▶ Study Report *Daphnia magna* immobilization test (OECD Guideline 202, 2004).
- ▶ Study Report Zebrafish (*Danio rerio*) embryo toxicity test (OECD Guideline 236, 2013).

These study reports are available at UBA upon request.

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