FACTORS TRIGGERING THE ECOTOXICITY OF METAL-BASED NANOPARTICLES TOWARDS AQUATIC INVERTEBRATES

by

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Declaration

I hereby declare that I autonomously conducted the work presented in this PhD thesis entitled "Factors triggering the ecotoxicity of metal-based nanoparticles towards aquatic invertebrates". All used assistances and involved contributors are clearly declared. This thesis has never been submitted elsewhere for an exam, as a thesis or for evaluation in a similar context to any department of this University or any scientific institution. I am aware that a violation of the aforementioned conditions can have legal consequences.

Landau in der Pfalz, _____

Place, date   Signature

The following parts of the thesis are published:

Appendix A.1: Frank Seitz, Mirco Bundschuh and Ralf Schulz conceived and designed the experiments. The experiments were conducted by the first, second and third author. The first author statistically analyzed the data. The first author wrote the first draft. All authors contributed to the final version of the manuscript.

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Appendix A.2: Frank Seitz, Mirco Bundschuh and Ralf Schulz conceived and designed the experiments. The experiments were conducted by the first, the second and third author. The first author statistically analyzed the data. The first author wrote the first draft. All authors contributed to the final version of the manuscript.

Seitz, F., Lüderwald, S., Rosenfeldt, R.R., Schulz, R., Bundschuh, M., 2015. Aging of TiO_2 nanoparticles transiently increases their toxicity to the pelagic microcrustacean *Daphnia magna*. PLoS ONE 10, e0126021.

Appendix A.3: The experiments were conceived and designed by all authors. Frank Seitz conducted parts of the experiments. All authors contributed to the writing of the article.

Bundschuh, M., Zubrod, J.P., Englert, D., Seitz, F., Rosenfeldt, R.R., Schulz, R., 2011. Effects of nano-TiO_2 in combination with ambient UV-irradiation on a leaf shredding amphipod. Chemosphere 85, 1563-1567.

Appendix A.4: Frank Seitz, Mirco Bundschuh and Ralf Schulz conceived and designed the experiments. The experiments were conducted by the first, the second and third author. The first author statistically analyzed the data. The first author wrote the first draft. All authors contributed to the final version of the manuscript.

Seitz, F., Rosenfeldt, R.R., Storm, K., Metreveli, G., Schaumann, G.E., Schulz, R., Bundschuh, M., 2015. Effects of silver nanoparticle properties, media pH and dissolved organic matter on toxicity to *Daphnia magna*. Ecotoxicology and Environmental Safety 111, 263-270.

Further contributions of Frank Seitz to peer reviewed articles can be taken from **Appendix A.5**: Curriculum Vitae

Acknowledgements

Table of content

List of abbreviations

ASTM-medium: Test medium

CI: Confidence interval

Cit nAg: Citrate coated nanoparticles

EC_{50}: Median effective concentration

LOEC: Lowest observed effect concentration

nAg: Silver nanoparticles

NOM: Natural organic matter

$nTiO_2$: Titanium dioxide nanoparticles

SD: Standard deviation

SE: Standard error

TOC: Total organic carbon

UV-light: Ultra violet light

Abstract

Nanoparticles are produced and used in huge amounts increasing their probability to end up in surface waters. There, they are subject to environmentally driven modification processes. Consequently, aquatic life may be exposed to different nanoparticle agglomerate sizes, while after sedimentation benthic organisms are more likely to be affected. However, most ecotoxicity studies with nanoparticles exclusively investigated implications of their characteristics (e.g. size) on pelagic organisms, ignoring environmentally modified nanoparticles. Therefore, a systematic assessment of factors triggering the fate and toxicity of nanoparticles under environmentally relevant conditions is needed. The present thesis, therefore, investigates the implications of nanoparticle related factors (i.e., inherent material-properties and nanoparticle characteristics) as well as environmental conditions towards the pelagic living organism *Daphnia magna* and the benthic species *Gammarus fossarum*. In detail, inert titanium dioxide ($nTiO_2$) and ion-releasing silver nanoparticles (nAg), both of varying particle characteristics (e.g. initial size), were tested for their toxicity under different environmental conditions (e.g. ultraviolet-light (UV-light)). The results indicate that the toxicity of $nTiO_2$ and nAg is mainly determined by: their adsorption potential onto biota, and their fate in terms of reactive oxygen species or Ag^+ ion release. Thus, inherent material-properties, nanoparticle characteristics and environmental conditions promoting or inhibiting these aspects revealed significant implications in the toxicity of $nTiO_2$ and nAg towards daphnids. Furthermore, the presence of ambient UV-light, for example, adversely affected gammarids at 0.20 mg $nTiO_2$/L, while under darkness no effects occurred even at 5.00 mg $nTiO_2$/L. Hence, the currently associated risk of nanoparticles might be underestimated if disregarding their interaction with environmental parameters.

Zusammenfassung

Heutzutage werden Nanopartikel in großem Maßstab produziert, weshalb deren Eintrag in Oberflächengewässer immer wahrscheinlicher wird. Dort angelangt unterliegen sie verschiedenen umweltbedingten (Oberflächen-)Modifikationen, die in letzter Konsequenz eine Vielfalt von Nanopartikel-Agglomeraten unterschiedlicher Größe hervorbringen. Direkt davon betroffen sind aquatische Lebewesen, die einer entsprechenden Nanopartikelexposition in der Wasserphase ausgesetzt sind. Nach Sedimentation der Agglomerate können aber ebenfalls benthische Organismen betroffen sein. Bisherige ökotoxikologische Untersuchungen haben solche umweltbedingten Einflüsse außer Acht gelassen und viel mehr nanopartikel-spezifische Charakteristika auf deren Wirkweise gegenüber pelagischen Vertretern untersucht. Aus diesem Grund ist eine systematische Untersuchung derer Faktoren von Nöten, die den Verbleib und das Verhalten aber auch die Toxizität von Nanopartikeln in der Umwelt maßgeblich beeinflussen. Die kumulative Arbeit dieser Dissertation macht sich dies zum Ziel und hinterfragt entsprechende Faktoren die einerseits durch Nanopartikel assoziierte Aspekte (definiert als i) inhärente Stoffeigenschaft des untersuchten Materials und ii) Nanopartikel Charakteristika)) und andererseits durch Umweltbedingungen in Oberflächengewässern geprägt sind. In diesem Kontext wurden verschiedene ökotoxikologische Untersuchungen mit inerten Titandioxid Nanopartikeln ($nTiO_2$) und Ionen freisetzenden Silber Nanopartikeln (nAg) unter Berücksichtigung verschiedener Nanopartikel Charakteristika (z.B. initiale Partikelgröße, Oberflächengröße) und Umweltbedingungen (z.B. Ionenstärke, ultraviolettes Licht (UV-Licht)), durchgeführt. Als Testorganismen dienten dazu die pelagischen bzw. benthischen Vertreter *Daphnia magna* und *Gammarus fossarum*. Die Ergebnisse deuten daraufhin, dass

die Toxizität von $nTiO_2$ und nAg gegenüber Daphnien maßgeblich durch das Adsorptionspotential (im Bezug auf das Anhaften der Partikel an die Organismenoberfläche) und das Umweltverhalten (Freisetzung von radikalen Sauerstoffspezies oder Metallionen) der Nanopartikel bestimmt wird. Darüber hinaus wurde die Nanopartikeltoxizität von jenen inhärenten Stoffeigenschaften, Nanopartikelcharakteritika und Umweltbedingungen am meisten beeinflusst, welche die zuvor genannten Aspekte entweder verstärken oder abschwächen. Hierfür beispielhaft ist der toxizitätsverstärkende Effekt von UV-Licht auf $nTiO_2$ in Experimenten mit *Gammarus*: Während eine Exposition der Organismen in absoluter Dunkelheit selbst bei 5,00 mg $nTiO_2$/L keine Effekt hervorrief, kam es in der Anwesenheit von UV-Licht schon bei 0,20 mg $nTiO_2$/L zu schwerwiegenden Effekten auf sublethaler und lethaler Ebene. Unter Berücksichtigung der Ergebnisse dieser Dissertation sowie bisherige Erkenntnisse der Wissenschaft im Allgemeinen, ist die derzeitige Risikoeinschätzung von Nanopartikeln möglicherweise unprotektiv, sofern eine Interaktion von Nanopartikeln und Umwelteinflüssen unberücksichtigt bleibt.

1.   Introduction

1.1   Nanoparticles: production, use, release and the aquatic life cycle

The field of nanotechnology has tremendously expanded over the last few years and nowadays contributes trillions of dollars to the global economy (CORDIS, 2006). This will continue with a steadily increasing demand (Scheringer, 2008) for nanoparticles, which can be attributed to their special physicochemical properties. These properties provide helpful functionalities, for instance, for (bio-) medical, cosmetic, textile, and environmental engineering purposes (Blaser et al., 2008; Morones et al., 2005; Nowack and Bucheli, 2007). As a consequence of their heavy use, metal-based nanoparticles, such as titanium dioxide ($nTiO_2$) or silver nanoparticles (nAg), especially (Gottschalk et al., 2009; Piccinno et al., 2012) are unintentionally released into aquatic environments (Gondikas et al., 2014; Klaine et al., 2011). The pathways nanoparticles travel to enter surface waters are most likely: wastewater treatment plant effluents, storm waters, landfill leaches, or in some cases major (car) accidents (Duester et al., 2014; Nowack et al., 2014; Westerhoff et al., 2011).

Once they have entered aquatic environments, nanoparticles are subjected to environmentally driven modification processes. Thereafter they may represent a distinct threat for various organisms, depending on the specific fate of the nanoparticle (Baun et al., 2008). Thus, in the initial phase of their aquatic life cycle they may pose a higher risk for pelagic species such as daphnids, when compared to organisms living at the bottom of surface waters. However, as most nanoparticles may quickly agglomerate and settle down (Petosa et al., 2010) after their release into surface waters, a bigger threat for benthic organisms (living in and on the substratum) may exist (Li et al., 2014a) during a subsequent aquatic life cycle phase

of the nanoparticles. The associated fate and resulting ecotoxicity of nanoparticles is likely controlled and affected by multiple factors. These are comprised of three main aspects, which are listed, and subsequently used throughout the entire thesis, as defined in the following:

i) Inherent material-properties: These are specific substance qualities that exist independently of the outer appearance of the material (e.g. in nano or bulk form). This includes, for instance, the intrinsic photocatalytical or ion-releasing abilities of $nTiO_2$ or nAg, respectively.

ii) Nanoparticle characteristics: These mainly determine the outer appearance but also comprise the composition and surface coating of nanoparticles (e.g. initial size, surface area, crystalline structure composites of nanoparticles).

iii) Environmental conditions: These are environmental parameters of surface waters, for example their ionic strength or level of pH.

Although the ultimate nanoparticle toxicity is determined by an interplay of these factors, knowledge on their interaction is patchy. Therefore, a systematic assessment, investigating the ecotoxicity of environmentally modulated nanoparticles for aquatic species of different habitats is urgently needed.

1.2 Inherent material-properties and nanoparticle characteristics: effects on fate and ecotoxicity of metal-based nanoparticles

1.2.1 Differentiating metal-based nanoparticles: inert vs. ion-releasing materials

In a more general point of view, two groups of metal-based nanoparticles can be differentiated by their suggested fate in water, which is, among other things, determined by their inherent material-properties:

i) Inert nanoparticles that cannot, or only in very limited (negligible) quantities, release toxic metal ions, such as $nTiO_2$.

ii) Metal-based nanoparticles that release high amounts of harmful ions during their aquatic life cycle as for instance nAg.

Consequently, the ecotoxicity of metal-based nanoparticles is directly affected by their fate. Besides the release of toxic ions, other inherent material-properties can also affect the toxic potential of nanoparticles, for instance, the photocatalytic activity of semi-conductors such as $nTiO_2$ (Fujishima et al., 2000). Particles exhibiting such properties can induce harmful reactive oxygen species (ROS) under ultraviolet light (UV-light) and thereby adversely affect aquatic organisms (Feckler et al., 2015; Kalčíková et al., 2014; Kim et al., 2010). However, irrespective of whether inert or ion-releasing nanoparticles, the extent of toxic potential not only depends on the material itself (inherent properties) but also on the nanoparticle characteristics (size, composites, coating) (Nel et al., 2006).

1.2.2 Particle size, composites, and coating: the role of particle characteristics

Nanoparticle characteristics have the potential to significantly influence their toxicity. For example, studies with inert $nTiO_2$ and ion-releasing nAg showed that smaller nanoparticles can reveal a higher toxicity for daphnids when compared to larger nanoparticles or respective bulk-material (Dabrunz et al., 2011; Kennedy et al., 2010). Whereas for $nTiO_2$ the reasons have not been fully uncovered yet (Dabrunz et al., 2011), explanations for ion-releasing nAg have been partly attributed to a higher surface area of the smaller nanoparticles. Amongst other things, this is suggested to induce a higher release of toxic metal ions and thereby potentially increasing the toxicity for nAg (Hoheisel et al., 2012). However, the ultimate reason for nAg toxicity is still under debate and therefore is not yet finally determined (sensu Völker et al., 2013). Moreover, existing studies with $nTiO_2$ and nAg have widely missed assessing the toxicity of nanoparticles systematically. Thus, the influence of single nanoparticle characteristics (especially size, surface area and composition), contributing to the overall toxicity, remains unclear.

For example, nanoparticle composites of different crystalline structure (anstase:rutile) may affect the extent of $nTiO_2$ ecotoxicity towards daphnids (Bang et al., 2011; Clément et al., 2013). Unfortunately, the experimental approaches used so far did not allow for a clear differentiation of particle size and product composition related effects. Therefore, the mechanisms behind the toxicity are not yet clarified. However, characteristics, such as the nanoparticle composition or surface coating, may either enhance or limit inherent material-properties of nanoparticles (Schaumann et al., in press). For instance, nanoparticle surface coatings can limit or increase the release of harmful ions (Chappell et al., 2011). This may, in the end, change the toxic

potential of a nanoparticle (Liu et al., 2010), which in turn also depends on the type of nanoparticle coating (e.g. Dobias and Bernier-Latmani, 2013).

1.3 Environmental conditions affecting the fate and ecotoxicity of nanoparticles

In addition to the inherent material-properties and nanoparticle characteristics, environmental conditions also determine the fate and ecotoxicity of nanoparticles for aquatic biota (Figure 1.1). Varying levels of ionic strength, particle interaction time (=aging), natural organic matter (NOM), pH and UV-light in the surrounding water, can significantly influence the nanoparticles fate and thus their bioavailability and toxicity (Schaumann et al., in press).

For instance, when considering the initial phase of the nanoparticles' aquatic life cycle, the ionic strength of the receiving water plays a very important role for the subsequent nanoparticle fate and toxicity. A high ionic strength facilitates an extensive nanoparticle agglomeration, which promotes a rapid deposition – as a function of aging duration – of nanoparticles (agglomerates) from the water phase to the sediment (Petosa et al., 2010). This in turn decreases their bioavailability for pelagic life, while increasing it for benthic organisms (Li et al., 2014b). Even though the particle size may have significantly increased at the time the agglomerates have settled to the bottom – reducing their total surface area and therefore their potential to release ROS or ions – the nanoparticles can still exhibit a certain toxic potential as a bottom layer (Seitz et al., 2013).
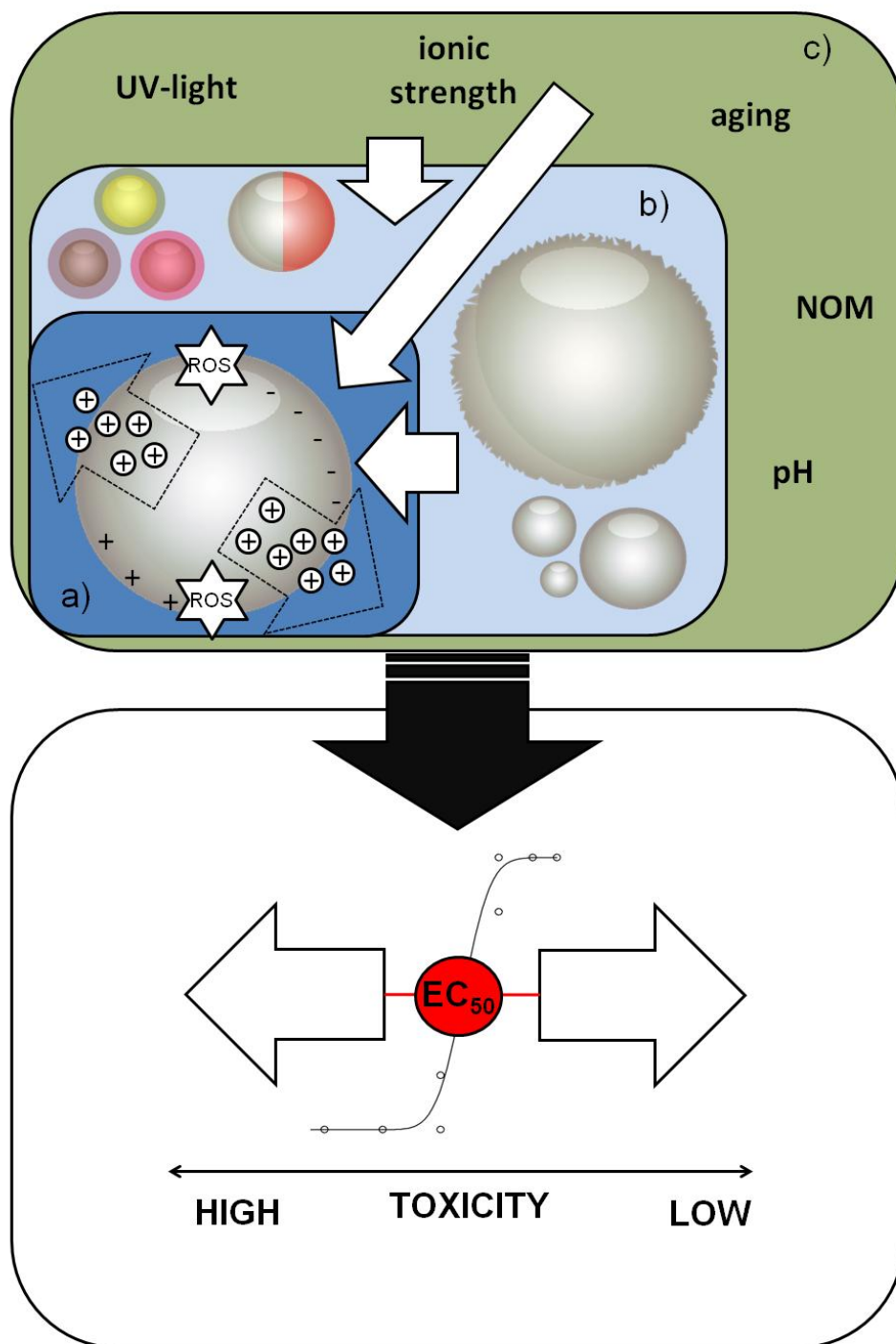
Figure 1.1: Factors interacting with and controlling the ecotoxicity of nanoparticles towards aquatic life: a) inherent material-properties (e.g. release of ROS or ions) b) particle characteristics (coating, composition, surface area and initial size) c) environmental conditions in surface waters (e.g. UV-light, aging (interaction time), natural organic matter (NOM)).

Furthermore, the presence of NOM can also significantly alter the fate and toxic potential of nanoparticles (Blinova et al., 2012; Hall et al., 2009). When NOM is present in sufficient quantities (Erhayem and Sohn, 2014a) it can build a natural coating around the nanoparticles' surface and thereby charge stabilize the material in the water phase (Hall et al., 2009). This comes along with an increased exposure period of nanoparticles for pelagic organisms. However, comparable to man-made coatings, NOM coatings can also significantly lower the toxic potential of nanoparticles (Schaumann et al., in press). For example, when NOM coats ROS or ion-releasing nanoparticles a reduced ecotoxicity can be assumed due to scavenging properties of NOM (Brame et al., 2014).

In addition, the predominant level of pH can impact a particle's fate and toxicity. Alterations in the pH level may directly influence the surface charge of nanoparticles (Badawy et al., 2010) and thus its potential for adsorption including homo or heteroagglomeration (Romanello and Fidalgo de Cortalezzi, 2013). Lower levels of pH may increase the toxic potential of certain metal nanoparticles by releasing higher amounts of harmful ions from their surface (Liu and Hurt, 2010)

In the case of photocatalytically active material the presence of UV-light can also significantly influence the toxic potential of nanoparticles by inducing the release of meaningful quantities of harmful ROS (Ma et al., 2012). However, after agglomeration and sedimentation the photocatalytically induced toxicity of the nanoparticles may be altered due to lower UV-light doses arriving at the bottom – as a function of water column height and presence of NOM – but also by a comparable smaller surface area of agglomerated particles (when compared to single particles). Thus, finally lower quantities of ROS may be released in a later phase of the

nanoparticles life cycle. However, a potential risk for benthic organisms cannot be excluded and thus needs to be assessed.

1.4 Factors and conditions triggering ecotoxicity: are results transferable among different nanoparticles and organisms?

The majority of studies dealing with the ecotoxicity of nanoparticles focus on a single factor modulating the toxicity of one specific nanoparticle product towards one test organism (Amiano et al., 2012; Campos et al., 2013; Fouqueray et al., 2012). This approach, however, widely disregards the existing variety of nanoparticles and their potential fate and impact under more realistic conditions. In nature, combinations of different factors determine the nanoparticle fate, which ultimately affects the toxicity for species of different habitats. The present work aims at counteracting this shortcoming by assessing single factors and combinations of factors affecting the fate and ecotoxicity of inert ($nTiO_2$) and ion-releasing (nAg) nanoparticles. Therefore, experiments with sensitive representatives from the pelagic (*Daphnia magna*) and benthic (*Gammarus fossarum*) zone were conducted. Thereby, the present thesis aims at evaluating, to which extent the results are transferrable among metal-based particles of different inherent material-properties and organisms from different aquatic habitats.

2.   Objective

The present dissertation was conducted within the subproject IMPACT as part of the larger DFG-project INTERNANO, that consists of several working groups and aims at investigating the "Mobility, aging and functioning of engineered inorganic nanoparticles at the aquatic-terrestrial interface". This dissertation has the main objective to point out single factors (nanoparticle- and environmental condition related) and factor combinations that significantly trigger the fate and ecotoxicity of metal-based nanoparticles. It further aims at assessing to which extent the observed results are transferrable among metal-based particles of different inherent material-properties (inert vs. ion-releasing) and organisms from pelagic and benthic habitats. In order to achieve the goals of this dissertation the following sub-objectives were developed:

- Assessment of fate and nanoparticle characteristics (size, surface area and crystalline structure composition) that trigger the acute ecotoxicity of $nTiO_2$ towards the pelagic and benthic organisms *D. magna* and *G. fossarum* [**Appendix A.1**].

- Assessment of fate and environmental conditions – including the impact of ionic strength and presence of NOM during nanoparticle aging – triggering the acute as well as chronic ecotoxicity of $nTiO_2$ in experiments with *D. magna* [**Appendix A.2**].

- Assessment of ambient UV-light triggering the acute ecotoxicity of inert $nTiO_2$ towards *G. fossarum* [**Appendix A.3**].

- Assessment of nanoparticle related factors (inherent material-properties and nanoparticle characteristics: ion release, coating, size) as well as

environmental conditions (presence and absence of NOM, level of pH) that trigger the fate and the acute as well as chronic ecotoxicity of ion-releasing nAg during experiments with *D. magna* [**Appendix A.4**].

3. Layout and methods

The present work is a cumulative thesis, which summarizes the results of four separate publications. These peer-reviewed publications are provided in **Appendix A.1 – A.4**. The studies within the present thesis systematically investigated implications of inherent material-properties, nanoparticle characteristics, and environmental conditions on the fate and ecotoxicity of inert and ion-releasing nanoparticles during experiments with representative pelagic and benthic organisms (Figure 3.1). Therefore, the inert and ion-releasing nanoparticles, $nTiO_2$ and nAg, both exhibiting different particle characteristics (e.g. crystalline structure composition, size, surface coating), were selected and applied during acute and chronic toxicity tests under varying environmental conditions (ionic strength, particle interaction time (=aging), NOM, UV-light, and pH). As test species *D. magna* and *G. fossarum* were chosen as representatives of two different aquatic habitats, namely pelagic and benthic zones. All toxicity tests were accompanied by a thorough particle characterization in terms of particle size measurements.
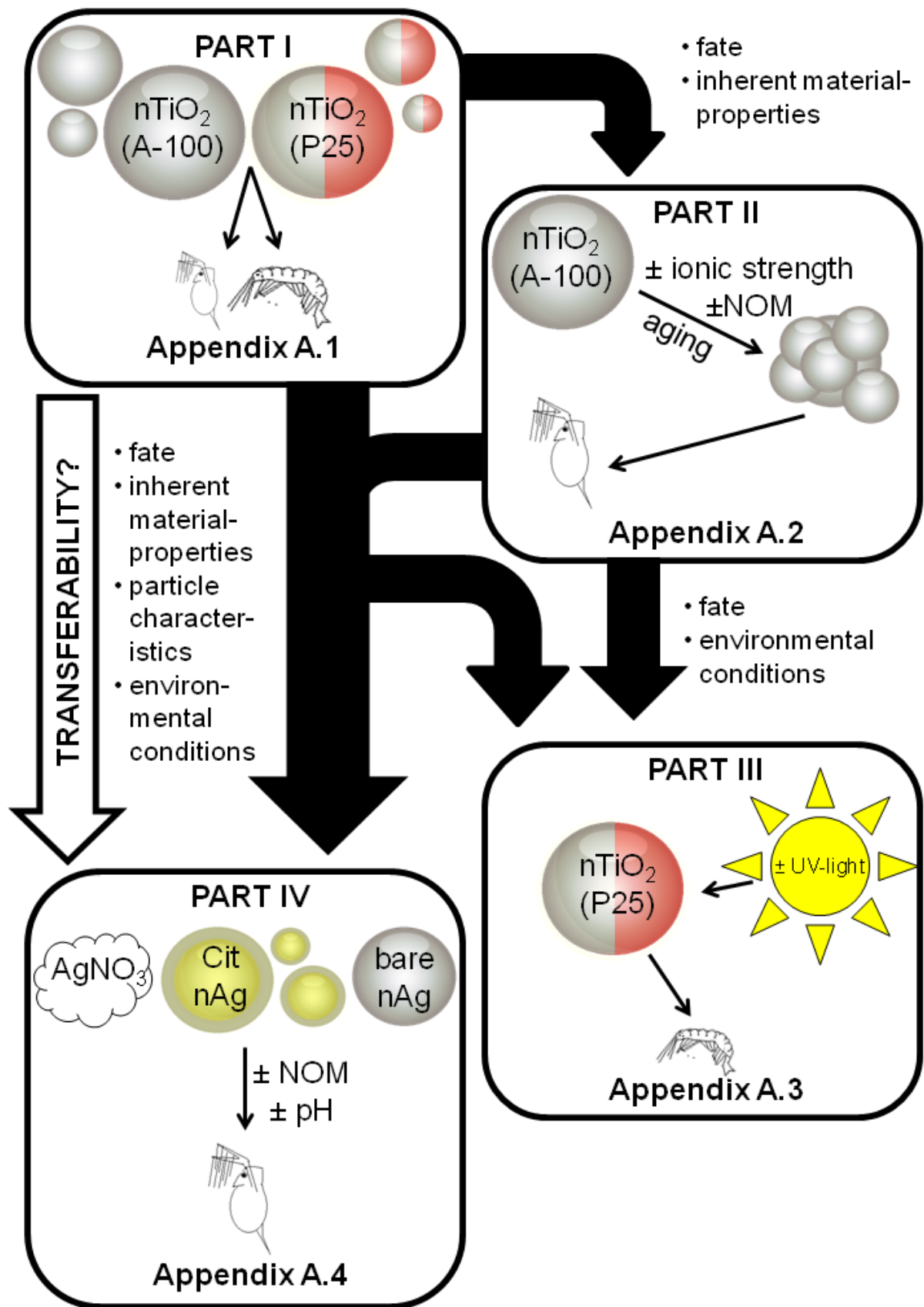
Figure 3.1: Flowchart visualizing the structure of the thesis and information transfer among included sub-objectives (PART I-IV).

1. PART I of the present thesis systematically assessed and differentiated the role of fate, inherent material-properties and varying particle characteristics, of nanoparticles during acute toxicity tests with inert $nTiO_2$. Therefore, investigations with two different $nTiO_2$ products (A-100: 99% anatase and P25: 70% anatase and 30% rutile), at three different average initial sizes (55; 100 and 140 nm) using the pelagic and benthic test organisms (Figure 3.2) *D. magna* and *G. fossarum*, respectively, were conducted [**Appendix A.1**]. During the acute toxicity tests with daphnids immobility was assessed after 96 h as recommended for nanoparticle testing with $nTiO_2$ (Dabrunz et al., 2011). The 7 d long experiments with gammarids focused on the animals' mortality and feeding activity as those are frequently used sensitive endpoints (Maltby et al., 2002). Each experiment was additionally accompanied by particle surface area determination.
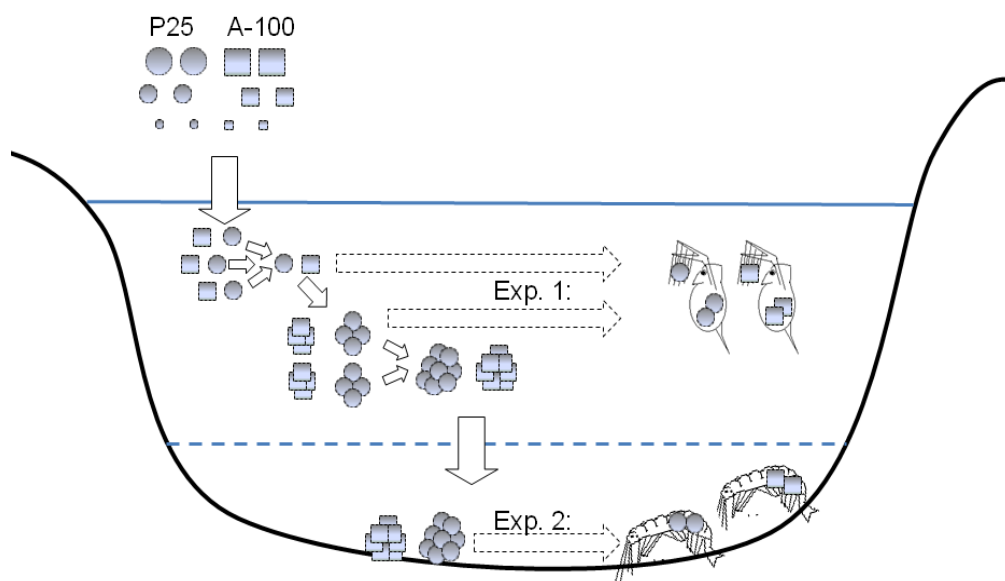
Figure 3.2: Experimental derivation based on the aquatic life cycle of $nTiO_2$ varying in initial size and crystalline structure composition (A-100 and P25). Experiment 1 of PART I covers potential particle characteristic and small agglomerate related effects of $nTiO_2$ towards pelagic living organisms at an early stage of nanoparticle life cycle. Experiment 2 of PART I focuses a later stage of the latter named and hence potential toxic effects on benthic organisms after nanoparticle agglomeration and sedimentation [**Appendix A.1**].

2. PART II focused on implications of different $nTiO_2$ aging scenarios on the fate and resulting ecotoxicity of the $nTiO_2$ product A-100 (~100 nm average diameter). The conditions were set at aging durations of 0, 1, 3 and 6 days while exhibiting varying levels of ionic strength (0.00 or 9.25 mmol/L) and NOM (0.00 or 8.00 mg total organic carbon/L). After aging, the material was assessed during acute and chronic toxicity tests with daphnids [**Appendix A.2**]. The endpoints were immobilization for the 96 h acute toxicity tests and mortality as well as reproduction for the 21 d chronic experiments.

3. PART III investigated the implications of environmental conditions on the fate and resulting ecotoxicity of the inert but photocatalytically active $nTiO_2$ product P25 (~100 nm average initial size). Therefore, effects of $nTiO_2$ on the mortality and feeding activity of the amphipod *G. fossarum* were assessed in absence and presence of ambient UV-light intensities (UV-A and UV-B: 28.0 W/m^2 and 0.9 W/m^2) [**Appendix A.3**].

4. PART IV assessed and differentiated the role of fate (in terms of inherent material-properties), varying particle characteristics, and environmental conditions for the ecotoxicological potential of ion-releasing nanoparticles during acute and chronic toxicity test with nAg and *Daphnia* [**Appendix A.4**]. In detail, experiments were carried out using different (n)Ag materials ($AgNO_3$, bare nAg and citrate coated nAg) exhibiting a variety of particle characteristics (e.g. surface coating but also different average initial particle sizes ranging from 20 to 140 nm). Additionally 48 h acute and 21 d chronic experiments were conducted under environmental conditions differing in pH (levels 6.5 and 8.0) and the absence and presence of NOM (0.00 or 8.00 mg total organic carbon/L). In order to evaluate the role of toxic ions, Ag^+ was quantified for each nAg type and environmental condition. Thus

multiple factors investigated during PART I and II were combined and assessed in PART IV, which allowed to evaluate for the transferability of results from inert nanoparticles to ion-releasing nanoparticles.

4. Assessment of factors influencing nanoparticle toxicity

4.1 Role of particle characteristics for $nTiO_2$ toxicity towards daphnids and gammarids

Results of the acute experiments with daphnids and $nTiO_2$ clearly displayed initial particle size related effects for both products. Thus, 55 nm sized particles showed, for both A-100 and P25, an up to 7-fold, and hence statistically significantly, higher toxicity compared 140 nm sized $nTiO_2$ (Figure 4.1 A) [**Appendix A.1**]. Findings for the surface area normalized 96-h EC_{50} values showed that smaller particles (55 and 100 nm) did not statistically differ, independent of the product investigated (A-100 and P25). In contrast, the surface area normalized 96-h EC_{50} values of 140 nm particles meaningfully differed from smaller (55 and 100 nm) nanoparticles (Figure 4.1 B).
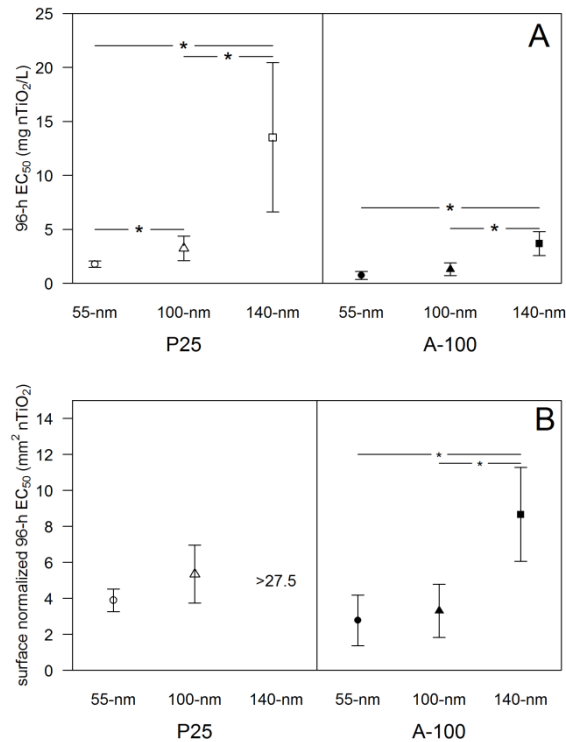
Figure 4.1: (A) 96-h EC_{50}-values with respective 95% CIs for the immobilization data of *D. magna* under either A-100 or P25 exposure. (B) Initial surface area normalized 96-h EC_{50}-values with respective 95% CIs for the immobilization data of *D. magna* under either A-100 or P25 exposure. Asterisks (*) denote statistically significant differences [**Appendix A.1**].

Also the product itself and thus the particle characteristic, in terms of crystalline structure composition, influenced the toxicity. In detail, the EC_{50} values of P25 showed for each initial particle size class an up to four times lower toxicity when compared to A-100 (Figure 4.2 A). The initial surface area normalized EC_{50} values of both products did not statistically significantly differ, even though values of A-100 were always smaller than those of P25 (Figure 4.2 B), which also points towards the importance of the surface area for the $nTiO_2$ toxicity. The experiments with gammarids did not reveal any statistically significant difference for the feeding activity of exposed animals, independent of the product or initial particle size applied [**Appendix A.1**].
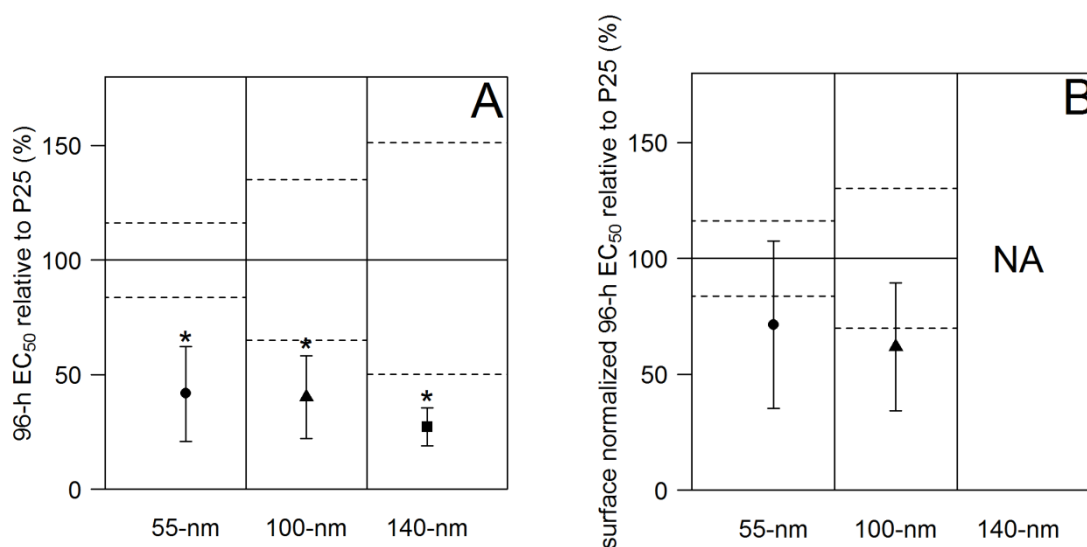
Figure 4.2: (A) percentage 96-h EC_{50} values (with 95% CIs) for the immobilization data of *D. magna* whereas gained 96-h EC_{50} values of A-100 were related to the respective 96-h EC_{50}-value of P25. (B) Percentage initial surface normalized 96-h EC_{50}-values (with 95% CIs) for the immobilization data of *D. magna* whereas gained 96-h EC_{50} values of A-100 were related to the respective 96-h EC_{50}-value of P25. Continuous and dashed lines indicate reference 96-h EC_{50}-values and respective 95% CI of P25, while filled symbols indicate the relativized 96-h EC_{50}-values of A-100. NA = not assessed due to missing initial surface normalized 96-h EC_{50} value for 140-nm sized P25. Asterisks (*) denote statistically significant differences [**Appendix A.1**].

4.2 Role of nanoparticle aging under varying environmental conditions for the fate and toxicity of nTiO$_2$ towards daphnids

Experiments with *Daphnia* showed that the aging of nTiO$_2$ (in different media, exhibiting varying levels of ionic strength and NOM) can significantly influence the particles' fate (in terms of agglomeration and sedimentation; Table 4.1) and induce acute as well as chronic toxicity [**Appendix A.2**].

Table 4.1: Nominal and mean measured (± SD; n=3) $nTiO_2$ concentrations after 0, 1, 3 and 6 d aging in the respective aging medium, namely ASTM-medium with and without NOM (8.00 mg TOC/L) [**Appendix A.2**].

Aging medium	Aging duration (d)	Nominal concentration	Mean measured concentration (±SD; mg/L)	
			Test start 0 h	Test end 96 h
Milli-Q without NOM	0	4.00	3.82 ± 0.05	0.04 ± 0.00
	1	4.00	3.80 ± 0.07	0.04 ± 0.00
	3	4.00	4.02 ± 0.08	0.06 ± 0.00
	6	4.00	3.90 ± 0.24	0.04 ± 0.01
Milli-Q with NOM	0	4.00	3.71 ± 0.04	0.04 ± 0.01
	1	4.00	3.80 ± 0.04	0.05 ± 0.00
	3	4.00	3.80 ± 0.03	0.14 ± 0.01
	6	4.00	3.61 ± 0.05	0.05 ± 0.00
ASTM without NOM	0	4.00	3.57 ± 0.07	0.05 ± 0.00
	1	4.00	3.56 ± 0.07	0.05 ± 0.00
	3	4.00	3.57 ± 0.05	0.09 ± 0.00
	6	4.00	3.43 ± 0.06	0.05 ± 0.00
ASTM with NOM	0	4.00	3.59 ± 0.06	2.59 ± 0.04
	1	4.00	3.60 ± 0.04	3.28 ± 0.05
	3	4.00	3.54 ± 0.05	3.41 ± 0.06
	6	4.00	3.42 ± 0.02	3.21 ± 0.06

A $nTiO_2$ aging, under conditions excluding implications of ionic strength (Milli-Q-water: 0.0 mmol/L) did not alter the acute toxicity compared to an unaged $nTiO_2$ control (Figure 4.3 A), irrespective of the aging duration and level of NOM applied. Contrary a 6 d aging in medium with high ionic strength (ASTM-medium, in absence of any NOM) statistically significantly reduced the toxicity by a factor of four (Figure 4.3 B).
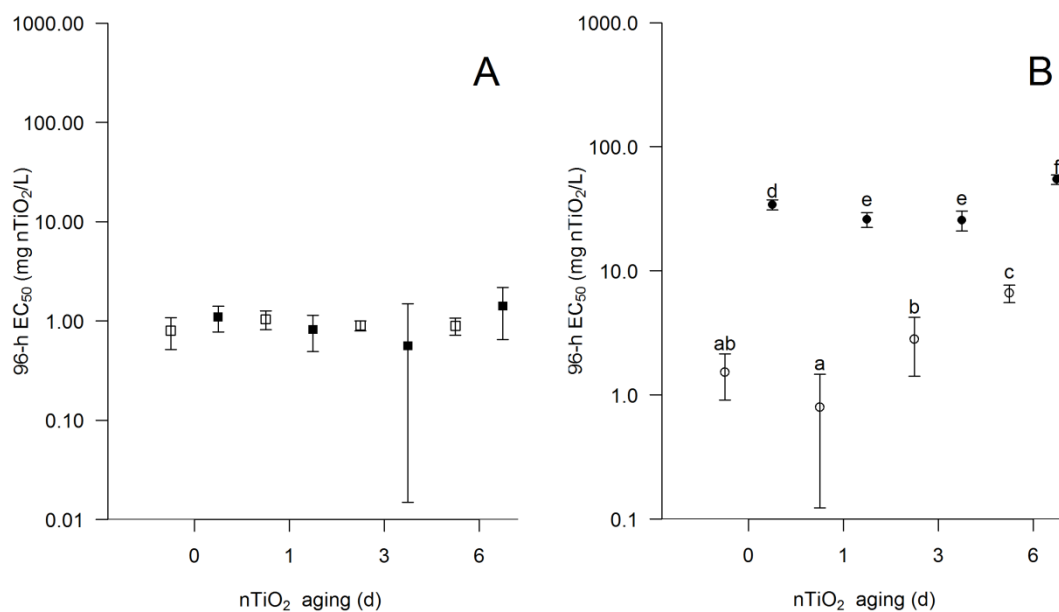
Figure 4.3: (A) 96-h EC_{50} values (± 95% CI) of $nTiO_2$ aged for 0, 1, 3 or 6 d in Milli-Q with (■) or without (□) NOM. (B) 96-h EC_{50} values (± 95% CI) of $nTiO_2$ previously aged for 0, 1, 3 or 6 d in ASTM-medium with (●) and without (○)NOM. 96-h EC_{50} values followed by different lower case letters are significantly different [**Appendix A.2**].

The presence of NOM during $nTiO_2$ aging in medium with high ionic strength generally reduced the nanoparticle toxicity for both, acute and chronic exposure scenarios [**Appendix A.2**]. However, if $nTiO_2$ was aged for only 1 or 3 days in medium of high ionic strength and in presence of NOM, a statistically significant increase in $nTiO_2$ toxicity (by ~ 30%) was observed if compared to unaged $nTiO_2$ (Figure 4.3 B). After 6 d of aging in the same medium the toxicity dropped again by ~60% when compared to its unaged control. For the chronic experiments with *Daphnia* comparable results were observed [**Appendix A.2**]. These chronic data displayed a higher mortality and lower fecundity of *Daphnia* when exposed to unaged rather than 3 d aged $nTiO_2$ in absence of any NOM. The presence of NOM during aging reduced the chronic toxicity significantly compared to its absence.

Nonetheless, a 3 d long aging in the presence of NOM significantly increased the toxicity when compared to a 0 d aging in the same medium [**Appendix A.3**].

4.3 Role of environmental conditions for the fate and toxicity of nTiO$_2$ towards gammarids

The experiments with nTiO$_2$ displayed significant implications for the survival and feeding activity of *Gammarus* in the presence of UV-light [**Appendix A.3**]. In this case the mortality of gammarids was by up to 90% statistically significantly increased (Figure 4.4) and the feeding activity was significantly reduced (≥50%; Figure 4.5).
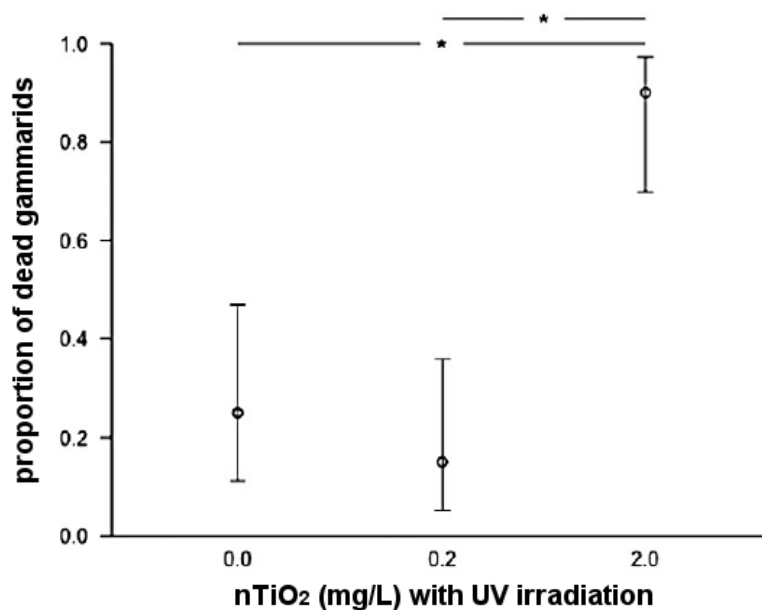
Figure 4.4: Proportion (with 95% CI) of dead gammarids exposed to different nTiO$_2$ concentrations in combination with UV-light. Asterisks denote significant differences between treatments [**Appendix A.3**].
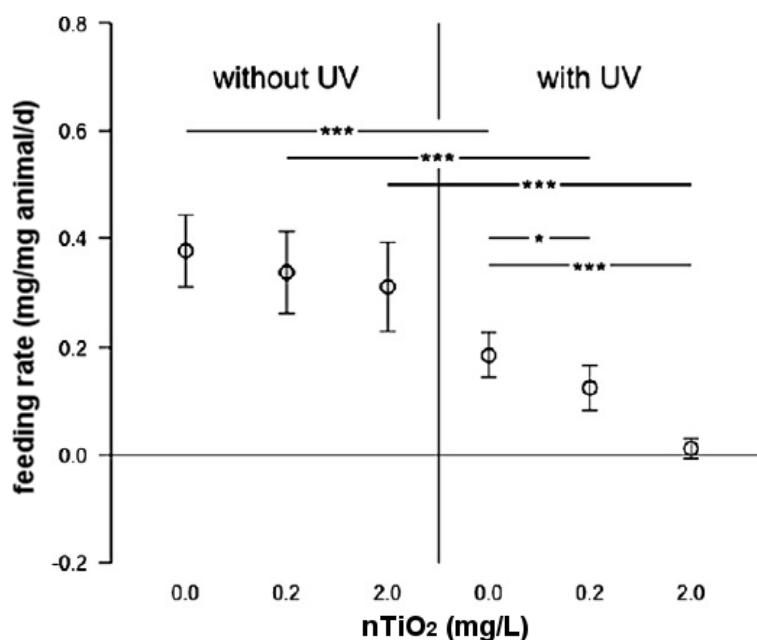
Figure 4.5: Mean (with 95% CI) feeding rate of *G. fossarum* exposed to 0.00, 0.20 or 2.00 mg $nTiO_2$/L for seven days in darkness or under ambient UV-light during the second feeding activity trial. Asterisks denote significant differences with $p < 0.05$ (*) and $p < 0.001$ (***) based on Dunnett's test for multiple comparisons (n = 19-20), respectively. Due to the 90% mortality recorded in the 2.00 mg $nTiO_2$/L with UV-light, this treatment was not included in the further statistical analysis [**Appendix A.3**].

4.4 Role of particle characteristics, environmental conditions and fate for nAg toxicity towards daphnids

Also the acute and chronic effects of nAg on *D. magna* were statistically significantly influenced by particle characteristics, environmental conditions and fate (Figure 4.6) [**Appendix A.4**]. Acute experiments showed that $AgNO_3$ – as a pure Ag ion source (Table 4.2) – was, with 48-h EC_{50} values ranging from 1.70 to 3.00 µg/L, the most toxic silver product independent of the environmental conditions (pH 6.5 or 8.0, NOM of 0.00 or 8.00 mg TOC/L). The 140 nm initial sized bare nAg, revealed 48-h EC_{50} values ranging from 3.90 (pH 6.5 in absence of NOM) to 33.40 µg/L (pH 8.0 in presence of NOM) and showed the highest release of Ag^+ among the nAg materials

tested (Table 4.2). Furthermore, the bare particles were significantly more toxic and released higher quantities of Ag^+ compared to citrate coated nAg (Cit nAg), independent of the Cit nAg initial size and environmental condition applied (Table 4.2; Figure 4.7 A). Comparisons among the different initial sizes of Cit nAg showed that particles of 20 nm were statistically significantly more toxic than 60 and 100 nm initial-sized particles (Figure 4.7 A). Also 60 nm particles displayed a higher toxicity compared to 100 nm Cit nAg. This particle-size-dependent toxicity of Cit nAg was only partly positively correlated with an increasing Ag^+ release of smaller particles compared to larger ones (Table 4.2).

Environmental conditions significantly altered the acute and chronic toxicity of the nAg materials tested. Generally higher levels of NOM and pH reduced the silver ion release (Table 4.2) and ecotoxicity (Figure 4.6 and 4.7 A-C) [**Appendix A.4**].
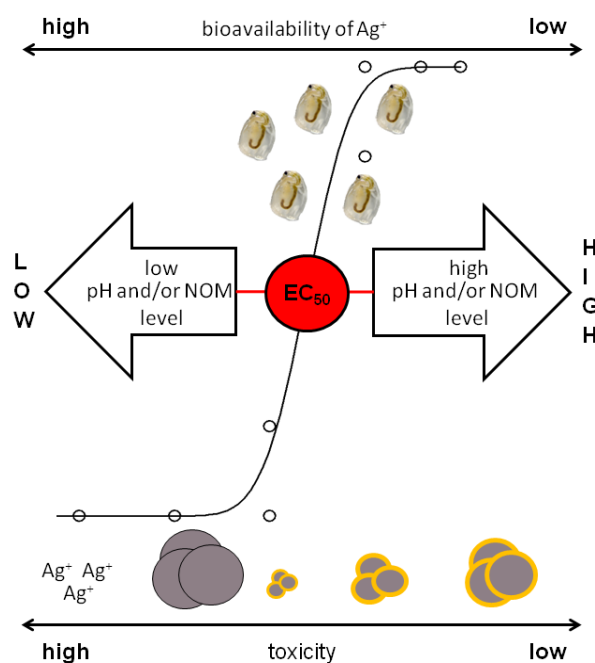
Figure 4.6: Schematic draft illustrating nanoparticle and environmental condition related factors that influence the silver (nanoparticle) toxicity [**Appendix A.4**].

Table 4.2: Mean (±SE; n=3) Ag concentrations (µg/L) for each silver material and environmental condition (NOM and pH level) investigated. Measurements were performed at different time intervals during the acute and chronic experiments by inductively coupled plasma mass spectrometry (Seitz et al., 2013). All samples of the acute toxicity tests were also subjected to an ultracentrifugation process to analyze a respective Ag^+ release after 48 h. NA: data not evaluated [**Appendix A.4**].

Acute toxicity test

Silver material	nominal	- NOM						nominal	+ NOM					
		pH 6.5			pH 8.0				pH 6.5			pH 8.0		
		0 h	48 h	48 h[a]	0 h	48 h	48 h[a]		0 h	48 h	48 h[a]	0 h	48 h	48 h[a]
AgNO$_3$	32.4	27.0 (± 0.1)	27.7 (± 0.2)	27.5 (± 0.1)	31.3 (± 0.1)	24.6 (± 0.1)	22.1 (± 0.1)	32.4	27.3 (± 0.1)	24.6 (± 0.1)	18.0 (± 0.1)	30.0 (± 0.1)	24.8 (± 0.14)	32.0 (± 0.2)
140 nm bare nAg	62.5	42.8 (±0.8)	39.5 (±0.6)	5.5 (±0.0)	38.1 (±0.4)	29.5 (±0.5)	6.0 (± 2.5)	62.5	70.2 (± 0.5)	58.9 (± 0.4)	3.7 (± 0.1)	64.1 (± 0.13)	40.7 (± 0.7)	3.1 (± 0.0)
20 nm Cit nAg	80.0	56.1 (± 0.6)	39.5 (±0.4)	3.4 (± 0.9)	50.8 (± 0.6)	37.1 (± 0.6)	5.0 (± 0.0)	80	62.2 (± 0.3)	67.0 (± 1.1)	2.0 (± 1.2)	67.5 (± 0.4)	62.0 (± 0.7)	0.8 (± 0.8)
60 nm Cit nAg	93.8	27.0 (± 0.7)	22.0 (± 0.6)	0.3 (± 0.0)	26.0 (± 0.5)	21.8 (± 0.5)	1.8 (± 0.0)	NA	NA	NA	NA	NA	NA	NA
100 nm Cit nAg	75.0	41.3 (± 0.8)	33.7 (± 0.8)	2.4 (± 0.0)	36.6 (± 0.7)	21.9 (± 0.6)	1.9 (± 0.1)	NA	NA	NA	NA	NA	NA	NA

Chronic toxicity test

	0 h	72 h	0 h	72 h	0 h	72 h	0 h	72 h
~30 nm Cit nAg	73.2 (± 0.1)	49.2 (± 0.1)	73.2 (± 0.1)	51.3 (± 0.1)	78.4 (± 1.5)	51.7 (± 1.3)	75.7 (±0.1)	69.5 (± 0.1)

[a]following centrifugation, resulting in an Ag concentration comprising of very small nAg (<2nm) and Ag^+ ions.
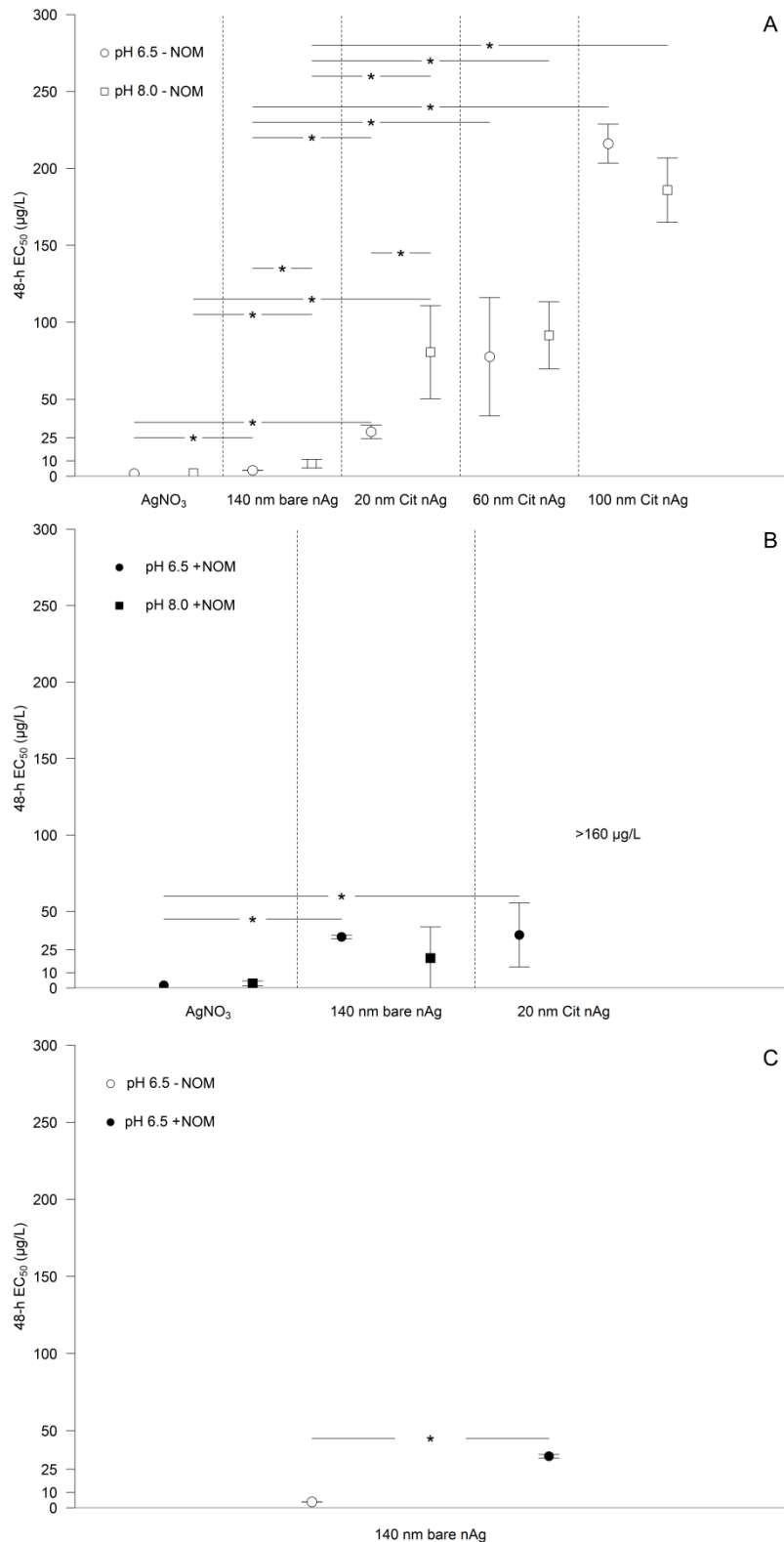
Figure 4.7: 48-h EC$_{50}$ values (with 95 % CIs) of different silver materials at varying pH levels 6.5 and 8.0 in the (+) presence and (-) absence of dissolved organic matter (NOM; 0.00 and 8.00 mg TOC/L). Asterisks (*) denote statistically significant differences between 48-h EC$_{50}$ values [**Appendix A.4**].

5. Synthesis

5.1 Effects of the inherent material-properties and nanoparticle characteristics

The results of the present thesis highlight the importance of inherent material-properties and particle characteristics for the fate and ecotoxicological potential of inert and ion-releasing nanoparticles.

As a consequence of their distinct inherent material-properties, resulting in different modes of toxic action, $nTiO_2$ and nAg displayed varying levels of toxicity during both acute and chronic experiments with daphnids [**Appendix A.1, A.2 and A.4**]. Even after 96 h, the inert $nTiO_2$ revealed higher EC_{50} compared to 48-h EC_{50} values of the ion-releasing nAg, independent of particle characteristics or environmental conditions applied [**Appendix A.1, A.2 and A.4**]. Explanations can be seen in the high toxicity of Ag^+ ions (Ratte, 1999), that were released in meaningful amounts during the experiments with nAg [**Appendix A.4**]. These ions are known to induce ROS, interact with cellular enzymes and have the potential to mimic endogenous ions (Bianchini et al., 2002; Völker et al., 2013), inducing adverse effects in daphnids rapidly (Lam and Wang, 2006; Rosenfeldt et al., 2014).

Furthermore, only limited quantities of harmful ROS may have been released under the light conditions in the experiments with daphnids and $nTiO_2$ [**Appendix A.1, A.2**] (Seitz et al., 2012). Thus, other modes of toxic action can be suggested for $nTiO_2$ during those experiments. For instance, a biological surface coating of test organisms affecting the mobility and molting of the organisms can be seen as a potential pathway of toxicity (Dabrunz et al., 2011; Noss et al., 2013). This suggests that the

adsorption potential of $nTiO_2$ on biota plays an important role during acute toxicity test with daphnids [**Appendix A.1 and A.2**]. Nevertheless, $nTiO_2$ may act differently during chronic experiments due to the presence of algae. There, $nTiO_2$ compete with algae and induces implications in the energy uptake after being consumed by *Daphnia*. In detail, ingested $nTiO_2$ agglomerates can lower the amount of consumed algae (Rosenkranz et al., 2009; Zhu et al., 2010), blocking the gut and ultimately affecting the fecundity of the animals [**Appendix A.2**].

However, besides the inherent material-properties the investigated particle characteristics also play an important role for the nanoparticle fate and toxicity. Thus, independent of the nanoparticle used, the initial particle size of $nTiO_2$ and (Cit) nAg statistically significantly affected the mobility of daphnids [**Appendix A.1 and A.4**]. For both materials, smaller initial particle sizes revealed a higher acute toxicity compared to larger ones. In the case of $nTiO_2$, presumably an adsorption of smaller relative to larger nanoparticles on the test organisms' carapace, may have led to a more dense biological surface coating of the animals, affecting the extent of toxic potential. This is in line with findings of the $nTiO_2$ surface area normalized EC_{50} values, showing statistically significant differences for nanoparticles of <100 nm sizes and 140 nm. Thus the nanoparticle surface area serves as explanatory variable for a higher $nTiO_2$ toxicity of particles smaller or equal to 100 nm.

In case of the ion-releasing nAg, the surface area also played an important role (Hoheisel et al., 2012). Those materials are, amongst others, suggested to induce toxic effects according to the amount of Ag^+ ions released (Völker et al., 2013; Yang et al., 2012). Other sole nanoparticle related aspects, such as size, surface, and shape are also suggested to induce nAg toxicity (Asharani et al., 2008; Fabrega et al., 2009). However, related to their particle size, smaller nAg exhibit a higher surface

to volume ratio, and therefore release a higher amount of Ag^+ ions in a shorter time, which finally results in a significantly higher toxicity, when compared to bigger particles (Hoheisel et al., 2012; Kennedy et al., 2010). This was also displayed for the different Cit nAg initial sizes in the work of the present thesis [**Appendix A.4**].

The particle composition, including the crystalline structure of $nTiO_2$, also significantly affected the extent of nanoparticle toxicity [**Appendix A.1**]. For those $nTiO_2$ products, that contain higher quantities of the crystalline structure anatase, a higher toxicity can be suggested compared to compositions including rutile [**Appendix A.1**]. Other researchers have also observed this phenomenon, while their experimental approach did not allow for a separation of particle size and product composition (Bang et al., 2011; Clément et al., 2013). The present work took care of this shortcoming and revealed clear differences in the toxicity of A-100 and P25, which can be mainly attributed to higher surface area [**Appendix A.1**] and reactivity of anatase when compared to rutile or a mixture of both (Cong and Xu, 2012). This may have promoted an increased toxicity for daphnids, by inducing a more dense biological surface coating or an elevated ROS release.

Nanoparticle coatings also play an important role for the resulting ecotoxicity of nanoparticles. In the present work $nTiO_2$ that was most likely naturally coated with NOM after its aging process revealed a significantly lower toxicity compared to bare $nTiO_2$ [**Appendiy A.2**]. Also, bare nAg released higher quantities of ions compared to Cit nAg and consequently displayed a higher toxic potential. Moreover, during experiments with nAg and NOM, most likely an additional coating with organic matter of the nanoparticles took place and further decreased the toxicity of the nAg [**Appendix A.4**]. Coatings can limit the release of harmful ROS and metal ions (Brame et al., 2014; Liu and Hurt, 2010) and thereby lower their toxic potential.

Natural coatings with humic or fulvic acid contents can affect the surface charge and thereby the adsorption potential of nanoparticles onto aquatic biota (sensu Seitz et al., 2013), [**Appendix A.2**]. This alters their interaction potential with biological surfaces and hence the ultimate toxicity as seen for both materials, namely $nTiO_2$ and nAg, in the present work [**Appendix A.2 and A.4**]. However, when the coating itself has toxic properties, a higher nanoparticle toxicity may also be observed (sensu Cho et al., 2009).

5.2   Effects of environmental conditions on fate and toxicity

Environmental conditions can diversely alter the fate and toxicity of inert and ion-releasing nanoparticles [**Appendix A.1, A.2, A.3 and A.4**]. For example, conditions exhibiting high ionic strengths (and low amounts of NOM) are known to induce a fast nanoparticle agglomeration (Petosa et al., 2010) and subsequent sedimentation (Dabrunz et al., 2011). Therefore, the concentration of nanoparticles during their suggested aquatic life cycle in surface waters may rapidly decrease in the water phase while increase at the bottom. Consequently, this alters the potential risk for pelagic and benthic life [**Appendix A.1, A.2, A.3, and A.4**]. The present work addresses this question, among others, by investigating: i) effects of unaged $nTiO_2$ towards pelagic (daphnids) and benthic organisms (gammarids) [**Appendix A.1**]; ii) effects of $nTiO_2$ after their interaction with environmental conditions (ionic strength and NOM) for different periods of time (=aging) on the more sensitive organism *Daphnia* [**Appendix A.2**]; iii) effects of $nTiO_2$ in the presence of UV-light using the benthic test organisms, namely *Gammarus* [**Appendix A.3**].

The pelagic organism *Daphnia* was more sensitive towards unaged $nTiO_2$ (96-h EC_{50} values of 55 nm sized A-100: 0.74 mg/L) when compared to gammarids [**Appendix A.1 and A.3**]. In contrast, *Gammarus* showed no adverse effects – irrespective of the $nTiO_2$ characteristics – on mortality and feeding activity at concentrations as high as 5.00 mg $nTiO_2$/L during PART I of the present work [**Appendix A.1**]. However, the findings of a combined exposure of gammarids to $nTiO_2$ and UV-light during PART III indicated significant implications on gammarid mortality and feeding activity at $nTiO_2$ concentrations as low as 0.20 mg/L [**Appendix A.3**]. This is in line with other studies using the same test organism and similar testing conditions, detecting effects of $nTiO_2$ only in the presence of UV-light (Kalčíková et al., 2014). The toxicity can be explained by the presence of harmful ROS, which are formed by the photocatalytically active $nTiO_2$ under the given UV-light conditions (Feckler et al., 2015). The ROS themselves may have either lowered the food quality (Feckler et al., 2015) and thus the feeding activity of the organisms or induced toxicity by damaging biomembranes and causing lipid peroxidation (Cabiscol et al., 2010) in gammarids.

Reasons for the difference in the sensitivity of *Daphnia* and *Gammarus* can be related to habitat specific adaptations. Whereas benthic life is most likely used to relatively high quantities of natural colloids or suspended sediments, pelagic living organisms might be more susceptible to ultra fine particles (in sensu Arruda et al., 1983; Levine et al., 2005).

Acute and chronic experiments with differently aged $nTiO_2$ (A-100) and *Daphnia* [**Appendix A.2**] highlighted the role of environmental conditions for the fate and extent of $nTiO_2$ toxicity. An aging in medium excluding implications of ionic strength (0.00 mmol/L) did not change the toxicity of A-100 independent from aging duration and the level of NOM applied (0.00 or 8.00 mg TOC/L). This can be attributed to a

largely unchanged particle size at the beginning of the respective experiments. In contrast, an aging in the presence of a high ionic strength (9.25 mmol/L) and absence of NOM induced a strong particle agglomeration during longer aging periods (6 d). This process reduced the toxicity drastically (by a factor of up to 4). Nevertheless, when the aging lasted 3 days and took place in presence of NOM an increased toxicity by up to 30% was observed, in comparison to the unaged control. Such an increase in toxicity after aging can be explained by a NOM induced stabilization of particles in a size range that is preferably ingested by daphnids (Figure 5.1). This may have led to an increased uptake of $nTiO_2$ agglomerates, which affected the fecundity and survival of *Daphnia* most likely by limiting their energy availability (Rosenkranz et al., 2009; Zhu et al., 2010) [**Appendix A.2**].
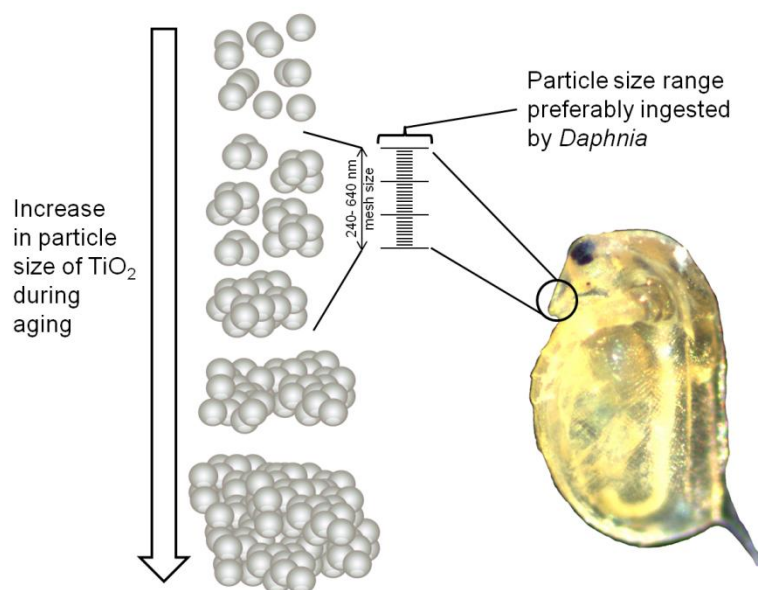
Figure 5.1: Schematic draft of the preferably ingested particle size range of *D. magna* [**Appendix A.2**].

The presence of NOM not only lowered the acute and chronic toxicity of the $nTiO_2$ product A-100 for *Daphnia*, it further decreased the toxicity of nAg [**Appendix A.2 and A.4**]. This mitigation like effect of NOM for nanoparticle toxicity is in line with other studies (Gao et al., 2012; Hall et al., 2009). The phenomenon may be explained by the adsorption (=coating) of NOM onto the nanoparticles surface. When the NOM surface coating is dense enough (depending on the NOM and nanoparticle concentration given) the nanoparticles become charge stabilized (Erhayem and Sohn, 2014b). Furthermore, when NOM also adsorbs onto the surface of biota, electro static repulsion forces may act between nanoparticle and the organism (Lin et al., 2012). As a consequence NOM prevents a biological surface coating of nanoparticles on the organisms and thus can reduce the nanoparticle toxicity. Moreover, NOM can also act as ROS or ion scavenging/complexing material (Brame et al., 2014), making them less bioavailable and therefore less harmful (Gao et al., 2012).

Also, pH affected the toxicity of nanoparticles [**Appendix A.4**]. For instance, pH levels of 6.5 revealed a significantly higher toxicity compared to identical exposures at pH 8.0 for 20 nm Cit nAg and 140 nm bare nAg [**Appendix A.4**]. Explanations can be related to an increased nAg dissolution rate under lower pH levels (Liu and Hurt, 2010). Thus under such environmental conditions higher amounts of toxic metal ions can be released in a shorter time from ion-releasing nanoparticles as e.g. displayed for zinc oxide or silver nanoparticles (Bian et al., 2011; Liu and Hurt, 2010). This increases their toxic potential immediately after their introduction in corresponding surface waters.

Finally, nanoparticle toxicity is significantly affected by the environmental conditions, ionic strength, interaction time (=aging duration), NOM content pH and UV-light.

These factors changed the extent of the inherent material-properties (ROS or ion release), the particle characteristics (size, surface charge or dissolution rate) as well as the fate and thereby the toxic potential of nanoparticles [**Appendix A.1, A.2, A.3 and A.4**].

6.   Conclusion and perspective

In summary the present thesis has comprehensively shown that nanoparticle toxicity not only depends on inherent material-properties and nanoparticle characteristics but also strongly on environmental conditions of the surrounding medium [**Appendix A.1, A.2, A.3 and A.4**]. Moreover, a partial transferability of results among inert and ion-releasing nanoparticles was uncovered. In this respect, the nanoparticle initial size was a main driver for $nTiO_2$ and nAg induced ecotoxicity towards *Daphnia* [**Appendix A.1 and A.4**]. Also, the presence of NOM meaningfully reduced nanoparticle toxicity, independent of the inherent material-properties or characteristics of the tested nanoparticles [**Appendix A.1, A.2 and A.4**].

Finally, when abstracting from the present thesis the lowest observed effect concentration (=LOEC; 0.20 mg $nTiO_2$/L in presence of UV-light; [**Appendix A.3**]) and comparing this value with predictions for environmental concentrations (e.g. 0.021 to 4.000 µg $nTiO_2$/L in surface waters and sewage treatment effluents (Gottschalk et al., 2009)) a risk for aquatic life cannot be excluded (see also Feckler et al., 2015). Moreover, it suggests that a risk for aquatic life already exists, especially when considering the steadily increasing demand for nanoparticles (Scheringer, 2008), the varying toxic potential of different nanoparticle products and the potentially higher sensitivity of other organisms [**Appendix A.1**]. Consequently,

the currently applied approaches that widely disregard environmentally relevant conditions during the ecotoxicological evaluation of nanoparticles, may underestimate their potential risk in nature.

Furthermore, the present thesis provides fundamental evidence that more research in the specialized field of "nanotoxicology" is urgently needed. Prospectively, further nanoparticle interactions considering various environmental conditions but also other chemical stressors should, due to its field relevant scenario, be systematically assessed. As a result of the tremendous diversity of nanoparticle characteristics they may further complicate the already existing challenge of mixture toxicology for classical chemicals (Schäfer et al., 2013). In this context, for instance, studies investigating the combined toxicity of $nTiO_2$ and the heavy metal copper showed different outcomes for the mobility of *Daphnia*. The $nTiO_2$ either enhanced (Fan et al., 2011) or mitigated (Rosenfeldt et al., 2014) the copper toxicity. Further environmental conditions may additionally affect interactions of nanoparticles and chemical stressors (Rosenfeldt et al., 2015). For instance, an UV-light irradiation of $nTiO_2$ and the carbamate Pirimicarb has shown to decrease the insecticide toxicity significantly compared to conditions of total darkness or absence of $nTiO_2$ (Seitz et al., 2012).

Thus, based on the findings of the present thesis and published data it seems sensible to revisit environmental risk assessment and adapt it to the special needs of nanoparticles, by considering, for instance, different characteristics and environmental conditions during their ecotoxicological testing. This would allow a more precise risk prediction of these novel stressors for aquatic life.

7. References

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Appendix

A.1: Size-, surface- and crystalline structure composition-related effects of titanium dioxide nanoparticles during their aquatic life cycle.

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A.2: Aging of TiO$_2$ nanoparticles transiently increases their toxicity to the pelagic microcrustacean *Daphnia magna*.

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A.3: Effects of nano-TiO$_2$ in combination with ambient UV-irradiation on a leaf shredding amphipod.

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A.4: Effects of silver nanoparticle properties, media pH and dissolved organic matter on toxicity to *Daphnia magna*.

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A.5: Curriculum vitae

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Appendix A.1

SIZE-, SURFACE- AND CRYSTALLINE STRUCTURE COMPOSITION-RELATED EFFECTS OF TITANIUM DIOXIDE NANOPARTICLES DURING THEIR AQUATIC LIFE CYCLE

Frank Seitz, Ricki R. Rosenfeldt, Sandra Schneider, Ralf Schulz, Mirco Bundschuh

Highlights

- nTiO$_2$ toxicity is triggered inter alia by its initial particle size and surface area

- Crystalline structure composition of nTiO$_2$ products affects its ecotoxicological potential

- Toxic potential of nTiO$_2$ decreases during its aquatic life cycle (=after sedimentation)

- nTiO$_2$ toxicity differs among representatives of different spatial and ecological niches

Abstract

Nanoparticle toxicity depends amongst others on particle characteristics and nanoparticle behavior during their aquatic life cycle. Aquatic organisms may be exposed to nanoparticle agglomerates of varying size, while lager agglomerates after settling rather affect benthic organisms. In this context, the present study systematically examined the role of particle characteristics, i.e. crystalline structure composition (anatase and mixture of anatase-rutile), initial particle size (55-, 100-, and 140-nm) and surface area, in the toxicity of titanium dioxide nanoparticles (nTiO$_2$) to the pelagic filter feeder *Daphnia magna* (n=4) and the benthic amphipod *Gammarus fossarum* (n=30). Smaller initial particles sizes (i.e. 55-nm) and anatase based particles showed an approximately 90% lower *Daphnia* EC$_{50}$-value compared to its respective counterpart. Most importantly, particle surface normalized EC$_{50}$-values significantly differed for nanoparticles equal to or below 100-nm in size from 140-nm sized particles. Hence, these data suggest that the reactive initial surface area may explain the ecotoxicological potential of different particle size classes only if their size is smaller or around 100 nm. In contrast to *Daphnia, Gammarus* was not affected by nTiO$_2$ concentrations of up to 5.00 mg/L, irrespective of their characteristics. This indicates fundamental differences in the toxicity of nTiO$_2$ during its aquatic life cycle mediated by alterations in their characteristics over time.

Keywords: *Daphnia magna*, *Gammarus fossarum*, crystallinity, toxicity, Crustacea

Introduction

The utilization of engineered nanoparticles is still increasing and expected to reach a $2.4 trillion contribution to the global economy by 2015 (Pearce, 2012). Amongst others titanium dioxide nanoparticles ($nTiO_2$) are heavily used as they have multiple advantageous properties (Fujishima et al., 2000; Schulz et al., 2002), making them a desirable additive for care-, remediation- and self-cleaning products (Di Paola et al., 2012; Kaegi et al., 2008; Sun et al., 2007). This frequent application at high quantities (Scheringer, 2008) inevitably results in $nTiO_2$-release into aquatic ecosystems for example through wastewater treatment plant effluents (Klaine et al., 2011; Westerhoff et al., 2011), wash off from facades (Kaegi et al., 2008) or major accidents during transport (Nowack et al., 2014).

In this context, scientists investigated the acute and chronic ecotoxicological potential of $nTiO_2$ on aquatic organisms mainly employing the standard test organism *Daphnia magna* (e.g. Dabrunz et al., 2011; Dalai et al., 2013). These studies exhibited median effective and lethal concentrations ranging from low mg/L to high g/L levels (cf. Dabrunz et al., 2011; Heinlaan et al., 2008). This broad range of $nTiO_2$ concentrations causing adverse effects among different studies is frequently attributed to varying particle properties such as initial particle size, surface area and crystalline structure composition, but was not yet empirically underpinned (cf. Dabrunz et al., 2011; Seitz et al., 2013). Moreover, once introduced into the aquatic environment, $nTiO_2$ start their aquatic life cycle being subjected to transformation processes that may have substantial implications on their fate and toxicity (Fig. 1). In this regard, the agglomeration of particles (triggered for instance by the ionic strength in the surface water (Petosa et al., 2010)) affect their sedimentation as previously

shown for instance by Dabrunz et al. (2011). This suggests that $nTiO_2$ pose initially,

and hence directly following their release into the aquatic ecosystem, a risk for

pelagic organisms such as daphnids (Dabrunz et al., 2011; Li et al., 2014a). At the

later stages of their aquatic life cycle, $nTiO_2$ will settle down as a result of

agglomeration processes potentially threatening benthic life (e.g. leaf shredding

amphipods) (Bundschuh et al., 2011b; Li et al., 2014b).
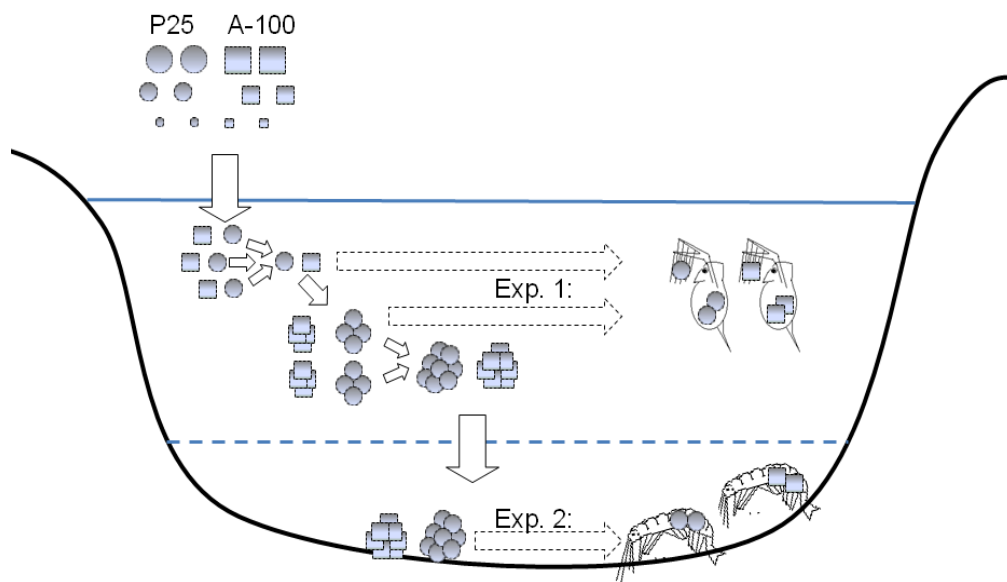
Figure 1: Experimental derivation based on the aquatic life cycle of $nTiO_2$ varying in initial size and crystalline composition (P25 and A-100). Experiment 1 covers potential particle characteristic and small agglomerate related effects of $nTiO_2$ towards pelagic living organisms at an early stage of nanoparticle life cycle. Experiment 2 focuses a later stage of the latter named and hence potential toxic effects on benthic organisms after nanoparticle agglomeration and sedimentation.

However, virtually nothing is known on how nanoparticles, in particular $nTiO_2$, with

differing initial characteristics (e.g. initial particle size, surface area and crystalline

structure composition) alter their ecotoxicological potential in the course of this

aquatic life cycle.

Therefore, the present study assessed the role of $nTiO_2$'s initial size, total initial surface area, and crystalline structure composition systematically on its toxicity to the pelagic filter feeder *D. magna* and the benthic leaf shredding amphipod *Gammarus fossarum*. The scenarios were achieved by applying the $nTiO_2$ products P25 and A-100, which differed in their crystalline structure composition either containing a mixture of anatase (70%) and rutile (30%) or exclusively anatase (99%), respectively, at three initial particle size classes each (55-, 100-, 140-nm), which were chosen based on published studies (Dabrunz et al., 2011). Both test species experienced similar static $nTiO_2$ exposure conditions. While daphnids were checked after 96 h for immobilization, for gammarids, a sublethal response in the species' feeding rate on leaf material was chosen as endpoint since it is robust, sensitive and ecologically meaningful (Maltby et al., 2002).

Materials and Methods

Nanoparticle characterization

Both titanium dioxide products were purchased as powders, either from Evonik (P25, Germany) or Crenox GmbH (A-100, Germany), featuring an advertised primary particle size of 21 nm and 6 nm, for P25 (~70% anatase and ~30% rutile) and A-100 (99% anatase), respectively. Their advertised surface area is approximately 50 (P25) and 230 m^2/g (A-100). In order to compensate for the differences in the respective advertised primary particle sizes among both materials for each product, dispersant and additive free, size homogenized, stable suspensions of three particle size classes (namely 55-, 100- and 140-nm) were obtained by stirred media milling (PML2, Bühler AG, Switzerland). Subsequently centrifugation was accomplished in order to remove residual coarse material. Prior to their application each stock suspension was analysed for its particle size distribution (intensity weighted) as well as its average initial particle surface area per volume (cf. Treuel et al., 2010) using dynamic light scattering (DelsaNano C, Beckman Coulter, Germany) and nanoparticle tracking analysis (LM20, NanoSight Ltd., United Kingdom), respectively (Tab. 1). Additionally, scanning electron microscope analyses were performed to verify the initial particle size of each applied $nTiO_2$ product (SI Fig. 1 A-F). Moreover, the average particle size in the test medium was monitored after 24 h and at test termination of all bioassays (Tab. 1). However for the particle size monitoring, 3-mL samples were taken 2 cm beneath the water surface (=middle of the water column) from the center of one randomly selected replicate of a 2 mg $nTiO_2$/L concentration (enabling a sufficient intensity) ensuring a reliable monitoring of $nTiO_2$ over the whole study duration.

Table 1: Mean particle size (±SD; n=3) and the respective polydispersity indices (=PI) of two different $nTiO_2$ products (P25 and A-100) with varying initial particle sizes (55-, 100- and 140-nm) applied to two different test media (ASTM-M and SAM-S5 medium) in the time course of 0, 24, 96 or 168 h. NA indicates invalid DLS measurements due to low scattered light intensities.

nominal initial particle size	ASTM medium							
	P25				A-100			
	measured size (nm)			PI	measured size (nm)			PI
	$t_{0\,h}$	$t_{24\,h}$	$t_{96\,h}$		$t_{0\,h}$	$t_{24\,h}$	$t_{96\,h}$	
55-nm	59.6 (± 1.9)	1576.3 (± 107.4)	NA	0.119 - 0.603	56.8 (± 3.7)	1947.8 (± 103.6)	NA	0.040 - 0.601
100-nm	95.4 (± 1.1)	1161.6 (±95.6)	NA	0.139 - 0.443	80.0 (± 3.5)	1397.8 (± 153.2)	NA	0.042 - 0.517
140-nm	145.2 (± 3.2)	1222.9 (± 59.3)	1461.1 (± 90.2)	0.133 - 0.509	126.9 (± 2.1)	855.4 (± 7.5)	NA	0.141 - 0.357
	SAM-S5 medium							
	$t_{0\,h}$	$t_{24\,h}$	$t_{168\,h}$		$t_{0\,h}$	$t_{24\,h}$	$t_{168\,h}$	
55-nm	59.6 (± 1.9)	946.8 (± 154.6)	NA	0.119 - 0.469	56.8 (± 3.7)	1016.1 (± 122.8)	NA	0.040 - 0.452
100-nm	95.4 (± 1.1)	522.8 (± 87.0)	NA	0.139 - 0.233	80.0 (± 3.5)	935.7 (± 150.9)	NA	0.042 - 0.480
140-nm	145.2 (± 3.2)	636.8 (± 83.2)	NA	0.133 - 0.331	126.9 (± 2.1)	360.6 (± 34.1)	NA	0.141 - 0.209

Test organisms

Daphnia magna (Eurofins-GAB, Germany) were kept in permanent culture within climate controlled chambers (Weiss Environmental technology Inc., Germany) with a 16:8 h (light:dark) photoperiod at 20±1°C. Organisms were cultured in groups of 25 animals using 1.5 L reconstituted hard freshwater (=ASTM-M) according to the ASTM International standard guide E729 (ASTM, 2007) enriched with selenium, vitamins (thiamine hydrochloride, cyanocobalamine, biotine) and seaweed extract (Marinure®, Glenside, Scotland; cf. Seitz et al., 2013). The medium was renewed three times a week, while daphnids were fed with the green algae *Desmodesmus* sp. on a daily basis with an equivalent of 200 µg C per organism.

Gammarus fossarum were obtained from the Hainbach (a near natural stream close to Landau, Germany; 49° 14' 19'' N, 8° 02' 59'' E) and stepwise acclimatized to reconstituted water (SAM-5S Borgmann et al., 1998) as well as given laboratory conditions. For toxicity testing only male gammarids (identified by their position in the precopular pair) with a cephalothorax diameter between 1.6 and 2.0 mm were used, whereas organisms were sorted using a passive underwater separation technique (Franke, 1977). During their acclimatization, gammarids were fed *ad libitum* with preconditioned black alder leafs (*Alnus glutinosa* L. Gaertn.).

Preparation of leaf discs

Leaf discs, which served as food during the feeding activity tests with *Gammarus*, were prepared similar to the method described by Bundschuh et al. (2011a). Briefly, senescent but undecomposed leaves of *A. glutinosa* were collected shortly before leaf fall in October 2011 from a pristine area close to Landau, Germany (49°33'N, 8°02'E). Leaves were frozen and stored at -20°C until further processing. After thawing, discs (2.0 cm diameter) were cut from leaves with a cork borer and subjected to a conditioning process using a nutrient medium (Dang et al., 2005) together with naturally inoculated alder leaves, previously exposed for two weeks in the Rodenbach (a near natural stream close to Mannheim, Germany; 49° 33' 59'' N, 8° 02' 33'' E). This procedure ensured the establishment of a near natural microbial community, *inter alia* bacteria and fungi, on the surface of the discs that enhances their palatability for shredding macroinvertebrates (Bärlocher, 1985). After a conditioning period of 10 d, leaf discs were dried at 60 °C to constant weight and weighed to the nearest 0.01 mg, which ensured a precise measurement of the amphipods' feeding rate (cf. Maltby et al., 2002) after the feeding activity tests. However, prior to the use of the leaf discs they were re-soaked for 48 h in SAM-5S to avoid floating on the surface of the test medium (Bundschuh et al., 2011b).

Toxicity testing

Experiment 1: acute toxicity tests with D. magna

During a series of acute toxicity tests, daphnids were exposed to P25 or A-100 of the particle size classes 55-, 100- and 140-nm (Tab. 1). Briefly, ASTM-M was used (see section *Test organisms*), while neither food nor seaweed extract was added. Each acute toxicity test was conducted according to the OECD guideline 202 (OECD, 2004), however, considering an elongated study duration of 96 h as proposed for nanoparticle testing (Dabrunz et al., 2011). Groups (n=4) of five juvenile daphnids (<24 h) were exposed to 0.0, 0.5, 1.0, 2.0, 4.0, 8.0 and 16.0 mg $nTiO_2$/L and checked for immobilization every 24 h. During an additional experiment (SI section 1) potential effects of $nTiO_2$ (P25 and A-100) on *Daphnia*'s molting behavior were investigated (SI Tab. 1). All tests were conducted under temperature and light conditions described in section *Test organism*.

Experiment 2: feeding activity tests with G. fossarum

Each of the six feeding activity bioassays carried out here was initiated by placing one specimen of *G. fossarum* together with two preconditioned and preweighed leaf discs in the respective $nTiO_2$ test solution. Therefore, 200-mL plastic beakers (n=30) were filled with 150 mL of SAM-5S and set to initial $nTiO_2$ concentrations, namely of 0.0, 0.1, 0.5, 1.0 and 5.0 mg $nTiO_2$/L, which were selected based on published data

(cf. Bundschuh et al., 2011b). Moreover, for each of the treatments, five further test vessels only containing SAM-5S and two preweighed leaf discs were set up in order to account and correct for microbial decomposition and handling losses of leaf material during the experiment. All experiments were conducted in total darkness at 20±1 °C, while each beaker was continuously aerated. After 7 d of exposure, all living animals, remaining leaf discs as well as any leaf tissue shredded off were removed and rinsed in distilled water. Afterwards, the leaf material was dried at 60 °C to constant weight and weighed to the nearest 0.01 mg. Finally, mortality, molting and the feeding rate were determined, whereas the latter was calculated as described in detail by Maltby et al. (2002).

Data analysis

In order to determine respective 96-h EC_{50}-values for $nTiO_2$, the immobilization data of each acute toxicity test with *Daphnia* was (if necessary) adjusted using Abbott's formula (correction for control mortality) and subsequently analyzed with adequate dose-response models (SI Fig. 2 A-F and 3 A-E), while considering Akaike's information criterion and visual expert judgment as quality control for model selection. Subsequently, gained EC_{50}-values were compared and assessed for statistically significant differences among particle sizes and crystalline structure via confidence interval (CI) testing (Wheeler et al., 2006). The respective surface normalized 96-h EC_{50}-values were similarly modeled, while the initially used independent variable "concentration" was replaced by a new variable, which resulted from a multiplication of the applied $nTiO_2$ stock solution volume and the respective

initial surface area gained by NTA measurements (see the section *Nanoparticle characterization*).

Feeding activity data of *Gammarus* was firstly checked for normal distribution and homoscedasticity by applying the Shapiro-Wilk and Bartlett's test, respectively. In case requirements for parametric testing were met, a one-way ANOVA was performed. If requirements were not met, a nonparametric alternative, namely the Kruskal-Wallis test, was accomplished. Finally, either Dunnett's (parametric) or pairwise Wilcoxon rank sum tests (nonparametric) were used as *post-hoc* analysis to assess for statistically significant differences ($p<0.05$) among the mean or median feeding rate of controls and the respective treatment groups. All tests were two sided, and if necessary, i.e. in case of multiple comparisons, Bonferroni adjusted (alpha-threshold). Statistical analysis and figures in the present study base on the statistics program R version 2.15.3 (2013) and the respective extension packages (Helms and Munzel, 2008; Lemon, 2010; Ritz and Streibig, 2005).

Results and Discussion

Experiment 1: acute toxicity tests with D. magna

The 96-h EC_{50} values for immobilized *D. magna* obtained from both $nTiO_2$ products with an initial particle size of approximately 100-nm (Fig. 2 A; SI Fig 2 A-F; SI Tab. 2) are well in line with earlier studies performed under similar conditions (e.g. Bundschuh et al., 2012; Dabrunz et al., 2011) indicating the reproducibility of test

results in our laboratory. Moreover, the data indicate meaningful implications on the test organisms' mobility, whereas the initial $nTiO_2$ size and crystalline structure composition played an important role in terms of effect thresholds (Fig. 2 A and 3 A). These effects may in general be attributed to a combination of $nTiO_2$ properties: $nTiO_2$ has the potential to bind to the outer surface of daphnids carapaces (=biological surface coating), which is assumed to disrupt molting and swimming behavior of *Daphnia* (cf. Dabrunz et al., 2011; Noss et al., 2013; SI Tab. 1). In addition, $nTiO_2$ can form reactive oxygen species (ROS), which cause oxidative stress (Kim et al., 2010). As at least small quantities of ROS may be released under the experimental conditions of the present study (cf. Seitz et al., 2012) this could be an additional trigger for the acute toxicity.

Irrespective of the mode of action responsible for the $nTiO_2$-induced toxicity, the EC_{50}-values from the present and some published studies deviate by several orders of magnitude (e.g. Heinlaan et al., 2008; Zhu et al., 2009). This inconsistency is frequently attributed to e.g. study design (including study duration and preparation of $nTiO_2$-stock suspensions prior to its application) but also particle size, surface area or crystalline structure composition of the product (cf. Dabrunz et al., 2011; Seitz et al., 2013; Zhu et al., 2009). However, a systematic assessment of their importance, especially of the three latter factors, is still missing.

The results of the present study suggest on the one hand, the initial particle size as one trigger of the $nTiO_2$ toxicity: $nTiO_2$ of the 55-nm particle size class displayed for both products with a factor of approximately seven (P25) and five (A-100) statistically significantly lower 96-h EC_{50}-values (Fig. 2 A) relative to their respective 140-nm sized counterparts. Additionally, the 100-nm $nTiO_2$ particles of both products were less toxic than the respective smallest size class – although only for P25 statistically

significant – and at the same time more toxic than the largest, i.e. 140-nm, $nTiO_2$ size class (Fig. 2 A). These results underpin findings of earlier rather initial studies by a more comprehensive assessment involving amongst others dose-response modeling: For instance, Dabrunz et al. (2011) observed an approximately two-fold difference in the acute toxicity of 100- and 200-nm sized A-100 for *Daphnia*. However, these findings are limited to one $nTiO_2$ product and one single concentration (2 mg/L). Nevertheless, the authors related the observed effects directly to the comparably higher surface area of smaller particles assuming a higher proportion of reactive sites attaching to the exoskeleton of *Daphnia,* which increased the probability of molting disruption (as also indicated in the present study (SI Tab. 1)) finally causing movement limitations (Noss et al., 2013), immobility and mortality. In addition, the potentially slightly elevated release of ROS (Seitz et al., 2012) as a result of a higher surface area reactivity of small $nTiO_2$ size classes may (at least partly) explain the observed difference in toxicity among particle size classes.

By normalizing $nTiO_2$ toxicity for *Daphnia* to the initial nanoparticle surface area, the present study addressed for the first time empirically the underlying hypothesis of the interpretation by Dabrunz et al. (2011), i.e. the nanoparticle initial surface area is the sole driver of toxicity. If this assumption is correct, initial surface area normalized 96-h EC_{50} values should be similar among particle size classes of the same $nTiO_2$ product. Indeed, small (55-nm) and medium (100-nm) sized $nTiO_2$ showed (irrespective of the crystalline structure composition) no meaningful deviation in the initial surface area normalized EC_{50} (Fig. 2 B; SI Fig 3. A-E). However the difference in initial surface area normalized EC_{50} of the small (55 and 100-nm) and large (140-nm) $nTiO_2$ particles of both products was, with a factor of five (A-100) or even higher (P25; no EC_{50} was definable due to too low immobilization in the highest treatment),

statistically significant (Fig. 2 B; SI Fig. 3 A-E). These data suggest for particles exhibiting a mean particles size of approximately 100-nm and below the initial surface area as one important factor explaining the ecotoxicity of different particle size classes. However, our results do not support this assumption if the particle size exceeds 100-nm – here: 140-nm. As the surface area measurements widely ignore the surface roughness of the material by assuming a spherical shape of particles (Treuel et al., 2010), the data presented in the present paper should be carefully interpreted. Nonetheless, these results strongly support an initial surface area dependent toxicity for nanoparticles equal to and below a particle size of 100 nm.
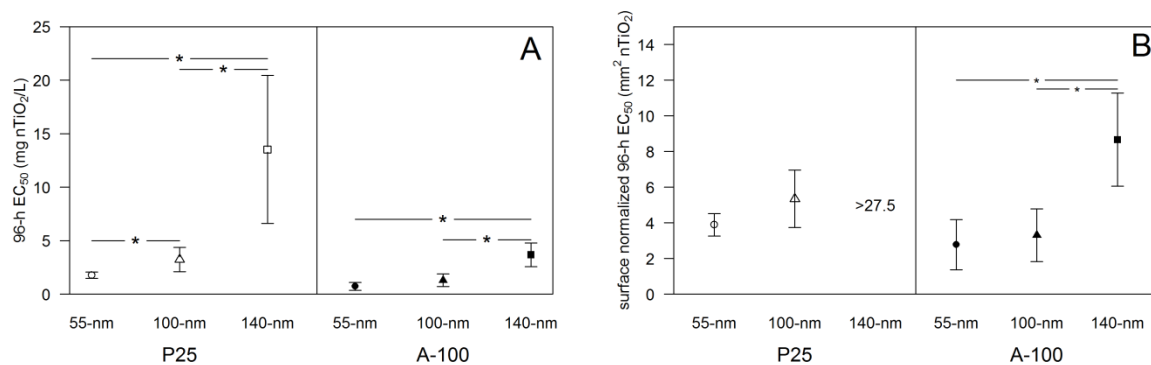
Figure 2: (A) 96-h EC_{50}-values with respective 95% CIs for the immobilization data of *D. magna* under either P25 or A-100 exposure. (B) Initial surface area normalized 96-h EC_{50}-values with respective 95% CIs for the immobilization data of *D. magna* under either P25 or A-100 exposure. Asterisks (*) denote statistically significant differences.

On the other hand the initial surface area may not represent a global trigger for the observed $nTiO_2$ toxicity. Hence, further factors such as size and number of individual particles might additionally contribute to the nanoparticle related ecotoxicity (cf. Warheit et al., 2007). In this context, especially the quantity of small particles might act as a very important factor driving the extent of ecotoxicity towards *Daphnia*. For instance, a review by Auffan et al. (2009) suggested that only nanoparticles smaller

than 30 nm are likely to be of ecotoxicological concern. This size fraction shows unique changes in particle properties as e.g. an enhanced interfacial reactivity due to notable changes in the crystalline structure of the particles (Auffan et al., 2009) potentially altering their toxicity. Indeed, own calculations analyzing the particle fraction ≤30 nm (SI Tab. 3) displayed much higher portions of either 17 (P25) or 8% (A-100) for the smallest initial size class of 55-nm if compared to the respective bigger size classes of 100- and 140-nm (in any case 0%). Thus, the nanoparticle fraction below ≤30 nm might have contributed to the observed effects of $nTiO_2$ with an initial size of 55-nm (Fig. 2 A). However, it is not able to explain either the differences between 100- and 140-nm sized $nTiO_2$ (Fig. 2 A) or between the two $nTiO_2$-products assessed (Fig. 3 A). Nevertheless, a high number of very small particles might significantly contribute to an enhanced uptake of reasonable particle amounts into more sensitive body areas or tissues. In this respect, a study of de Jong et al. (2008) displayed that intravenously injected gold nanoparticles of initially small particle size (10-nm) showed the most widespread organ distribution in the body of rats, when compared to bigger nanoparticles (50-, 100- and 250-nm). However, whether a similar explanation holds true for the present study can, due to the fundamental deviations in the experimental design, not reliably be concluded.

On the other hand, also the product itself and therefore the crystalline structure composition significantly influenced the $nTiO_2$ toxicity (Fig. 3 A). The $nTiO_2$ product P25 (containing a mixture of anatase (~70%) and rutile (~30%)) revealed 96-h EC_{50}-values indicating for each initial particles size class an up to four times lower toxicity than A-100 (Fig. 3 A). At the same time P25 was with 30% more toxic than an additionally tested rutile product (R050P, MKnano, Canada; see also SI section 2), although the latter was only assessed with a particle size of 100 nm (SI Tab. 2).

Such product dependent toxicity of nTiO$_2$, on the basis of 72-/96-h acute toxicity tests with daphnids, was also assumed by other authors (Bang et al., 2011; Clément et al., 2013), while their experimental approach did not allow for a separation of particle size and product composition effects. In contrast, the present study eliminated the confounding effect of (primary advertised) particle size allowing for the conclusion that the two products exhibiting a differing crystalline structure composition deviated up to four-fold in their ecotoxicological potential independent of the particle size class (see also Seitz et al., 2013).
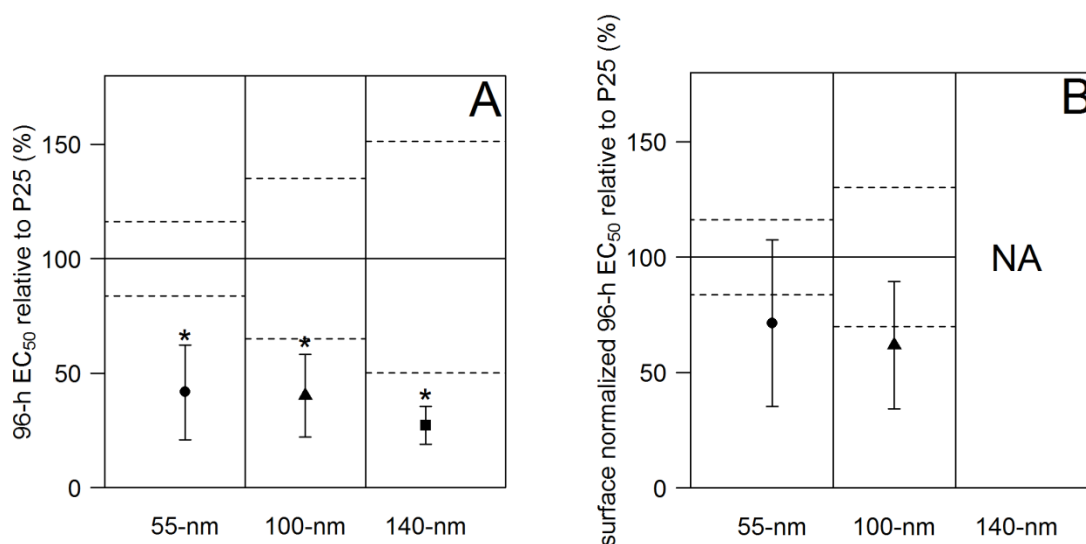
Figure 3: (A) percentage 96-h EC$_{50}$ values (with 95% CIs) for the immobilization data of *D. magna* whereas gained 96-h EC$_{50}$ values of A-100 were related to the respective 96-h EC$_{50}$-value of P25. (B) Percentage initial surface normalized 96-h EC$_{50}$-values (with 95% CIs) for the immobilization data of *D. magna* whereas gained 96-h EC$_{50}$ values of A-100 were related to the respective 96-h EC$_{50}$-value of P25. Continuous and dashed lines indicate reference 96-h EC$_{50}$-values and respective 95% CI of P25, while filled symbols indicate the relativized 96-h EC$_{50}$-values of A-100. NA = not assessed due to missing initial surface normalized 96-h EC$_{50}$ value for 140-nm sized P25. Asterisks (*) denote statistically significant differences.

The initial surface area normalized 96-h EC_{50} of A-100 (≤100-nm) showed always a lower value relative to the respective P25 particles, while these deviations were not statistically significant (Fig. 3 B). This observation may be explained by the higher surface reactivity (e.g. biological surface coating, ROS release) of anatase compared to rutile (Cong and Xu, 2012). Furthermore, the higher toxicity of A-100 relative to P25 observed in the present study (see also Seitz et al., 2013) may also be a result of the different properties of each crystalline structure of $nTiO_2$. Braydich-Stolle et al. (2009) explained the comparably lower toxicity of rutile, by its formation of ROS, which may be controlled by defense mechanisms such as the production of antioxidants. In contrast, anatase leads to membrane leakage, which could not be controlled for by the immune system of the cell line investigated. Analogously, Sayes et al. (2006) explained a lower cytotoxicity of rutile-anatase mixtures (60:40 and 20:80) compared to pure anatase by a higher photocatalytic potential of the latter, while the ecotoxicological potential of the $nTiO_2$ composition seems, as shown in the present study, largely driven by its initial surface area if the particles are smaller or around 100 nm in size.

Experiment 2: feeding activity tests with G. fossarum

None of the investigated endpoints revealed lethal (SI Tab. 4) or sublethal (Tab. 2) effects of $nTiO_2$ on *Gammarus*, while each experiment was conducted at least twice (data not shown). This contradicts earlier studies using the same experimental design and $nTiO_2$ product (P25, 100 nm) as stressor as well as *Gammarus* as test species (Bundschuh et al., 2011b). In this previous study gammarids' feeding rate was

reduced by about 40% already at 0.2 mg $nTiO_2$/L. However, this discrepancy between the present study and the study of Bundschuh et al. (2011b) may be explained by the substantial variation in sensitivity of *Gammarus* among seasons. Prato and Blandolino (2009) uncovered a ten-fold higher tolerance of *Gammarus aequicauda* if collected in autumn compared to spring, summer or winter, which was attributed to a higher lipid content in autumn. As the present study was performed in autumn and those conducted by Bundschuh et al. (2011b) took place during winter, a similar mechanism may hold for the deviation between both studies.

Table 2: Mean feeding rate (in percent relative to the respective control; ±SD) of *Gammarus* being exposed for 7 d to different concentrations of either nTiO$_2$ P25 or A-100, both exhibiting initial particle sizes of 55-, 100- or 140-nm.

initial particle size	P25										A-100									
	0.0 mg/L	n	0.1 mg/L	n	0.5 mg/L	n	1.0 mg/L	n	5.0 mg/L	n	0.0 mg/L	n	0.1 mg/L	n	0.5 mg/L	n	1.0 mg/L	n	5.0 mg/L	n
55-nm	100.00 (± 75.00)	25	83.33 (± 80.00)	26	87.50 (± 76.20)	19	104.17 (± 60.00)	24	100.00 (± 58.33)	25	100.00 (± 36.36)	26	109.10 (± 36.11)	26	90.91 (± 60.00)	22	93.94 (± 61.29)	27	93.94 (± 51.61)	27
100-nm	100.00 (± 25.81)	27	96.77 (± 31.67)	28	85.48 (± 26.42)	23	87.10 (± 31.48)	26	98.34 (± 39.34)	28	100.00 (± 52.17)	23	108.70 (± 56.00)	21	86.96 (± 65.00)	19	121.74 (± 57.14)	23	86.96 (± 80.00)	25
140-nm	100.00 (± 34.88)	29	97.67 (± 38.10)	30	67.44 (± 51.72)	22	102.33 (± 38.64)	29	104.65 (± 40.00)	28	100.00 (± 45.45)	25	87.88 (± 51.72)	22	96.97 (± 62.50)	19	124.24 (± 36.59)	27	100.00 (± 48.48)	25

The implication of the statistical tools used for the analyses of both studies may as well explain the results: A type II error (not detecting a statistically significant effect even though there is one) may have occurred in the present study. This however, seems rather unlikely as in none of the six experiments presented any tendency for adverse effects could be measured. It seems more likely that in the earlier study (Bundschuh et al., 2011b) a type I error (displaying a statistically significant effect although there is none) occurred. Hence, the present study probably uncovered falsely detected effects of P25 on *Gammarus* in an earlier study (Bundschuh et al., 2011b), which is also supported by Li et al. (2014b) who reported 96-h median lethal concentrations of 631 mg $nTiO_2$/L (P25, ~30 nm), a factor of 100 above the highest concentration tested in the present study, for another benthic amphipod, i.e. *Hyalella azteca*. Thus, the present study accentuates to seriously consider confounding biological as well as statistical implications during the choice of experimental designs. This however, is certainly not limited to nanoparticle related research.

Contrasting effects of $nTiO_2$ over its aquatic life cycle

The anticipated aquatic life cycle of $nTiO_2$, which covers introduction of nanoparticles into the surface water where they are subjected to agglomeration and sedimentation (e.g. Bundschuh et al., 2011b; Dabrunz et al., 2011; Kalčíková et al., 2014; Noss et al., 2013), calls for assessment of potential implications in pelagic and benthic species. A similar fate of $nTiO_2$ was observable in the present study: Regardless of the initial particle size, product and test system, $nTiO_2$ agglomerated within the first 24 h (>360 nm; Tab. 1). Moreover, at the termination of each experiment the particle

size was, due to low particle densities, not measureable. This in turn indicates a considerable sedimentation of $nTiO_2$, which is underpinned by a visually observable nanoparticle bottom layer and further by experiments conducted earlier in our laboratory (Noss et al., 2013).

During the outlined aquatic life cycle of $nTiO_2$ the exposure concentration decreases for pelagic and increases for benthic organisms. This, however, is not reflected by the ecotoxicological results for the species tested in the present study. Although *Gammarus* was shown to be more sensitive towards other chemical stressors, as for instance fungicides (Zubrod et al., 2014), the pelagic species *D. magna* seems (on the basis of the endpoints used in the present study) more sensitive than the benthic amphipod in the present study. This may be explained by the higher accessibility of the initially smaller particles for pelagic filter feeding organisms. In contrast, the deposited agglomerates are less bioavailable for pelagic organisms and seem to be (up to a concentration of 5.0 mg/L) uncritical for benthic species. On the other hand habitat specific adaptations of both species may have contributed to the observed differences. Pelagic organisms (here: from standing water bodies) mainly live in the water column, where they do usually experience exposure to micro- and macroscopic algae but not to relatively high quantities of e.g. natural colloids, inorganic fine particulate matter or suspended sediments (*sensu* Arruda et al., 1983; Levine et al., 2005). Benthic species (here: from running water bodies), however, live in and on the substratum (Statzner and Bittner, 1983) having potentially developed strategies to cope with such conditions, which may explain the higher sensitivity of daphnids. Furthermore, an interspecies variability in sensitivity is likely to be an important factor explaining the observed difference in toxicity among the tested species. In addition, some minor differences in the experimental design may explain the observed effects:

during the experiments with *Daphnia* the presences of visible light may have initiated the formation of ROS at a low but relatively continuous level (cf. Seitz et al., 2012). Since the feeding activity experiments with *G. fossarum* were performed in complete darkness this particular process could not have taken place.

Conclusion

The present study comprehensively assessed potential effects of $nTiO_2$ during its aquatic life cycle and thereby underpinned the importance of nanoparticle fate and characteristics, namely initial size, initial surface area and crystalline structure composition, for their ecotoxicological potential. In this context, the results indicate the importance of initial surface area as explanatory variable for the ecotoxicological potential of $nTiO_2$ towards *Daphnia*, particularly if the particles size is ≤100 nm. The findings for the tested particle sizes within this study further motivate the frequently applied size-threshold separating nanoparticles (defined as material with at least one dimension ≤100 nm) from bigger particles (The Royal Society & The Royal Academy of Engineering, 2004; Foss Hansen et al., 2007). Moreover, the presented data displayed a substantially lower sensitivity of *Gammarus* towards $nTiO_2$ relative to *Daphnia*, indicating the global need to identify critical exposure and effect pathways for nanoparticles. This in turn will allow selecting the most sensitive test species further supporting a reliable risk assessment of nanoparticles. Finally, the currently applied approach for toxicity testing of nanoparticles seems to be improvable as differences in specific particle properties of apparently similar materials are frequently ignored even though they are decisively involved in the ecotoxicological potential of the respective product.

Supporting Information

The supporting information contains further data on the characterization of P25, A100 and R050P. Additionally a table of exact 96-h EC_{50} values and associated model figures as well as molting data of *Daphnia* is provided. Furthermore, additional data on the mortality and molting of *Gammarus* is included.

Acknowledgements

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SUPPLEMENTARY MATERIAL

of Appendix A.1

SIZE-, SURFACE- AND CRYSTALLINE STRUCTURE COMPOSITION-RELATED EFFECTS OF TITANIUM DIOXIDE NANOPARTICLES DURING THEIR AQUATIC LIFE CYCLE

Frank Seitz, Ricki R. Rosenfeldt, Sandra Schneider, Ralf Schulz, Mirco Bundschuh
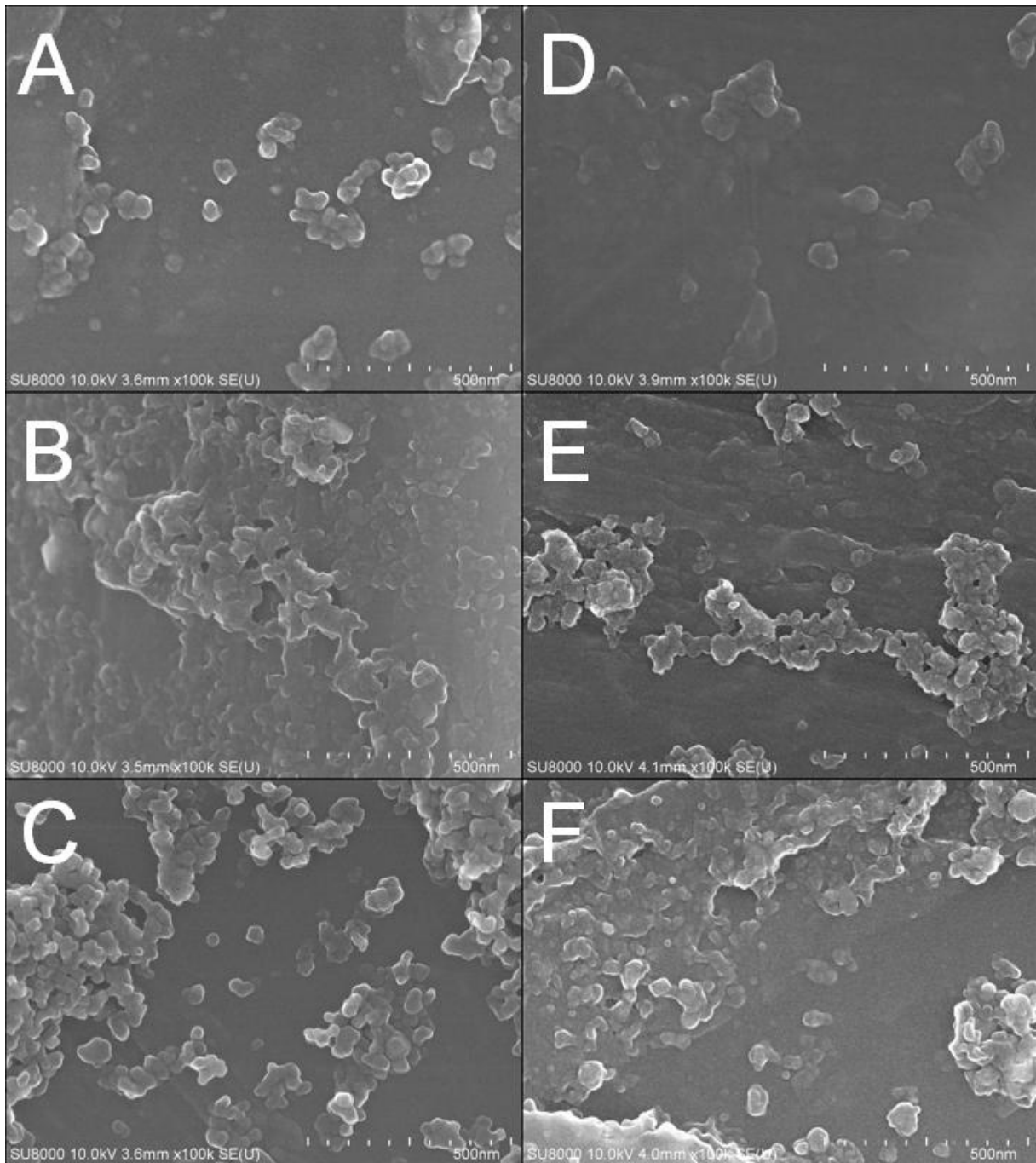
Figure S-1 A-F: Scanning electron microscopy images of all nTiO$_2$ stock suspension tested (Hitachi SU8030): Either (A) 55- (B) 100- and (C) 140-nm nominal sized P25 or (D) 55- (E) 100- and (F) 140-nm nominal sized A-100.
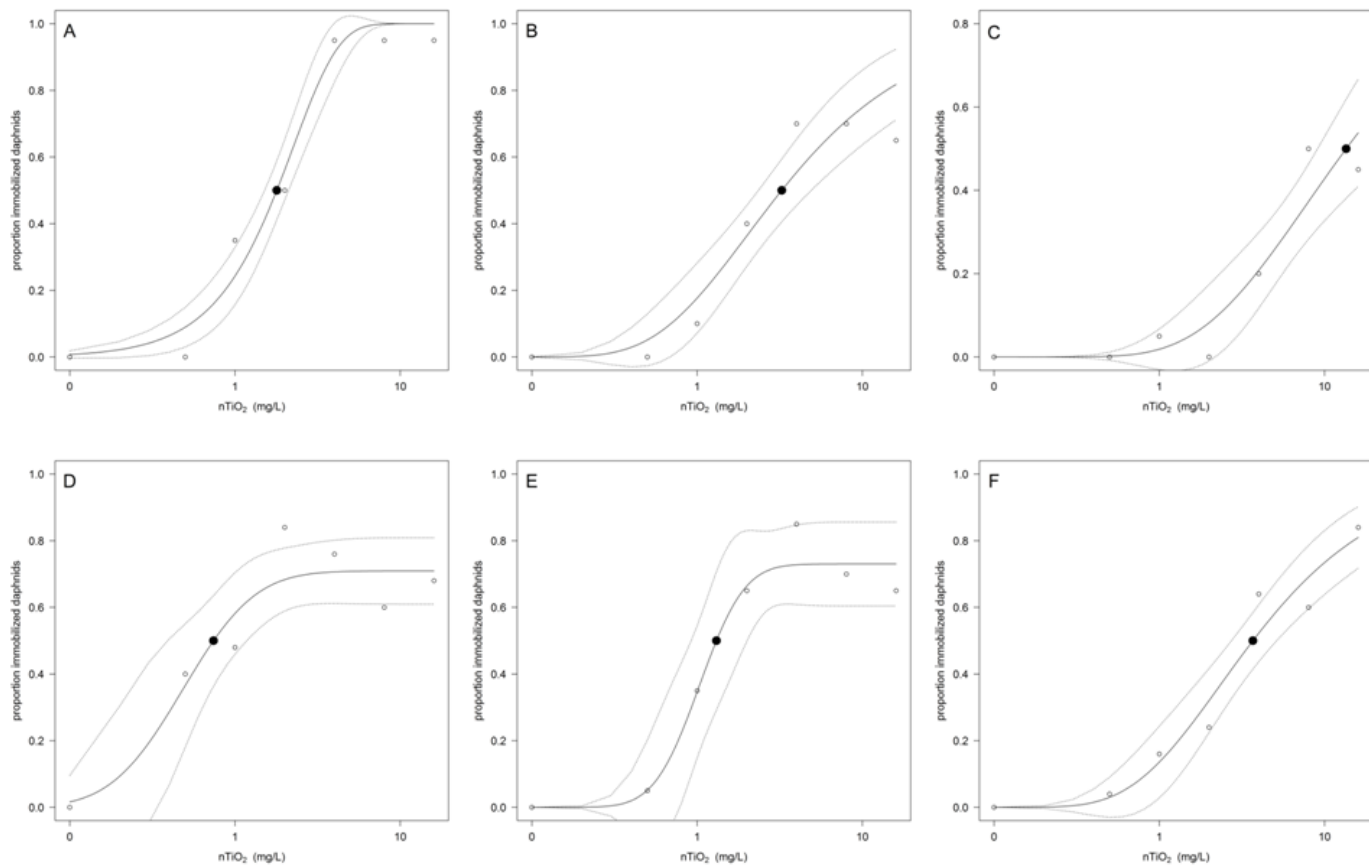
Figure S-2 A-F: Concentration (mg nTiO$_2$/L) dependent dose response curves and corresponding 96-h EC$_{50}$ values (●) with respective 95% CI (dashed line) for the immobilization data (○) of *Daphnia magna* either exposed to P25 (A-C) and A-100 (D-E) at different initial particle size classes of 55-, 100-, 140-nm, respectively.
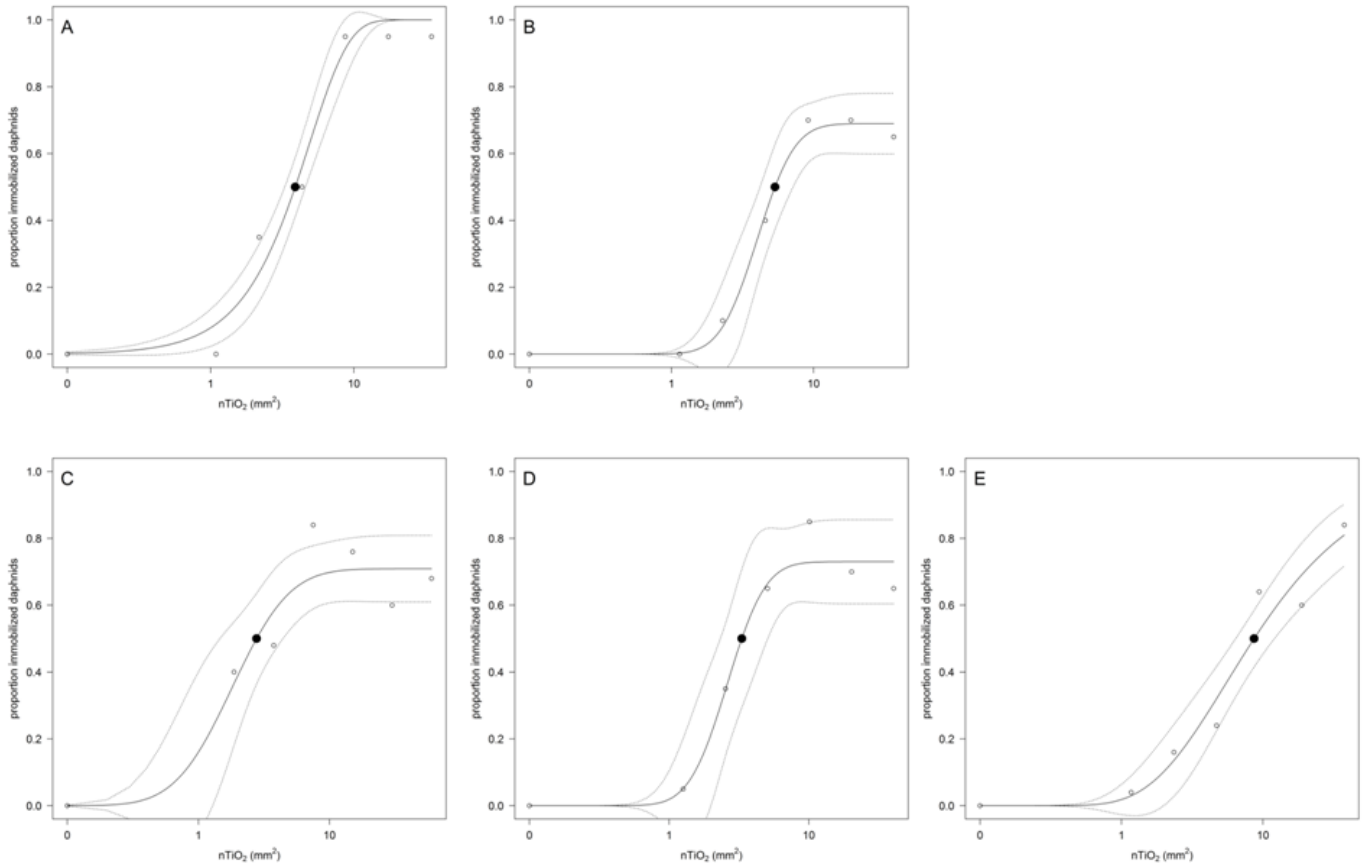
Figure S-3 A-E: Surface (mm^2 nTiO$_2$) dependent dose response curves and corresponding 96-h EC$_{50}$ values (●) with respective 95% CI (dashed line) for the immobilization data (○) of *D. magna* either exposed to P25 and A-100 at different initial particle size classes of (A) 55-nm and (B) 100-nm for P25 and (C) 55-nm (D) 100-nm and (E) 140 nm for A-100. For P25 (140-nm) no EC$_{50}$ could be calculated due to too low immobilization in the highest treatment.

Supplementary data Section S-1. Molting test with *Daphnia magna*

During the acute toxicity test (96 h) the molting behavior (frequency) of *Daphnia* was investigated. Therefore, groups of five juveniles (<12 h; n=23) were exposed to either 1.75 mg/L of P25 or A-100, while each product was applied at an initial particle size of 55-, 100- and 140-nm, respectively. As a consequence of pseudo replication, *Daphnia's* molting behavior was not perfectly relatable to their individual level. However the observed results are comparable to those gained during an earlier study in our laboratories (Dabrunz et al., 2011) considering the latter named difficulty.   The molting and immobility of *Daphnia* was recorded every 12 h, whereas the test was conducted under temperature and light conditions described in the manuscript.

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Dabrunz A, Duester L, Prasse C, Seitz F, Rosenfeldt R, Schilde C, et al. Biological surface coating and molting inhibition as mechanisms of TiO_2 nanoparticle toxicity in *Daphnia magna*. PLoS ONE 2011; 6: e20112.

Table S-1:   Percentage of second molting events and immobilization of *Daphnia magna* (n=23) after being exposed for 96 h to 0.00 (control) or 1.75 mg/L of different nTiO$_2$ products (P25 or A-100) exhibiting varying initial particle sizes (55-, 100-, 140-nm).

product	initial particle size (nm)	2nd molting (%)	immobilized (%)
control	-	50	0
P25	55	1	35
	100	8	14
	140	28	4
A100	55	0	42
	100	3	41
	140	47	3

Supplementary data Section S-2. Preparation and characterization of R050P

The nTiO$_2$ product R050P (99% rutile) was purchased as powder from MKnano Canada, exhibiting an advertised primary particle size of 50 nm. Similar to P25 and A-100, also for R050P a dispersant and additive free, size homogenized stable suspension (100-nm) was obtained using the method described in the material and method section. Also the product and sample preparation of R050P was carried out as described in the manuscript. However, for R050P only the initial particle size, i.e. at the start of the experiment, was assessed.

Table S-2: 96-h EC_{50}-values and respective 95% CIs of different $nTiO_2$ products (P25, A-100 and R050P) with varying initial particle size (55-, 100-, 140-nm) for *Daphnia magna*.

product	initial particle size (nm)	96-h EC_{50} (mg/L)
	55	1.79 (1.50 - 2.08)
P25	100	3.25 (2.11 - 4.39)
	140	13.52 (6.62 - 20.44)
	55	0.74 (0.38 - 1.11)
A-100	100	1.31 (0.72 - 1.89)
	140	3.69 (2.58 - 4.80)
R050P	100	4.36 (2.70 - 6.03)

Table S-3: Mean particle size (±SD; n=3) for the nTiO$_2$ products P25 and A-100 with nominal initial particle sizes of 55-, 100- and 140-nm. Displayed are the respective percentiles (10th, 50th and 90th) of their particle size distribution as well as the percentage of particles below or equal to a size of 30 nm.

nominal initial particle size	P25					A-100				
	measured initial size (nm)	10th percentile of the particle size distribution (nm)	50th percentile of the particle size distribution (nm)	90th percentile of the particle size distribution (nm)	percentage of particles ≤30 nm	measured initial size (nm)	10th percentile of the particle size distribution (nm)	50th percentile of the particle size distribution (nm)	90th percentile of the particle size distribution (nm)	percentage of particles ≤30 nm
55-nm	59.6 (± 1.9)	31.2 (± 8.2)	63.6 (± 1.1)	138.0 (± 35.6)	17.4	56.8 (± 3.7)	35.8 (± 4.7)	57.7 (± 3.2)	94.6 (± 6.3)	7.9
100-nm	95.4 (± 1.1)	59 (± 2.2)	99.4 (± 0.4)	169.0 (± 8.0)	0.0	80.0 (± 3.5)	51.6 (± 7.7)	79.7 (± 1.6)	117.6 (± 7.5)	0.0
140-nm	145.2 (± 3.2)	87 (± 4.9)	150.8 (± 1.6)	264.3 (± 7.9)	0.0	126.9 (± 2.1)	75.7 (± 3.5)	132.7 (± 1.6)	236.4 (± 7.9)	0.0

Table S-4: Percentage molting and mortality events of *Gammarus fossarum* during 7-d feeding activity tests with two different $nTiO_2$ products (P25 and A-100) of varying primary particle size (55-, 100-, 140-nm).

product	initial particle size (nm)	conc. (mg/L)	molts (%)	mortality (%)	n
		0.00	13.79	3.33	29
		0.10	14.29	6.67	28
	55	0.50	20.69	3.33	29
		1.00	13.33	0.00	30
		5.00	27.59	3.33	29
		0.00	20.00	0.00	30
		0.10	16.67	0.00	30
P25	100	0.50	16.67	0.00	30
		1.00	13.33	0.00	30
		5.00	23.33	0.00	30
		0.00	40.00	0.00	30
		0.10	35.71	6.67	28
	140	0.50	26.67	0.00	30
		1.00	34.48	3.33	29
		5.00	20.69	3.33	29
		0.00	16.67	0.00	30
		0.10	17.24	3.33	29
	55	0.50	10.34	3.33	29
		1.00	13.79	3.33	29
		5.00	3.45	3.33	29
		0.00	24.14	3.33	29
		0.10	30.00	0.00	30
A-100	100	0.50	23.33	0.00	30
		1.00	20.69	3.33	29
		5.00	20.00	0.00	30
		0.00	18.52	10.00	27
		0.10	15.38	13.33	26
	140	0.50	8.00	16.67	25
		1.00	10.00	0.00	30
		5.00	18.52	10.00	27

Appendix A.2

AGING OF TIO$_2$ NANOPARTICLES TRANSIENTLY INCREASES THEIR TOXICTY TO THE PELAGIC MICROCRUSTACEAN *DAPHNIA MAGNA*

Frank Seitz, Simon Lüderwald, Ricki R. Rosenfeldt, Ralf Schulz, Mirco Bundschuh

Abstract

During their aquatic life cycle, nanoparticles are subject to environmentally driven surface modifications (e.g. agglomeration or coating) associated with aging. Although the ecotoxicological potential of nanoparticles might be affected by these processes, only limited information about the potential impact of aging is available. In this context, the present study investigated acute (96 h) and chronic (21 d) implications of systematically aged titanium dioxide nanoparticles ($nTiO_2$; ~90 nm) on the standard test species *Daphnia magna* by following the respective test guidelines. The $nTiO_2$ were aged for 0, 1, 3 and 6 d in media with varying ionic strengths (Milli-Q water: approx. 0.00 mmol/L and ASTM: 9.25 mmol/L) in the presence or absence of natural organic matter (NOM). Irrespective of the other parameters, aging in Milli-Q did not change the acute toxicity relative to an unaged control. In contrast, 6 d aged $nTiO_2$ in ASTM without NOM caused a fourfold decreased acute toxicity. Relative to the 0 d aged particles, $nTiO_2$ aged for 1 and 3 d in ASTM with NOM, which is the most environmentally-relevant setup used here, significantly increased acute toxicity (by approximately 30%), while a toxicity reduction (60%) was observed for 6 d aged $nTiO_2$. Comparable patterns were observed during the chronic experiments. A likely explanation for this phenomenon is that the aging of $nTiO_2$ increases the particle size at the start of the experiment or the time of the water exchange from <100 nm to approximately 500 nm, which is the optimal size range to be taken up by filter feeding *D. magna*. If subjected to further agglomeration, larger $nTiO_2$ particles, however, cannot be retained by the daphnids' filter apparatus ultimately reducing their ecotoxicological potential. This non-linear pattern of increasing and decreasing $nTiO_2$ related toxicity over the aging duration, highlights the knowledge gap regarding the

underlying mechanisms and processes. This understanding seems, however, fundamental to predict the risks of nanoparticles in the field.

Introduction

The enormous production of engineered nanoparticles is suggested to contribute trillions of dollars to the global economy [1]. This high production comes along their increasing use [2], which inescapably leads to their release into aquatic ecosystems via, for instance, wastewater treatment plant effluents [3]. On their way into as well as within aquatic ecosystems, nanoparticles are subject to environmentally driven modifications of their surface characteristics (e.g. size, surface area or charge) over time (=aging), which potentially alter their fate and toxicity. In this context, aging can include agglomeration and also coating of the particles' surface with omnipresent natural organic matter (NOM) [4]. These processes are, for instance, triggered by the ionic strength and the quantity of NOM in the medium. In detail, high cation levels (high ionic strength) increase agglomeration speed [5], whereas NOMs stabilize or even disagglomerate particles [6] by inducing electrostatic repulsion [7].

Although these modifications are inevitable during the aquatic life cycle of such engineered nanoparticles, the resulting modification of their ecotoxicological potential is largely unknown. Only a few studies have documented the acute [8,9] or chronic [10] effects of aged nanoparticles, and the single chronic experiment considered only one particular aging condition [10], hampering extrapolation of the findings. To overcome this limitation, the present study systematically varied the properties of the medium during the aging of the nanoparticles, prior to testing their acute and chronic ecotoxicological potential. In the context of the present study, the standard test

organism *Daphnia magna* was used as model species to assess for both acute and chronic effects, while titanium dioxide nanoparticles (nTiO$_2$, ~90 nm, ~99% anatase) served as model nanoparticles. This selection was motivated by (i) their frequent application in various products [2], (ii) the relatively good characterization of the potential effects on aquatic life (in particular *D. magna*) in an unaged form [e.g. 11,12] as well as (iii) their potential to cause adverse effects in aquatic organisms at environmentally relevant concentrations [13].

It was hypothesized that higher ionic strength [14], which is considered as representative for natural freshwater environments, and the presence of a environmentally relevant NOM level [6,15] during aging as well as the longer duration of aging may decrease the nanoparticle-induced ecotoxicity to *D. magna*. Therefore, nTiO$_2$ were aged for 0, 1, 3 and 6 d in media with varying ionic strength (Milli-Q water: approx. 0.00 mmol/L and ASTM: 9.25 mmol/L) with and without NOM and subsequently assessed for their acute toxicity (immobility). Based on these data, four scenarios were selected for the chronic studies, which showed particularly strong alterations in ecotoxicity. Accordingly, nTiO$_2$ were aged for 0 or 3 d in ASTM with or without NOM prior to the evaluation of their respective chronic effects (reproduction, mortality) in daphnids. Both experimental setups were supplemented by particle size characterization before (acute toxicity tests) but also during the exposure (chronic) period.

Material and Methods

nTiO₂ preparation and characterization

The titanium dioxide product A-100 (99% anatase) was provided as powder by Crenox GmbH (Germany), exhibiting an advertised primary particle size of 6 nm and a surface area of approximately 230 m^2/g. Using this powder, a dispersant and additive free, size homogenized, stable suspension of ~90 nm was obtained by stirred media milling (PML 2, Bühler AG, Switzerland), and the resulting suspension was subsequently centrifuged (7500 rpm, ~20°C; Universal 320, Hettich, UK) in order to remove residual coarse material. Prior to its application the stock suspension (2 g nTiO₂/L) was analysed for its particle size distribution (intensity weighted) using dynamic light scattering (n=3 á 60 measurements; temperature: 20°C; pinhole: 100 µm; DelsaNano C, Beckman Coulter, Germany), which revealed a mean particle size of 87±1 nm (polydispersitiy index: 0.10-0.26). Moreover, before the start of each acute toxicity test and thus directly after (max. 3 min) the nTiO₂ aging process (0, 1, 3, 6 d; Table 1) the mean initial particle size (particle size at the start of the experiment) was also determined in the respective aging medium. For all chronic studies the particle size was additionally monitored during the bioassay, on three consecutive days (representative of the time interval between the two water exchanges; Table 2). In order to exclude any measurement bias (e.g. a shifted particle size distribution induced by algal food, animal excrements), 3-mL samples were taken from one additional replicate without daphnids of a 2.00 mg nTiO₂/L concentration at the center of the water column. Further, samples of test medium with NOM but without nTiO₂ were analyzed to determine any background signals, which were not quantifiable. Moreover, during additional experiments the concentrations of

aged nTiO$_2$ were measured in the 4.00 mg/L treatment after 0 h and 96 h, representative of the start and the end of each acute toxicity test. For this purpose, inductively coupled plasma mass spectrometry (Table 3) was used according to methods described in detail by Rosenfeldt et al. (2014). As our chemical analysis revealed no substantial differences relative to the nominal concentrations, the present study is based on the nominal TiO$_2$ concentrations exclusively.

Table 1: $nTiO_2$ size after aging. Mean initial particle size (± SD; n=3) of $nTiO_2$ aged for 0, 1, 3 and 6 d in different aging media with and without NOM (8 mg TOC/L), prior to its application in the respective acute toxicity test.

Aging medium	Acute toxicity tests							
	0 d		1 d		3 d		6 d	
	Initial particle size	PI[a]	Initial particle size	PI[a]	Initial particle size	PI[a]	Initial particle size	PI[a]
Milli-Q without NOM	82 (± 2)	0.11-0.23	81 (± 1)	0.13-0.19	82 (± 1)	0.16-0.17	81 (± 1)	0.16-0.17
Milli-Q with NOM	83 (± 1)	0.16-0.20	84 (± 1)	0.12-0.19	85 (± 1)	0.13-0.18	84 (± 1)	0.18-0.20
ASTM without NOM	1593 (± 53)	0.46-0.50	3712 (± 223)	0.89-1.00	2921 (± 103)	0.68-0.83	2530 (± 28)	0.67-0.74
ASTM with NOM	576 (± 11)	0.25-0.27	587 (± 27)	0.27-0.36	548 (± 11)	0.24-0.30	571 (± 10)	0.24-0.27

[a]Polydispersity index

Table 2: Particle size during chronic toxicity tests. Mean particle size (± SD; n=3) of aged $nTiO_2$ (0 or 3 d) measured over 3 consecutive days (representative for the time between a water exchange) in the respective aging-/test medium, namely ASTM with and without NOM (8 mg TOC/L), during all chronic experiments.

Aging conditions		Chronic toxicity tests					
		0 d		1 d		2 d	
Aging medium	Aging duration (d)	Initial particle size	PI[a]	Initial particle size	PI[a]	Initial particle size	PI[a]
ASTM without NOM	0	1334 (± 73)	0.30-0.41	1448 (± 90)	0.43-0.58	1453 (± 89)	0.48-0.60
ASTM without NOM	3	5567 (± 709)	0,74-1.00	2992 (± 357)	0.42-1.00	2583 (± 380)	0.38-1.00
ASTM with NOM	0	181 (± 68)	0.12-0.36	221 (± 46)	0.11-0.28	116 (± 1)	0.15-0.25
ASTM with NOM	3	498 (± 36)	0.26-0.30	351 (± 21)	0.20-0.32	311 (± 14)	0.21-0.28

[a]Polydispersity index

Table 3: Measured concentrations of nTiO2. Nominal and mean measured (± SD; n=3) nTiO$_2$ concentrations after 0, 1, 3 and 6 d aging in the respective aging medium, namely ASTM with and without NOM (8 mg TOC/L).

Aging medium	Aging duration (d)	Nominal concentration	Mean measured concentration (±SD; mg/L)	
			Test start 0 h	Test end 96 h
Milli-Q without NOM	0	4.00	3.82 ± 0.05	0.04 ± 0.00
	1	4.00	3.80 ± 0.07	0.04 ± 0.00
	3	4.00	4.02 ± 0.08	0.06 ± 0.00
	6	4.00	3.90 ± 0.24	0.04 ± 0.01
Milli-Q with NOM	0	4.00	3.71 ± 0.04	0.04 ± 0.01
	1	4.00	3.80 ± 0.04	0.05 ± 0.00
	3	4.00	3.80 ± 0.03	0.14 ± 0.01
	6	4.00	3.61 ± 0.05	0.05 ± 0.00
ASTM without NOM	0	4.00	3.57 ± 0.07	0.05 ± 0.00
	1	4.00	3.56 ± 0.07	0.05 ± 0.00
	3	4.00	3.57 ± 0.05	0.09 ± 0.00
	6	4.00	3.43 ± 0.06	0.05 ± 0.00
ASTM with NOM	0	4.00	3.59 ± 0.06	2.59 ± 0.04
	1	4.00	3.60 ± 0.04	3.28 ± 0.05
	3	4.00	3.54 ± 0.05	3.41 ± 0.06
	6	4.00	3.42 ± 0.02	3.21 ± 0.06

Test organism

Daphnia magna (Eurofins-GAB, Germany) were kept in permanent culture within a climate controlled chamber (Weiss Environmental Technology Inc., Germany) at 20±1°C with a 16:8 h (light:dark) photoperiod (visible light intensity, 3.14 W/m^2; UVA, 0.109 W/m^2; UVB, 0.01 W/m^2). Thereby, groups of 25 organisms were cultured in 1.5 L of reconstituted hard freshwater (=ASTM) according to the ASTM International standard guide E729 [17]. The medium was additionally enriched with selenium, vitamins (thiamine hydrochloride, cyanocobalamine, biotine) and seaweed extract (Marinure®, Glenside, Scotland; cf. [18]) and was renewed three times a week.

Animals were fed on a daily basis with the green algae *Desmodesmus* sp. (200 µg C per organism).

nTiO₂ aging process

Prior to the start of any bioassay, nTiO$_2$ were aged for different time intervals, i.e., 0, 1, 3 or 6 d (acute toxicity tests) and 0 or 3 d (chronic toxicity tests). Either Milli-Q water (solely used for the nTiO$_2$ aging prior to the acute toxicity tests) or ASTM (as part of both: acute and chronic toxicity tests) was used as aging medium. The first represents a medium without ions (Milli-Q; nominally: 0.00 mmol/L) and the second a comparably high ionic strength (ASTM; nominally: 9.25 mmol/L; Table S1). Additionally, for both media, the absence and presence of NOM was obtained using seaweed extract addition (cp. section: test organism) at concentrations of 0.0 or 8.0 mg TOC/L. The selection of this organic matter was based on (i) its recommendation as a medium additive during chronic metal toxicity tests with *Daphnia* [19,20] and (ii) its relatively balanced composition in terms of chromomorphic dissolved organic carbon, which is also representative for NOM released from waste water treatment plants (Table S2). The aging of nTiO$_2$ in Milli-Q (with and without NOM) took place in 15 mL centrifuge vials with a nominal concentration of 1.00 g nTiO$_2$/L. In contrast, the nTiO$_2$ (nominal concentrations: 0.00 - 128.00 mg nTiO$_2$/L) aging in ASTM with and without NOM was accomplished in a 500 mL glass vessel. Independent of the medium, each aging process was performed in total darkness (excluding photoactivation of nTiO$_2$ to avoid the oxidation of NOM during aging) on a horizontal shaker (50 rpm; VKS-B-50, Edmund Bühler GmbH, Germany). Prior to toxicity testing

all aged and unaged suspensions were vortexed for 30 seconds ensuring a homogenous distribution of $nTiO_2$ (Table 3).

Acute toxicity tests

During all acute toxicity tests, groups (n=4) of five juvenile (<24 h) daphnids each were exposed for 96 h to different concentrations of 0, 1, 3 or 6 d aged $nTiO_2$. Each acute toxicity test was conducted according to the OECD guideline 202 [21], during which daphnids were checked for immobilization every 24 h. In a first experiment ($nTiO_2$ aged in Milli-Q with and without NOM), measured amounts of the aged and unaged $nTiO_2$ stock suspension were added to ASTM (without NOM), resulting in a series of nominal $nTiO_2$ concentrations with 0.0 (=control), 0.5, 1.0, 2.0, 4.0, 8.0 and 16.0 mg/L. Subsequently, daphnids were carefully transferred to 50 mL of each treatment. In contrast, for experiments with $nTiO_2$ aged in ASTM with and without NOM, juvenile daphnids were directly placed in the aged and unaged $nTiO_2$ ASTM suspensions, which were evenly distributed in 50 mL volumes (using concentrations from 0.00 (=control) to 128.00 mg $nTiO_2$/L). All acute toxicity tests were conducted at 20±1°C with a 16:8 h (light:dark) photoperiod (visible light intensity, 3.14 W/m^2; UVA, 0.109 W/m^2; UVB, 0.01 W/m^2).

Chronic toxicity tests

Each chronic toxicity test was conducted according to the OECD guideline 211 [19]. Briefly, during all chronic toxicity tests, daphnids were exposed for 21 d to different nominal concentrations (i.e., 0.00 (=control), 0.02, 0.06, 0.20, 0.60, 2.00 or 6.00 mg/L) of 0 or 3 d aged $nTiO_2$ using ASTM with and without NOM as aging medium. The aging duration was selected based on the outcome of the acute experiments, where $nTiO_2$ aging for 3 d (ASTM with NOM) displayed an increased toxicity compared to unaged (0 d) $nTiO_2$ in the presence of NOM. In detail, ten daphnids (<24 h) were individually placed in 50 mL of aged and unaged $nTiO_2$ and fed daily with *Desmodesmus* sp. (from 50 to 100 µg C/organism with increasing age). Dead animals as well as released offspring were counted and removed daily. The test medium was renewed three times a week, while adult daphnids were carefully transferred using plastic pipettes. Dissolved oxygen (median: 7.8 mg/L) and pH (median: 8.2) fulfilled the requirements of the guideline [19] (Table S3). Each chronic toxicity test was – similar to the acute toxicity tests – conducted at 20±1°C with a 16:8 h (light:dark) photoperiod (visible light intensity, 3.14 W/m^2; UVA, 0.109 W/m^2; UVB, 0.01 W/m^2).

Statistical analysis

Acute toxicity of differently aged $nTiO_2$ suspensions was analyzed for the respective 96-h EC_{50} values (concentration at which half of the tested organisms are affected). Therefore, immobilization data of each acute toxicity test was corrected for control mortality (never exceeding 20%) using Abbott's formula. Subsequently, adequate dose-response models were fitted to these data (Figure S1-4). The model selection was based on Akaike's information criterion and expert judgment (Table S4). Finally, EC_{50} values were assessed for statistically significant differences among aging conditions using confidence interval testing [22].

For each chronic reproduction test, the cumulative mean offspring (after 21 d) was calculated separately (considering each treatment and aging process). Afterwards, differences in effect sizes (for the control and the highest $nTiO_2$ concentrations, respectively; i.e., 2.00 and 6.00 mg $nTiO_2$/L) among the different aging processes were statistically compared also using confidence interval testing [23]. Higher numbers indicate a higher effect size in terms of a decreased cumulative reproduction relative to the control. Additionally, a time to event analysis was accomplished by separately applying the Kaplan-Meier estimator for the data of each aging condition at the highest $nTiO_2$ concentrations (2.00 or 6.00 mg/L). For all statistical analyses and figures the statistical software environment R for Windows [24] and corresponding packages [25,26,27] were used.

Results

Toxicity of aged and unaged nTiO$_2$: absence of NOM

We detected similar 96-h EC$_{50}$ outcomes (0.9 – 1.4 mg nTiO$_2$/L; Figure 1A) when using Milli-Q without NOM as an aging medium, independent of the applied aging duration. Aging of nTiO$_2$ in ASTM without NOM revealed a lower ecotoxicity compared to Milli-Q without NOM (up to 7.5-fold; Figure 1A and 1B).

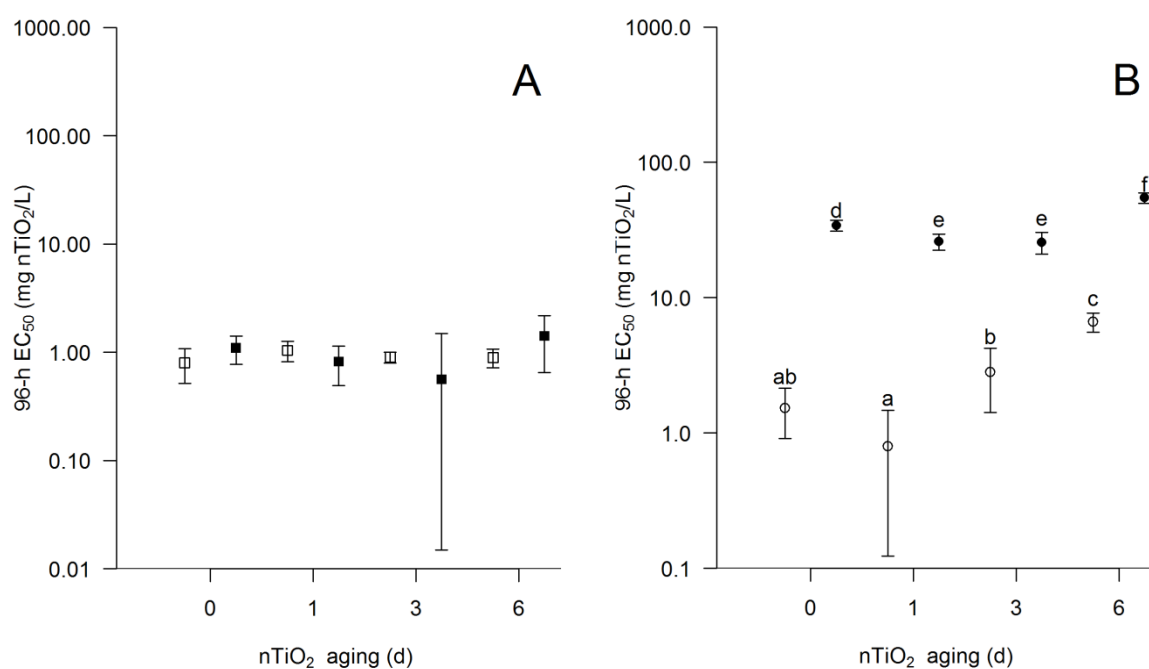Figure 1:   Acute toxicity of aged nTiO$_2$. (A) 96-h EC$_{50}$ values (half maximal effective concentration ; ± 95% CI) of nTiO$_2$ aged for 0, 1, 3 or 6 d in Milli-Q with (■) or without (□) NOM. (B) 96-h EC$_{50}$ values (± 95% CI) of nTiO$_2$ previously aged for 0, 1, 3 or 6 d in ASTM with (●) and without (○) NOM. 96-h EC$_{50}$ values followed by different lower case letters are significantly different.

Further, the nTiO$_2$ toxicity decisively dropped for a 3 and 6 d aging (~1.7 and ~4-fold; Figure 1B; see also supporting information Figure S5), when compared to the 0 d aging in ASTM without NOM. Similarly, 3 d aging of nTiO$_2$ in ASTM without NOM reduced the chronic toxicity. In detail, 2.00 mg/L of 0 d aged nTiO$_2$ caused 100% mortality in daphnids after six days of exposure (Figure 2) and thus there was no reproductive outcome. In contrast, at the same concentration of nTiO$_2$ but aged for 3 d the mortality dropped to only 50% at the termination of the experiment (see supporting information Figure S6) accompanied by an approximately 60% reduced fecundity compared to the respective control (Figure 3A).
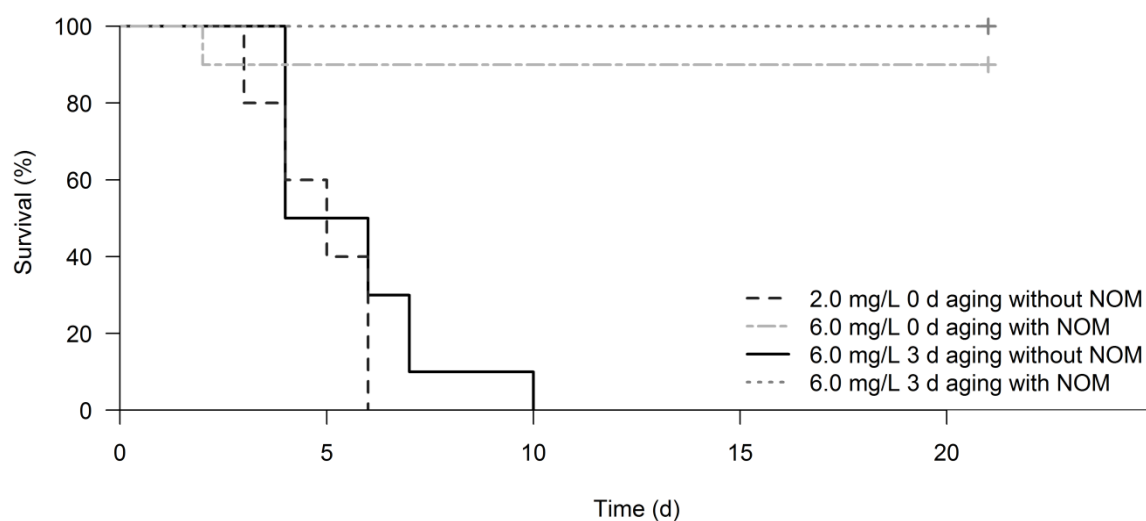

Figure 2: Survival time analysis during chronic exposure. Survival (%) of daphnids during the 21 d chronic toxicity tests with nTiO$_2$. Lines represent the exposure to nTiO$_2$ (i.e. 2.0 or 6.0 mg/L) aged under varying conditions (0 or 3 d) in ASTM with and without NOM.

When aging nTiO$_2$ in Milli-Q with NOM, no statistical significant change in the acute toxicity became evident relative to the unaged control and the aging in Milli-Q without NOM (Figure 1A). In contrast, a 0, 1, 3 or 6 d aging of nTiO$_2$ in ASTM with NOM, revealed a significantly lower acute toxicity (8 to 33-fold; Figure 1B, see also supporting information Figure S5) when compared to the aging in ASTM without NOM. This reduction in toxicity was also observed during the chronic experiment (ASTM with and without NOM). For instance, a 0 and 3 d aging of 2.00 and 6.00 mg/L nTiO$_2$ in ASTM without NOM displayed 100% mortality of daphnids after 21 days (see supporting information Figure S6A and B), whereas the aging in presence of NOM led to a reduced mortality, which is – depending on the nTiO$_2$ concentration – equal to or below 10% (Figure 2). Moreover, while the cumulative reproduction of *Daphnia* was significantly reduced (~60%; Bonferroni adjusted pairwise Wilcoxon rank sum test: p$_{=0.027}$) at 2.00 mg/L of 3 d aged nTiO$_2$ in ASTM without NOM when compared to the respective control, no reproductive implications became evident for the same concentration using ASTM with NOM as aging medium (Bonferroni adjusted pairwise Wilcoxon rank sum test: p=1; Figure 3A).

When aged in ASTM with NOM, the acute toxicity of nTiO$_2$ displayed a nonlinear pattern: relatively short aging durations of 1 or 3 d increased the acute toxicity of nTiO$_2$ by up to 27% relative to the 0 d scenario (Figure 1B), whereas an extension of the aging duration to 6 d significantly reduced the acute nTiO$_2$ toxicity (~two-fold) relative to all other scenarios (Figure 1B). In accordance with the acute toxicity data, chronic toxicity also increased for 3 d aged nTiO$_2$ in ASTM with NOM (Figure 3A and B). In detail, an approximately 50% decline in reproduction of *Daphnia* was observed

at 6.00 mg $nTiO_2$/L aged for 3 d compared to the respective control (Figure 3B; see supporting information Figure S7D, Bonferroni adjusted pairwise Wilcoxon rank sum test; $p=0.003$). In contrast, the same concentration of 0 d aged $nTiO_2$ did not affect the animals' reproduction significantly (Figure 3B; see supporting information Figure S7C; Dunnett test: $p=0.554$). Moreover, a direct comparison of both scenarios (i.e. 0 d aging vs 3 d aging) revealed a 60% higher effect size for a 3 d aging (confidence interval testing: $p<0.05$; Figure 3B).
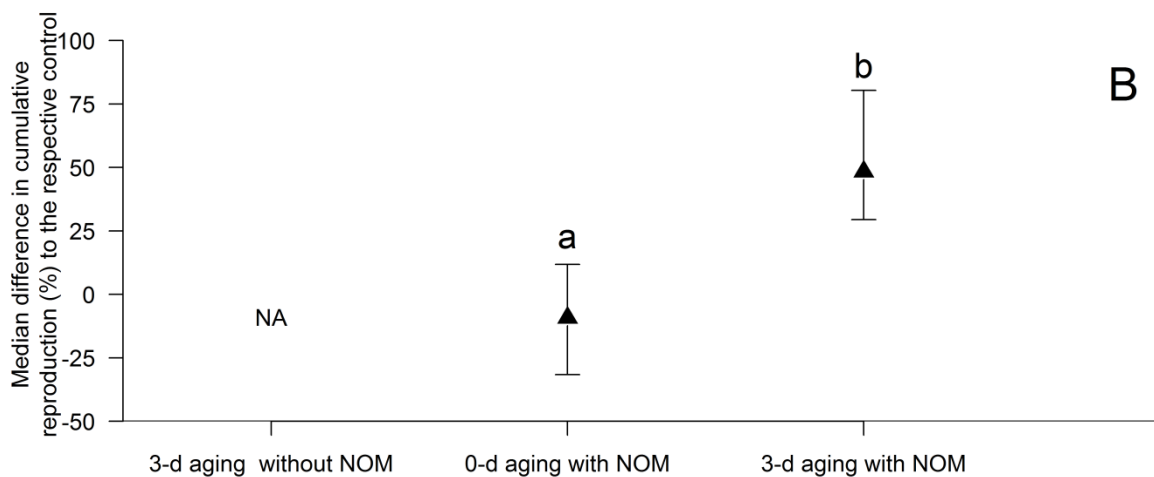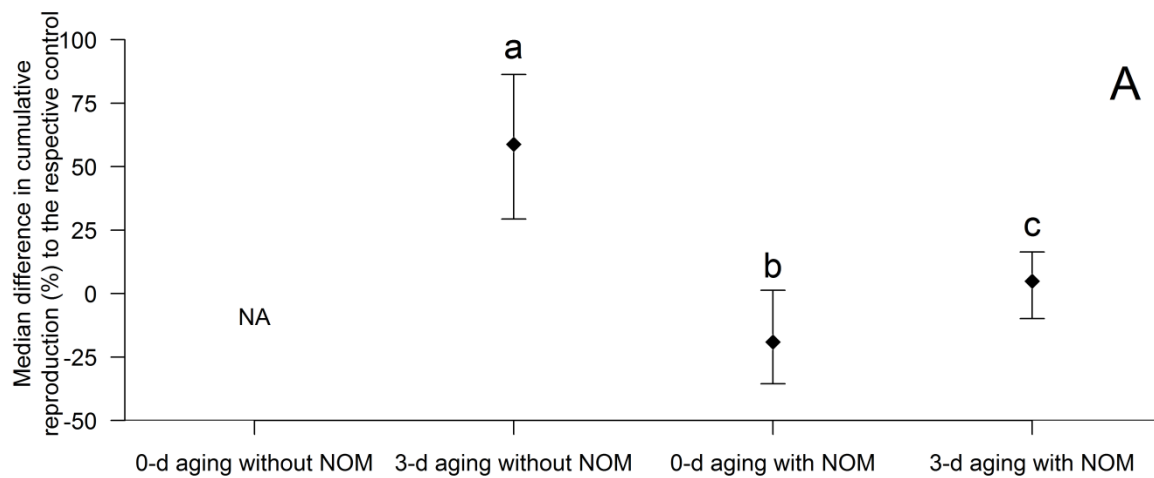
Figure 3:   Fecundity of *Daphnia*. Median difference in the reproduction of *Daphnia* (± 95% CI) after 21 d of exposure to (A) 2.0 and (B) 6.0 mg nTiO$_2$/L expressed relative to the respective control containing 0.0 mg/L nTiO$_2$. Higher numbers indicate a decreased cumulative reproduction compared to the control. NA=not assessed due to 100% mortality in the nTiO$_2$ treatment. Values followed by different lower case letters denote a statistically significant.

Discussion

Toxicity of aged and unaged nTiO$_2$: absence of NOM

Results of our acute toxicity tests showed that nTiO$_2$ aged in Milli-Q without NOM did not influence the nanoparticles toxicity even after elongated aging periods (1, 3 and 6 d) and thus revealed comparable 96-h EC$_{50}$ values for *Daphnia*'s immobility (Figure 1A). These results can be attributed to largely unchanged nTiO$_2$ characteristics after aging in Milli-Q without NOM. In particular, the nTiO$_2$ initial size – which has been suggested as an important factor driving the extent of nanoparticle toxicity [28,29] – was similar to the original nTiO$_2$ stock solution irrespective of the aging duration (see Table 1). These observations may be attributed to a lack of ions during aging (ionic strength: approx. 0.00 mmol/L), which accelerates nTiO$_2$ agglomeration in liquid media [30].

The importance of ions in the medium is also supported by the results of the present study. In contrast to the stable toxicity of nTiO$_2$ aged in Milli-Q (aging medium of low ionic strength), nanoparticles aged in ASTM (aging medium of high ionic strength) showed a significant shift in their toxicity with aging duration. In particular, acute as well as chronic toxicity of nTiO$_2$ aged in ASTM without NOM decreased with increasing aging duration (Figure 1B; Figure 2, Figure 3A and B). This reduced toxicity may be explained by altered nTiO$_2$ characteristics, particularly the particle size at the initiation of the exposure of daphnids (Table 1). In other words, the relatively high ecotoxicity of 0 and 1 d aged particles can be associated with a potentially higher share of small sized nTiO$_2$ in the water phase [see 11,28] when

compared to longer aging periods. As a result of the elongated aging duration, the particle size at the test initiation increases (e.g. after 6 d aging; ~2500 nm; Table 1) facilitating a fast sedimentation of $nTiO_2$ agglomerates (as visually observed) and hence reduction of the $nTiO_2$ concentration in the water column (the location where daphnids mainly dwell; Table 3). Moreover, such bigger particles are less likely to coat the surface of daphnids outer shell, a mode of toxic action of these nanoparticles suggested by Dabrunz et al. [11]. This in turn might affect the test species molting success [11] as well as their movement [31] and ultimately the mortality of *Daphnia*.

Toxicity of aged and unaged $nTiO_2$: presence of NOM

The particle size of $nTiO_2$ at the initiation of the bioassay did not change with aging duration in Milli-Q with NOM medium (Table 1), which probably also explains the absence of any difference in the EC_{50} values relative to $nTiO_2$ aged in Milli-Q without NOM (Figure 1A). The rather stable particle sizes over 6 d of aging in Milli-Q with NOM can be attributed to the low ion concentration (ionic strength: approx. 0.00 mmol/L) together with a NOM-induced particle size stabilization. In contrast to an aging in Milli-Q with NOM, the aging in ASTM with NOM generally reduced the acute and chronic toxicity of nanoparticles relative to ASTM without NOM (Figure 1B, Figure 2, Figure 3A and 3B). This result may directly be related to NOM coating both the nanoparticles (indicated by an decreased zeta potential of $nTiO_2$; see [32]) and the test organisms [33]; coating which was largely absent for $nTiO_2$ aged in Milli-Q with NOM due to the lower NOM concentrations in the test medium. In ASTM with

NOM, the electrosteric repulsion [34] kept $nTiO_2$ in the water phase and prevented an attachment to the surface of *Daphnia* [33]. In addition, NOM coating may have limited irradiation of the nanoparticle surfaces and potentially scavenged harmful reactive oxygen species [35], which are usually formed by $nTiO_2$ under the visible light conditions in our experimental facilities [36]. Moreover, the NOM may have served as a energy source for *Daphnia* [37], which may have lowered the overall toxicity of aged and unaged $nTiO_2$ indirectly as a result of an increased fitness of the test organisms [38]. This assumption is also supported by an approximately 40% higher reproductive output of daphnids cultured under control conditions but in presence of NOM relative to its absence (see supporting information Figure S7A and C).

Irrespective of the general tendency of NOM to reduce the ecotoxicity of $nTiO_2$, especially after 6 d of aging in ASTM with NOM, an aging of these particles for 1 and 3 d in the same medium induced an increased acute as well as chronic toxicity relative to the respective unaged particles (Figure 1B, Figure 2, Figure 3A and B). This pattern may be explained by (i) a relatively high number of small and ecotoxicological potent particles – while the predominance of this size fraction likely decreased with increasing aging duration as well as (ii) size stabilized $nTiO_2$ agglomerates of ~500 nm size (due to NOM; Table 1). The smaller particles might have mainly contributed to the acute $nTiO_2$ toxicity after 0 and 1 d of aging, while for 3 d aged $nTiO_2$ bigger agglomerates may have induced adverse effects. The latter suggestion may be explained by the mesh size of *D. magna*'s filter apparatus – displaying a range of 0.24-0.64 µm [39] – which facilitates an uptake of ~500 nm agglomerates [in sensu 40,41] (Figure 4). However, the size range of the organisms filter apparatus also indicates that a limited amount of smaller particles of unaged $nTiO_2$ (with a mean particle size of approximately 180 nm; Table 2) were actively

ingested by the test species (due to their filter passage). Hence, a higher mass of $nTiO_2$ might have been taken up by daphnids if exposed to $nTiO_2$ aged for 3 d (mean particle size: ~500 nm; ASTM with NOM; Table 2) relative to the same product aged for 0 d (mean particle size: ~180 nm; Table 2). During the acute experiments this hypothesized increased accumulation of $nTiO_2$ in the gut [42,43] may have reduced their mobility [31] ultimately increasing the mortality (immobility) of daphnids. Similarly, during the chronic experiments such $nTiO_2$ agglomerates may have decisively lowered the amount of ingested algae [cf. 40,43] limiting at the same time the energy availability for daphnids. Such implications in the energy processing might have led to a decreased fecundity [44] during the chronic investigations at $nTiO_2$ concentrations as high as 6.00 mg/L (Figure 3B). This is further supported by findings of previous investigations [13,18] where, $nTiO_2$ agglomerates of ~330 nm revealed statistically significant implications in *Daphnia's* reproduction output [18], while smaller agglomerates (~140 nm) did not affect the animals fecundity [13]. However, our findings are widely contrary to the common scientific assumption (especially when considering results obtained with $nTiO_2$ aged for up to 3d), which expects stable or decreased toxicity of nanoparticles with aging duration and thus elevating agglomeration and sedimentation [8,14,45,46].
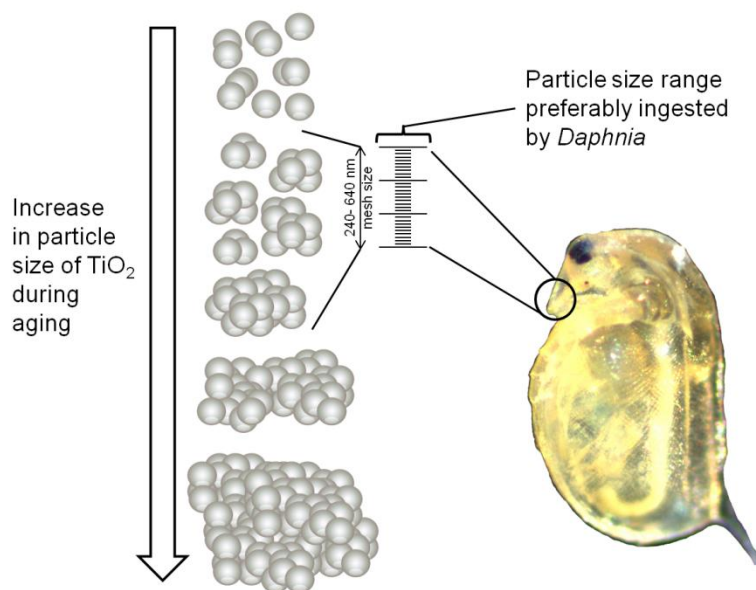
Figure 4:   Particle uptake of *Daphnia*. Schematic draft of the preferably ingested particle size range of *Daphnia magna.*

Results of the present study thus showed that the aging duration as well as the properties of the medium (in terms of ionic strength and NOM content) alter the nTiO$_2$-related toxicity. As the effect-size and -direction strongly varied, the hypothesis, that aging reduces nTiO$_2$ toxicity, is not completely supported. Although it is obvious that the presence of NOM reduced the toxicity of nanoparticles substantially, the ultimate risk associated with their release may by underestimated when ignoring the aging history. Since similar patterns may also be relevant for other types of nanoparticles, it seems sensible to uncover the underlying mechanisms and assess for their transferability among different classes of nanoparticles. In the light of recent literature and the present study, it seems moreover crucial to consider the implications of environmental parameters such as differing ionic strengths (representing for instance fresh- and seawater) and NOM levels (being present in nature) – on the ecotoxicological potential of nTiO$_2$ in particular and nanoparticles in

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general. Moreover this may also account for ultraviolet irradiation, which likely potentiates negative effects [47]. Overall, the present study provides an example on how nanoparticle-typical environmental processes, such as agglomeration during aging, lead to a varying toxicity profile over time, which may be explained when coupled with ecological information, such as particle size selectivity of *Daphnias'* filtering apparatus.

Acknowledgements

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SUPPLEMENTARY MATERIAL

of Appendix A.2

AGING OF TIO$_2$ NANOPARTICLES TRANSIENTLY INCREASES THEIR TOXICTY

TO THE PELAGIC MICROCRUSTACEAN *DAPHNIA MAGNA*

Frank Seitz, Simon Lüderwald, Ricki R. Rosenfeldt, Ralf Schulz, Mirco Bundschuh
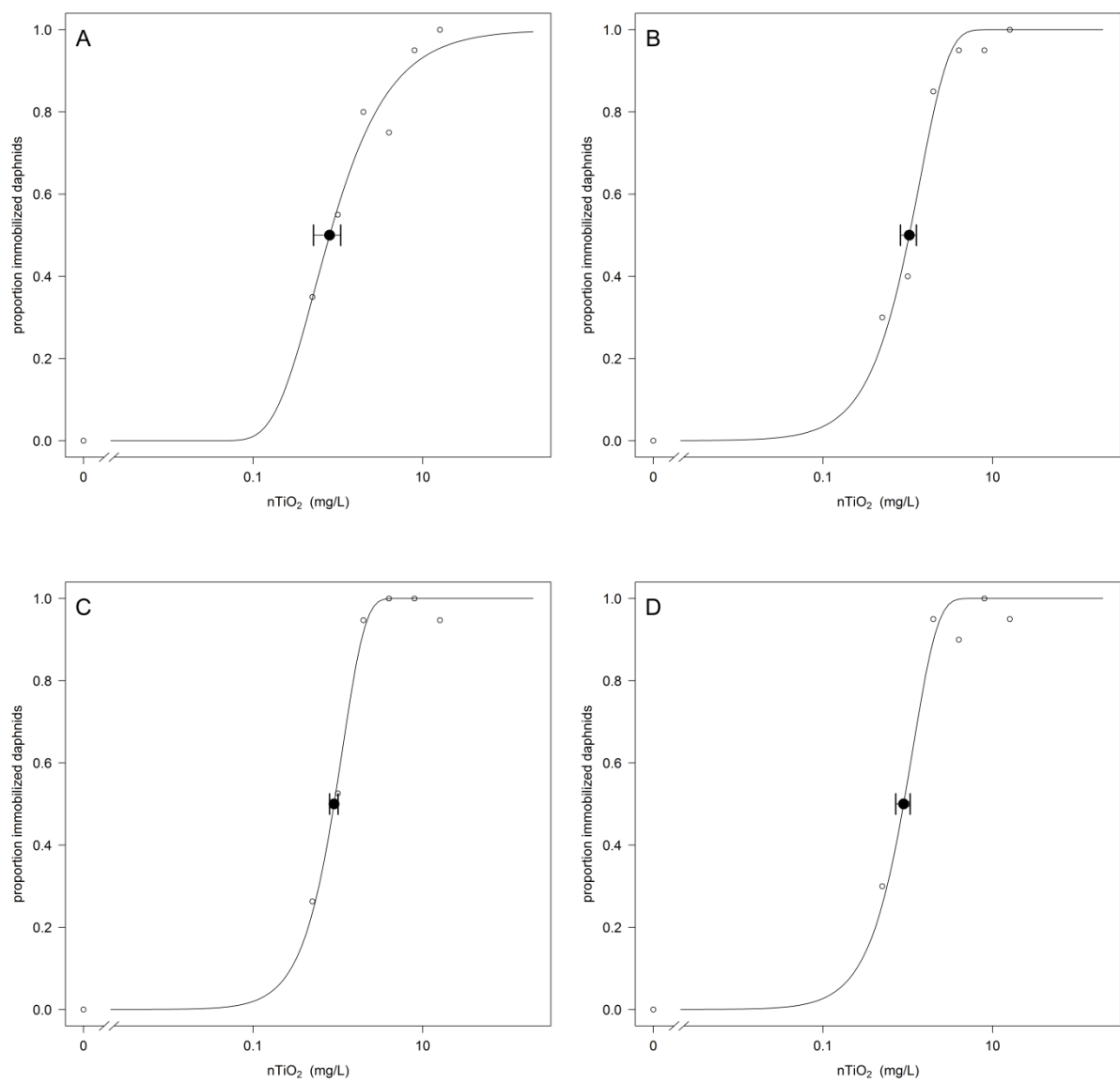
Figure S1: Dose-Response curves underlying the 96-h EC_{50} calculations for (A) 0, (B) 1, (C) 3 and (D) 6d aged $nTiO_2$ in Milli-Q without NOM.
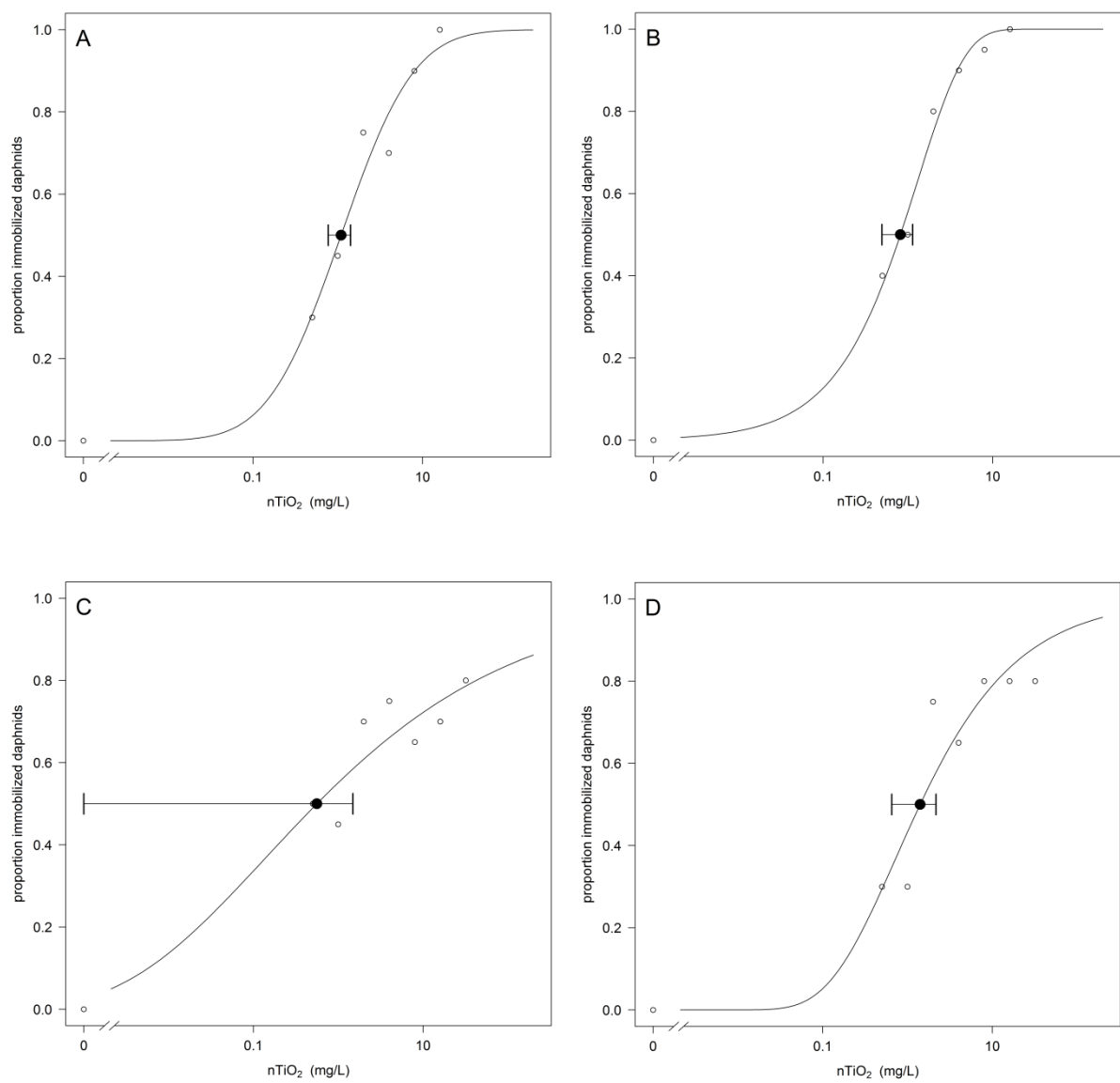
Figure S2:   Dose-Response curves underlying the 96-h EC$_{50}$ calculations for (A) 0, (B) 1, (C) 3 and (D) 6d aged nTiO$_2$ in Milli-Q with NOM.
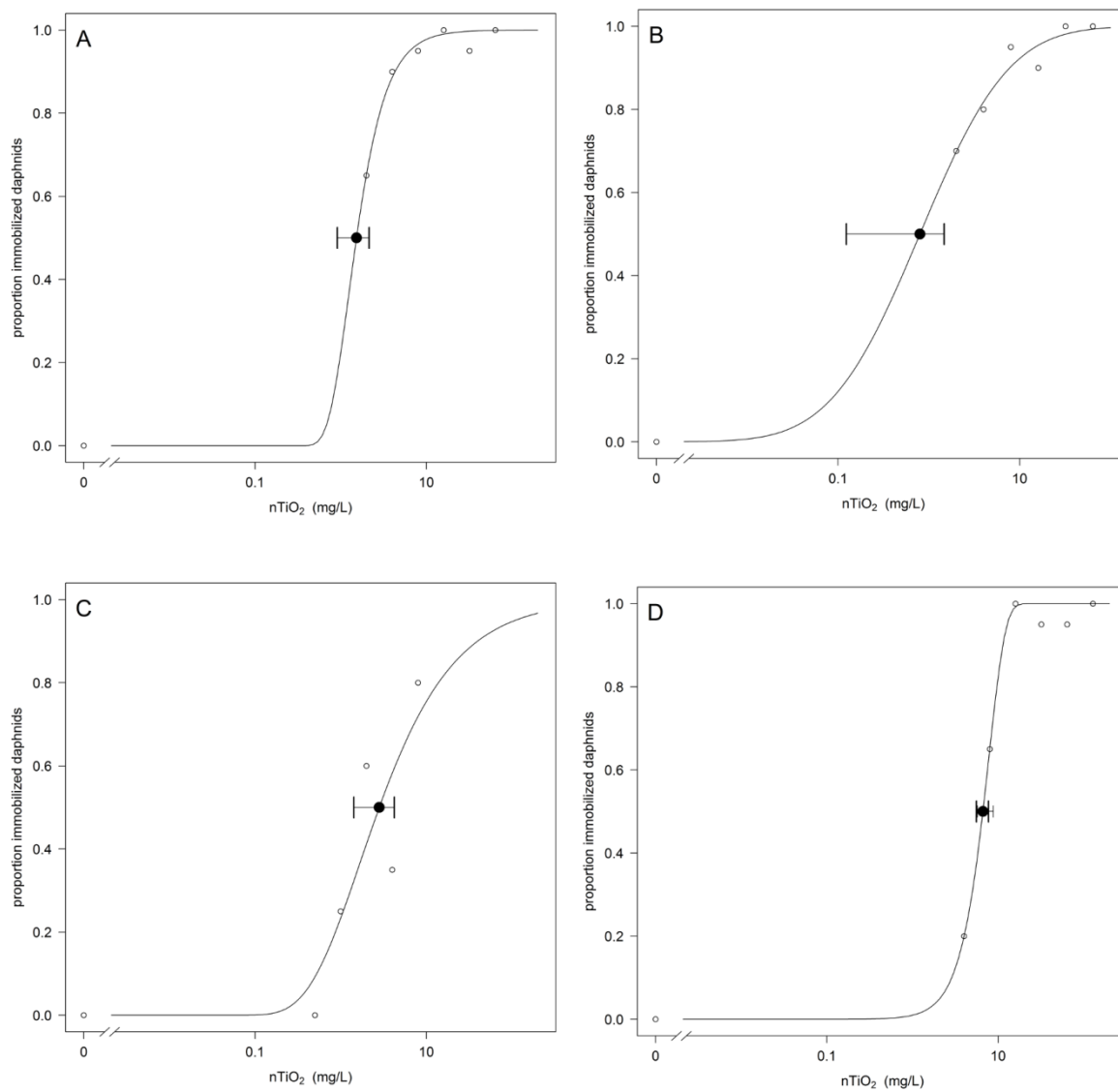
Figure S3: Dose-Response curves underlying the 96-h EC$_{50}$ calculations for (A) 0, (B) 1, (C) 3 and (D) 6d aged nTiO$_2$ in ASTM without NOM.
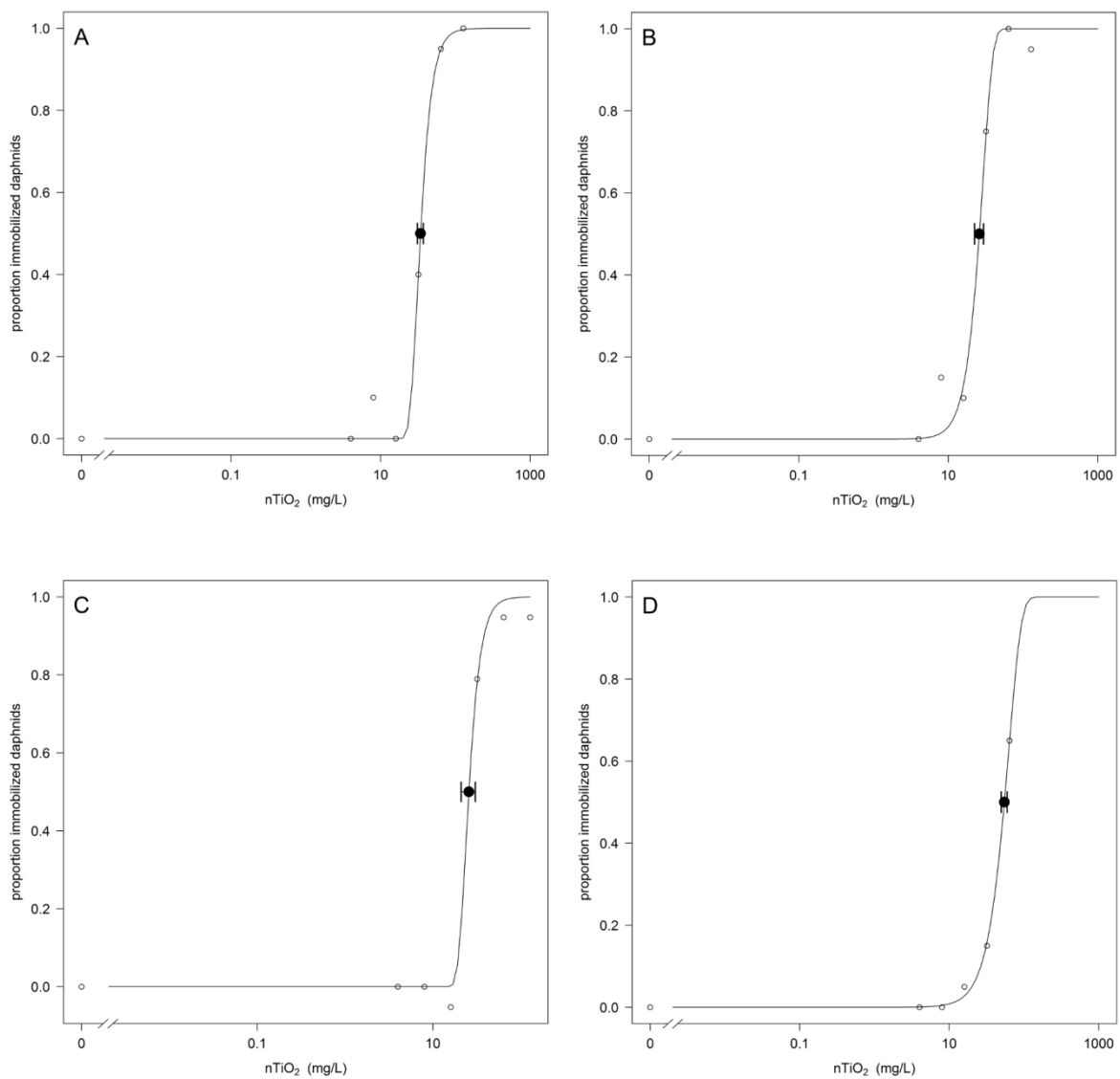
Figure S4: Dose-Response curves underlying the 96-h EC$_{50}$ calculations for (A) 0, (B) 1, (C) 3 and (D) 6d aged nTiO$_2$ in ASTM with NOM.
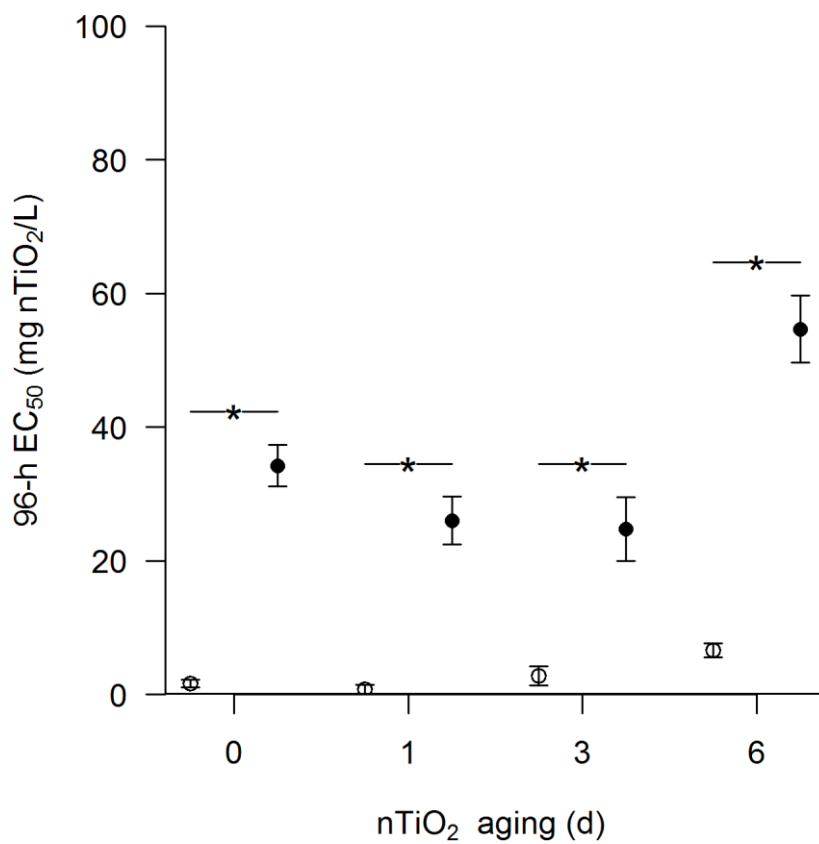
Figure S5: 96-h EC_{50} values (half maximal effective concentration; ± 95% CI) of nTiO$_2$ previously aged for 0, 1, 3 or 6 d in ASTM with (●) and without (○) NOM. Asterisk (*) denotes statistical significant difference to the respective 96-h EC_{50} value.
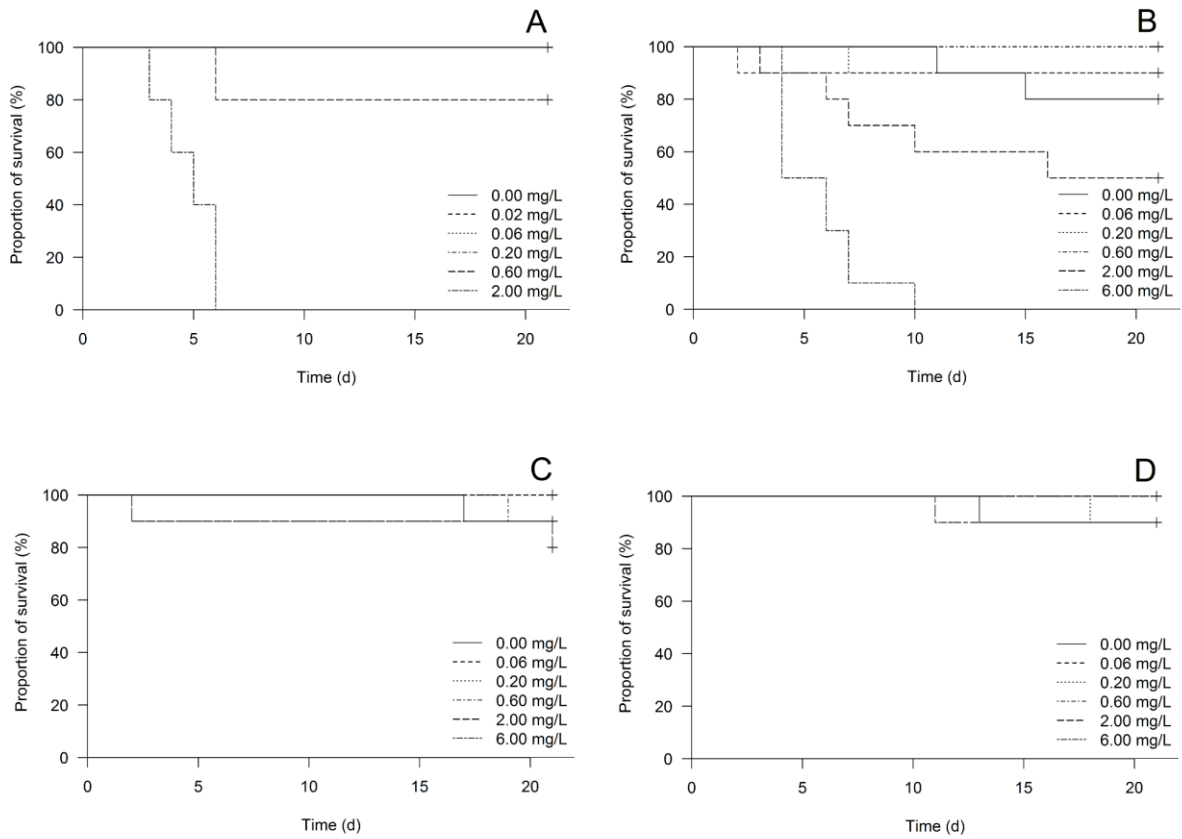
Figure S6: Survival (%) of daphnids during 21 d of $nTiO_2$ exposure. Different lines represent response of *Daphnia* in the respective $nTiO_2$ treatment. (A) Animals exposed to $nTiO_2$ previously aged for 0 d in ASTM without NOM media. (B) Animals exposed to $nTiO_2$ previously aged for 3 d in ASTM without NOM media. (C) Animals exposed to $nTiO_2$ previously aged for 0 d in ASTM with NOM media. (D) Animals exposed to $nTiO_2$ previously aged for 3 d in ASTM with NOM media.
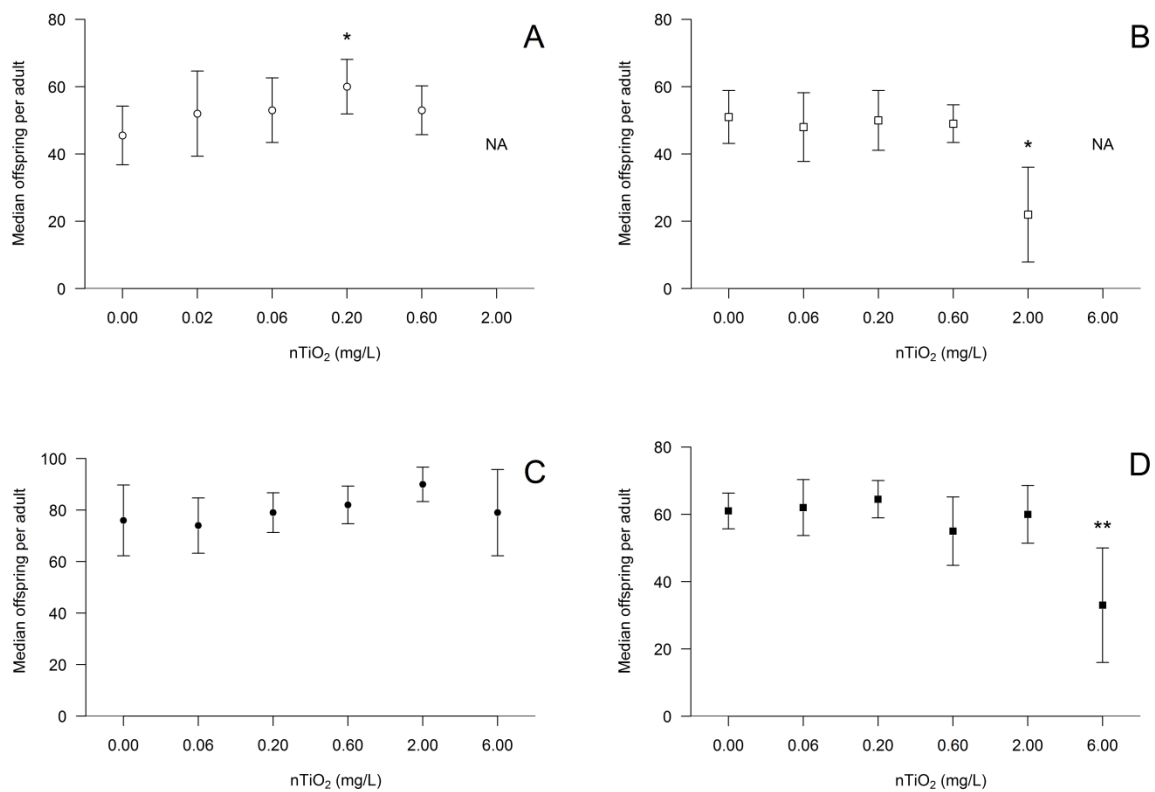
Figure S7: Cumulative median (± SD) reproduction per test-organism after 21 d exposure to differently aged $nTiO_2$. (A) Animals exposed to $nTiO_2$ previously aged for 0 d in ASTM without NOM media (○). (B) Animals exposed to $nTiO_2$ previously aged for 3 d in ASTM without NOM media (□). (C) Animals exposed to $nTiO_2$ previously aged for 0 d in ASTM with NOM media (●). (D) Animals exposed to $nTiO_2$ previously aged for 3 d in ASTM with NOM media (■). Asterisks denote statistical significant difference to the respective control; $p < 0.05$ (*), $p < 0.01$ (**). NA indicates not calculable reproduction output due to 100% mortality of adult daphnids in the respective treatment.

Table S1:   Composition and ionic strength of ASTM test medium.

Component	Concentration in mg/L
$NaHCO_3$	192
$CaSO_4 \cdot 2H_2O$	120
$MgSO_4$	120
KCl	8
Na_2SeO_3	0.00219
Thiamine hydrochloride (B_1)	0.075
Biotin (B_7)	0.00075
Cyanocobalamine (B_{12})	0.001
Ionic strength	9.25 mmol/L

Table S2:   Dissolved organic carbon analysis (µg/L-C) for seaweed extract (SW).

NOM	total	HOC[b]	CDOC[c]								SUVA[a] (L/mg*m)
		total	total	BIO-polymers	Humic Substance			Building Blocks	LMW[d] neutrals	LMW[c] acids	
				total	total	aromaticity (L/mg*m)	Mol-weight (g/mol)				
SW	1276	366	911	71	196	4.96	954	285	312	48	2.13

[a] specific UV absorbance; [b] hydrophobic organic carbon; [c] hydrophilic organic carbon; [d] low molecular weight

Table S3: Mean (±SE; n = 3) water quality parameters measured over the entire test duration of each experiment.

Aging conditions	1st Week			2nd Week			3rd Week		
	pH	Oxygen (mg/L)	Temperature (°C)	pH	Oxygen (mg/L)	Temperature (°C)	pH	Oxygen (mg/L)	Temperature (°C)
0 d aging ASTM without NOM	8.15 (±0.03)	7.41 (±0.60)	19.97 (±0.09)	8.14 (±0.05)	6.77 (±1.03)	19.67 (±0.27)	8.73 (±0.30)	8.32 (±0.11)	19.57 (±0.24)
3 d aging ASTM without NOM	8.29 (±0.01)	7.06 (±0.21)	19.57 (±0.23)	8.27 (±0.08)	7.67 (±0.66)	19.8 (±0.26)	8.35 (±0.08)	8.47 (±0.12)	19.47 (±0.20)
0 d aging ASTM with NOM	8.23 (±0.04)	8.06 (±0.35)	20.00 (±0.31)	8.1 (±0.03)	7.48 (±0.26)	19.37 (±0.32)	8.18 (±0.02)	7.95 (±0.09)	19.83 (±0.09)
3 d aging ASTM with NOM	8.35 (±0.00)	8.04 (±0.12)	19.80 (±0.12)	8.22 (±0.05)	7.59 (±0.11)	19.43 (±0.48)	8.2 (±0.01)	8.11 (±0.09)	19.8 (±0.12)

Table S4: Model specification and Akaike's information criterion used for the calculation of each EC_{50} value.

Aging medium	Aging duration (d)	Model		Akaike's information criterion	Lack of fit
Milli-Q without NOM	0d	two-parameter Weibull function	W1.2	-15.93	0.82
	1d	two-parameter Weibull function	W2.2	-31.02	0.59
	3d	two-parameter Weibull function	W2.2	-58.13	0.67
	6d	two-parameter Weibull function	W2.2	-32.02	0.44
Milli-Q with NOM	0d	log-normal dose-response model	LN.2	-31.62	0.40
	1d	two-parameter Weibull function	W2.2	-21.28	0.96
	3d	two-parameter Weibull function	W1.2	-5.68	0.84
	6d	two-parameter Weibull function	W1.2	-6.49	0.46
ASTM without NOM	0d	two-parameter Weibull function	W1.2	-34.85	0.98
	1d	log-normal dose-response model	LN.2	-51.06	0.76
	3d	two-parameter Weibull function	W1.2	-3.18	0.03
	6d	two-parameter Weibull function	W2.2	-26.68	0.97
ASTM with NOM	0d	two-parameter Weibull function	W1.2	-37.23	0.74
	1d	two-parameter Weibull function	W2.2	-29.73	0.45
	3d	two-parameter Weibull function	W1.2	-38.00	0.84
	6d	two-parameter Weibull function	W2.2	-44.37	0.99

Appendix A.3

EFFECTS OF NANO-TIO$_2$ IN COMBINATION WITH AMBIENT UV-IRRADIATION ON A LEAF SHREDDING AMPHIPOD

Mirco Bundschuh, Jochen Zubrod, Dominic Englert, Frank Seitz, Ricki R. Rosenfeldt,

Ralf Schulz

Abstract

Production and use of engineered nanoparticles, such as titanium dioxide nanoparticles (nTiO$_2$), is increasing worldwide, enhancing their probability to enter aquatic environments. However, direct effects of nTiO$_2$ as well as ecotoxicological consequences due to the interactions of nTiO$_2$ with environmental factors like ultraviolet (UV) irradiation on representatives of detrital food webs have not been assessed so far. Hence, the present study displayed for the first time adverse sublethal effects of nTiO$_2$ at concentrations as low as 0.2 mg L^{-1} on the leaf shredding amphipod *G. fossarum* both in presence and absence of ambient UV-irradiation following a seven-day exposure. In absence of UV-irradiation, however, the effects seemed to be driven by accumulation of nTiO$_2$ at the bottom of the test vessels to which the gammarids were potentially exposed. The adverse sublethal and lethal effects on gammarids caused by the combined application of nTiO$_2$ and ambient UV-irradiation are suggested to be driven by the formation of reactive oxygen species. In conclusion, both the accumulation of nTiO$_2$ at the bottom of the test vessel and the UV induced formation of reactive oxygen species clearly affected its ecotoxicity, which is recommended for consideration in the environmental risk assessment of nanoparticles.

Keywords:

nanoparticle – titanium dioxide – ultraviolet irradiation – *Gammarus fossarum* – accumulation – reactive oxygen species

Abbreviations:

nTiO$_2$ Titanium dioxide nanoparticles

ROS   Reactive oxygen species

UV   Ultraviolet

ANOVA   Analysis of variance

PNEC   Predicted no effect concentration

Research Highlights:

- effects of nTiO$_2$ and ambient UV-irradiation affect representatives of detrital food webs

- accumulation of nTiO$_2$ at the bottom of the test vessel seems to affect ecotoxicity

- nTiO$_2$ & ambient UV-irradiation increases ecotoxicity due to the formation of ROS

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Introduction

Nanotechnological industry is emerging worldwide and is predicted to become a trillion US dollar industry in the near future (Schmidt, 2009). The resulting increased production of nanoparticles will finally enhance the likelihood of such compounds to enter the aquatic environment in meaningful quantities (Scown et al., 2010). Titanium dioxide nanoparticles ($nTiO_2$), for instance, are widely used as ingredients in personal care products (Zhu et al., 2010a) like sunscreens and cosmetics but also as façade paints (Serpone et al., 2007; Kaegi et al., 2008). Moreover, technologies involving the combined application of $nTiO_2$ and ultraviolet (UV) irradiation are appropriate for decontamination of air, soil and (waste)water (Fujishima et al., 2000; Herrmann, 2005) due to the formation of reactive oxygen species (ROS) (Fujishima et al., 2000).

Despite this wide range of $nTiO_2$ applications, no study was so far technically capable of quantifying environmental $nTiO_2$ concentrations. But they were predicted to be up to 0.021 µg L^{-1} in surface waters and up to 4 µg L^{-1} in European and American wastewater treatment plant effluents (Gottschalk et al., 2009). These $nTiO_2$ concentrations are relatively low and may hence not cause any direct ecotoxicological effect, which is indicated by e.g. a 21-d EC_{50}-value of 0.46 mg L^{-1} for the reproduction of *Daphnia magna* (Zhu et al., 2010b). However, this and comparable studies have not taken the potentially increasing $nTiO_2$ concentrations at the bottom of the test vessels into account, which can be assumed due to the rapid agglomeration (Velzeboer et al., 2008) and sedimentation of $nTiO_2$ (Dabrunz et al., 2011). This may result in the exposure of benthic invertebrates. It is however questionable whether $nTiO_2$ agglomerates have the potential to exert appreciable toxic effects,

In addition, published ecotoxicological studies, which assess effects on aquatic organisms, consider exclusively species involved in food webs based on primary production in aquatic environments, like daphnids and algae (Scown et al., 2010). Hence, detrital food webs are so far largely ignored. Moreover, effects caused by the potential formation of ROS during ambient UV-irradiation of $nTiO_2$ on invertebrates were indicated only by one study (Hund-Rinke and Simon, 2006), although UV is an important environmental factor (Häder et al., 2007). Hence, ecotoxicological consequences of the combined stress of $nTiO_2$ and ambient UV-irradiation can also not satisfactorily be evaluated.

The present study, therefore, investigated direct effects of $nTiO_2$ on the feeding rate of the leaf shredding benthic amphipod *Gammarus fossarum*. One experiment considered the potential increase of the nanoparticle concentrations at the bottom of the test vessel. Another experiment assessed by means of a two factorial test design the combined effects of $nTiO_2$ and ambient UV-irradiation. As *G. fossarum* is considered as a key species in the ecosystem function of leaf litter breakdown (Dangles et al., 2004), alterations in its feeding rate, which is a frequently used sublethal, ecotoxicological endpoint (e.g. Bundschuh et al., 2011a), may perpetuate to shifts in this ecosystem service and hence the energy provision for local and downstream communities (Bundschuh et al., 2011b).

Material and Methods

Preparation and analysis of $nTiO_2$

Titanium dioxide nanoparticles (P25; Degussa, Essen, Germany) were purchased as powder (anatase 80%, rutile 20%) to prepare a dispersant and additive free, size

homogenized stable suspension. This suspension was obtained by stirred media milling (PML2, Bühler AG, Switzerland). Particle size distributions in undiluted and monodisperse stock suspension were determined via dynamic light scattering (Delsa^{TM} Nano C, Beckman Coulter, Germany) prior to each medium exchange. The stock suspension had a mean (± 95% confidence interval (CI)) particle diameter of approximately 97.16 (± 1.96) nm and a concentration of 6.9 g $nTiO_2$ L^{-1}. Prior to its application and characterization the $nTiO_2$ suspension was sonicated for 10 min to ensure a homogeneous distribution of particles. The nominal test concentrations were achieved by serial dilution.

Preparation of leaf discs

Leaf discs were prepared as described in detail in Bundschuh et al. (2011a). Briefly, senescent but undecomposed black alder (*Alnus glutinosa* L. Gaertn.) leaves were collected shortly before leaf fall in October 2008 from a group of trees near Landau, Germany (49°11' N; 8°05' E), and stored frozen at -20°C until further use. After thawing, discs (2.0 cm diameter) were cut from each leaf with a cork borer. To establish a microbial community on these leaf discs, they were conditioned in a nutrient medium together with alder leaves exhibiting a natural microbial community consisting of bacteria and fungi due to a three weeks exposure in the Rodenbach, located near Mannheim, Germany (49° 33' N, 8° 02' E). Prior to the laboratory conditioning period, the field conditioned leaves were kept for several weeks at 15±1°C in aerated stream water from the same site. Following a conditioning period of 10 d, the discs were dried at 60°C to constant weight (~24 h), which ensured an accurate measurement of the amphipods' feeding rate, and weighed to the nearest 0.01 mg. After that the leaf discs were, if not stated otherwise, soaked in test medium

(= SAM-S5 medium) described by Borgmann (1996), for 48 h and randomly allocated to the respective treatment.

Test organisms

G. fossarum were chosen as test organisms since they are known as key species in the ecosystem function of leaf litter breakdown (Dangles et al., 2004). They were obtained from a near natural stream (Hainbach) near Landau, Germany (49°14' N; 8°03' E), one week before the start of each bioassay and were checked visually for infection with acanthocephalan parasites. Infected specimens were excluded from the experiment as parasites may affect the behavior, amongst others the feeding rate, of its host (Pascoe et al., 1995). The remaining *G. fossarum* were divided into three size classes using a passive underwater separation technique (Franke, 1977). Only adults with a cephalothorax length between 1.2 and 1.6 mm were used. Subsequently, those animals were kept in test medium until the start of the experiment while preconditioned black alder leaves were provided *ad libitum*.

Feeding activity trials

Two feeding activity trials were conducted in the present study (Bundschuh et al., 2011a). During the first experiment, one specimen of *G. fossarum* was placed together with two randomly allocated preconditioned leaf discs in a 250-mL glass beaker filled with 200 mL of test medium containing 0.0, 0.2, 2.0 or 20.0 mg $nTiO_2$ L^{-1} for seven days in total darkness. Each vessel was aerated during the whole period. For each treatment 30 replicates were set up.

In contrast to the first experiment, the test medium of the second experiment

containing 0.0, 0.2 or 2.0 mg nTiO$_2$ L^{-1} was renewed every 24 h. To ensure a careful transfer of the test organisms and leaf discs into the fresh medium, stainless steel (mesh size = 0.5 mm) cages were used. These cages additionally guaranteed that the gammarids and leaf discs never entered the lowest 1.0 cm medium layer (= test vessel bottom). The second feeding activity trial assessed in addition to nTiO$_2$ toxicity the potential adverse effects of the interaction of nTiO$_2$ and ambient UV-irradiation. Therefore, again 0.0, 0.2 or 2.0 mg nTiO$_2$ L^{-1} were assessed together with UV-A and UV-B irradiation at an intensity of 28.0 W m^{-2} and 0.9 W m^{-2} (measured with a RM12 radiometer; Dr. Gröbel UV-Elektronic GmbH, Ettlingen, Germany), which is 25% below peak intensities measured during summer time in Central Europe (Häder et al., 2007). The UV-irradiation period was set at 12 h per d. Mortality of the test organism *G. fossarum* was monitored every 24 h. Each treatment was replicated 20 times during the second feeding activity trial. Five additional beakers containing two leaf discs without *G. fossarum* were established to correct for microbial decomposition and handling losses in leaf mass for each treatment during both experiments. After seven days of exposure, the test organisms, remaining leaf discs and any leaf tissue shredded off were removed and dried at 60°C to constant weight and weighed to the nearest 0.01 mg. The feeding rate was calculated as described in Maltby et al. (2000).

Food choice trial

Preconditioned leaf discs were soaked for 48 h in seven mL of test medium containing 0.0 and 20.0 mg nTiO$_2$ L^{-1}, while the medium was exchanged after 24 h. Following this soaking period, one leaf disc soaked in the control and one disc soaked in medium containing 20.0 mg nTiO$_2$ L^{-1} were paired and offered to a single

specimen of *G. fossarum*, which was starved for 96 h prior use in the food choice trial. Two other leaf discs soaked in test medium containing 0.0 and 20.0 mg nTiO$_2$ L^{-1}, respectively, were also introduced to the same feeding arena but protected from amphipod feeding by a 0.5-mm nylon mesh screen to account *inter alia* for microbial decomposition and abiotic leaf mass loss. The trial (n = 49) ran for 24 h at 20 ± 1 C in total darkness to avoid phototactic response of the test animals. After the feeding period, all leaf discs and animals were individually removed, dried separately to constant mass, weighed, and used to calculate leaf consumption per mg of *Gammarus* body dry mass and day (cp. Bundschuh et al., 2009).

Statistical analysis

Data were check for normal distribution and variance homogeneity using Kologmorov-Smirnov and Bartlett's test, respectively. Subsequently, differences in the mean feeding rates of gammarids exposed to different nTiO$_2$ concentrations measured during the first feeding activity trial were assessed for statistical significance by ANOVA followed by Dunnett's test for multiple comparisons. A two factorial ANOVA was applied to judge statistical significance of UV-irradiation, nTiO$_2$ as well as their interaction term during the second feeding activity trial. This two factorial ANOVA was supplemented by one-way ANOVAs, followed by Dunnett's tests, separately for situation with and without ambient UV-irradiation among nTiO$_2$ treatments. Moreover, unpaired Student's t-tests were performed to assess statistical significance between exposure scenarios with and without ambient UV-irradiation for each nTiO$_2$ concentration separately. The proportion of dead gammarids following the seven days of exposure were compared among treatments exposed to nTiO$_2$ and ambient UV-irradiation using the corresponding methods described by Altman et al.

(2000) by considering adjustments for multiple comparisons. If CIs of differences between two proportions did not include zero the test outcome was judged as significant. Finally, for the food choice trial also an unpaired Student's t-test was used to assess statistical significance regarding the feeding preference of gammarids following the two leaf discs soaking scenarios. All tests were two-sided and significance level was set at $p < 0.05$. In the following chapter the expression "significant(ly)" is exclusively used in terms of "statistical significance".

Results and Discussion

The present study assessed for the first time potential adverse effects of $nTiO_2$ alone and in combination with ambient UV-irradiation on a key species in the ecosystem function of leaf litter breakdown, i.e. *G. fossarum* (Dangles et al., 2004). The first feeding activity trial of the present study revealed for all $nTiO_2$ concentrations tested, namely 0.2, 2.0 or 20.0 mg $nTiO_2$ L^{-1}, mean feeding rates significantly reduced (Dunnett; p < 0.05, n = 30; Fig. 1) by approximately 40%. These effects seem to be caused by direct exposure of the test organism *G. fossarum*, as leaf associated $nTiO_2$ did not affect the food selection of this species during the food choice trial (Fig. 2). The lowest concentration displaying a significantly reduced mean feeding rate of *G. fossarum*, 0.2 mg $nTiO_2$ L^{-1}, is at the same level as those causing reduced numbers of living offspring for *D. magna* after 21 d of exposure (Zhu et al., 2010b). Accordingly, Dabrunz et al. (2011) reported a time weighted 96-h EC_{50}-value of 0.24 mg $nTiO_2$ L^{-1} for *D. magna*. In contrast, the second feeding trial of the present study displayed only a slight, non-significant decrease in the mean feeding rate of *G. fossarum* exposed in total darkness to 0.2 and 2.0 mg $nTiO_2$ L^{-1} if compared to the

respective control.

Figure 1: Mean (±95% CI) feeding rate of *G. fossarum* exposed to 0.0, 0.2, 2.0 or 20.0 mg nTiO$_2$/L for seven days in darkness during the first feeding activity trial. Asterisks denote significant differences at $p < 0.05$ based on Dunnett's test for multiple comparisons compared to the control (n = 30).
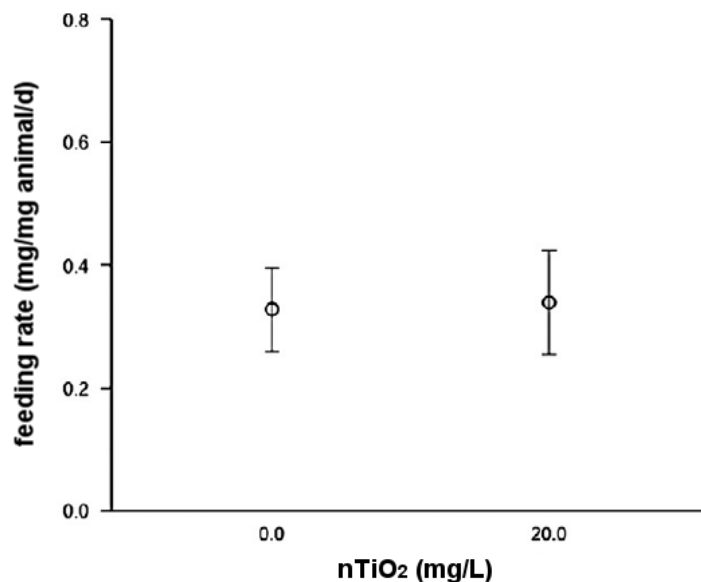
Figure 2: Mean (±95% CI) feeding rate of *G. fossarum* during the food choice trial following the 48 h soaking period in test medium containing 0.0 or 20.0 mg nTiO$_2$/L. No significant difference was obvious (t-test; $p = 0.73$; n = 49).

As Dabrunz et al. (2011) have shown that $nTiO_2$ and their agglomerates disappear from the water phase of the test medium during the first 24 h, it can be assumed that they accumulated on the bottom of the test vessels. However, during our second experiment, cages ensured that both the benthic test organism *G. fossarum*, which acts negatively phototactic (Franke, 1977), and the leaf discs were held approximately 1.0 cm above the bottom of the glass beakers. This procedure hence reduced the exposure of the test organism to $nTiO_2$ or their agglomerates and finally any adverse ecotoxicological response.

The two factorial design of the second feeding activity trial allowed the additional assessment of potential adverse effects due to ambient UV-irradiation in combination with different $nTiO_2$ concentrations. This UV-irradiation alone - and hence without any $nTiO_2$ – caused with approximately 50% a significantly reduced mean feeding rate compared to the control (= without UV-irradiation and $nTiO_2$) (t-test; $p < 0.0001$; n = 20/19; Fig. 3).
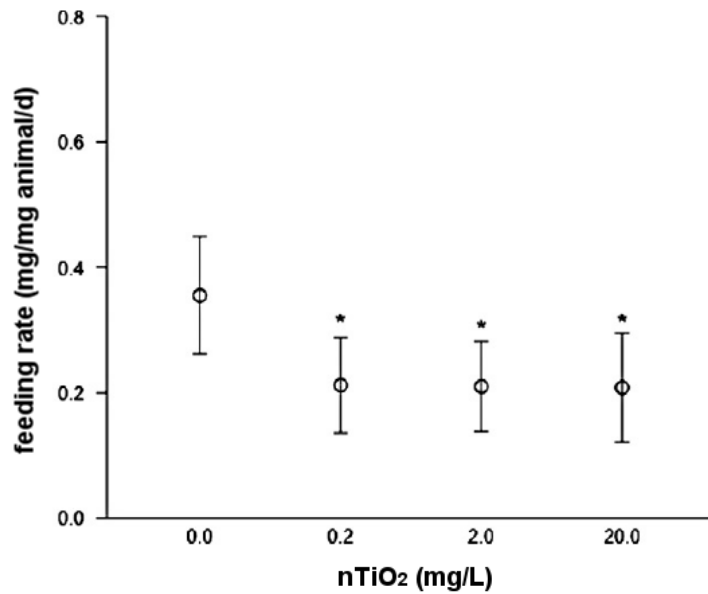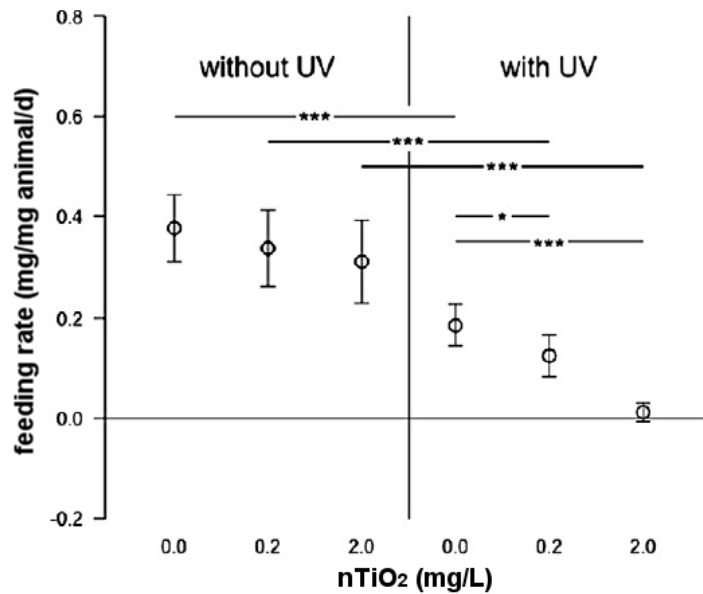
Figure 3: Mean (±95% CI) feeding rate of *G. fossarum* exposed to 0.0, 0.2 or 2.0 mg nTiO$_2$/L for seven days in darkness or under ambient UV-irradiation during the second feeding activity trial. Asterisks denote significant differences with $p < 0.05$ (*) and $p < 0.001$ (***) based on Dunnett's test for multiple comparisons (n = 19-20), respectively. Due to the 90% mortality recorded in the 2.0 mg nTiO$_2$/L with UV-irradiation, this treatment was not included in the further statistical analysis

This reduced feeding rate may be explained by the photoperiod applied in the present study, since UV-irradiation, especially UV-B (280 – 315 nm), is acutely toxic to amphipods and other invertebrates as shown by Cywinska et al. (2000) at 0.32 W m^{-2}. Hurtubise et al. (1998) suggest that a shelter would reduce the adverse effects. Hence, it can be assumed – also based on the observations during the present study - that *G. fossarum* hid under and do not feed on the leaf discs provided as food during the 12 h UV-irradiation period, which finally resulted in the observed 50% reduced feeding rate. However, in presence of 0.2 and 2.0 mg nTiO$_2$ L^{-1} and ambient UV-irradiation a significant difference to the respective control (Dunnett; $p = 0.029$ & $p < 0.0001$; n = 19/20; Fig. 3) and the same nTiO$_2$ concentrations not subjected to UV-irradiation were present (t-test; both $p < 0.0001$; n = 20/19; Fig. 3). This suggests, especially as the interaction term of UV-irradiation and nTiO$_2$ concentrations were

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significant (Tab. 1), a synergistic adverse effect of both stressors, which was e.g. also

shown for a marine amphipod by Liess et al. (2001) with regards to copper and UV-B.

Moreover, 90% of the gammarids exposed to 2.0 mg $nTiO_2$ L^{-1} in combination with

UV-irradiation died during the study duration. This mortality rate was significantly

higher than for all other treatments with UV-irradiation (Fig. 4).

Table 1: Output of the two-factorial ANOVA assessing differences in the mean feeding rate of *G. fossarum* due to exposure to three levels of $nTiO_2$ (0.0, 0.2 or 2.0 mg $nTiO_2$ L^{-1}) in combination with or without UV irradiation.

	df	SS	MS	F-value	p-values
UV irradiation	1	2.315	2.315	126.436	<0.0001
$nTiO_2$	2	0.434	0.217	11.840	<0.0001
UV-irradiation x $nTiO_2$	2	0.168	0.084	4.589	0.0121
Residuals	114	2.087	0.018		

df = degrees of freedom; SS = sum of squares; MS = mean squares
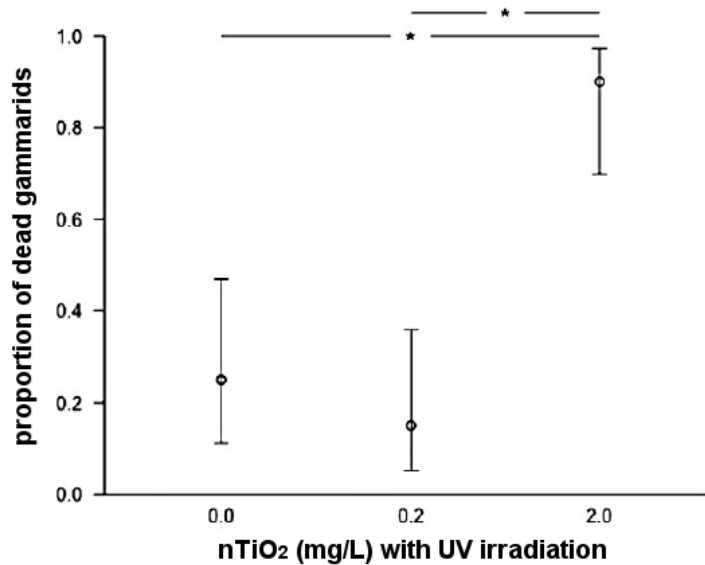
Figure 4:   Proportion (with 95% CI) of dead gammarids exposed to different nTiO$_2$ concentrations in combination with UV-irradiation. Asterisks denote significant differences between treatments.

Furthermore, it is obvious that both nTiO$_2$ concentrations assessed during the second experiment resulted in an increased ecotoxicity if applied in combination with UV-irradiation. The mechanism of toxicity proposed during the first experiment of the present study was, however, excluded here as a reason. Although not analyzed, formation of ROS may be assumed to have occurred in the present study as the band gap energy of 3.0 – 3.2 eV for nTiO$_2$, which is necessary to form ROS, is provided by photons, i.e. UV-irradiation (Ahmed et al., 2010). Hence, the observed increase in toxicity may be caused by this formation of ROS that damage amongst others cellular lipids and proteins (Kahru et al., 2008) as well as adversely affected the bacteria *Bacillus subtilis* (Adams et al., 2006). Moreover, Hund-Rinke and Simon (2006) attributed an increased immobilization of *D. magna* following a 30 min UV-pre-irradiation in the presence of nTiO$_2$ also to ROS. However, the authors were not able to identify a clear dose response relationship. As no further study was identified that addresses potential synergistic or antagonistic ecotoxicological effects of UV-

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irradiation and $nTiO_2$ on aquatic invertebrates, the present study fills a relevant scientific knowledge gap by providing a clear reference that most data currently available in the literature may have underestimated toxic effect potentially associated with the $nTiO_2$ exposure, since field relevant UV irradiation conditions were not considered.

In conclusion, the present study displayed that the unavoidable accumulation of $nTiO_2$ and other metal oxide nanoparticles at the bottom of test beakers may be the driving factor for adverse effects on test organisms. This is especially important for benthic organisms, which should also be considered in future experiments with nanoparticles. Moreover, it was empirically shown, by avoiding the exposure of *G. fossarum* to accumulated $nTiO_2$ at the bottom of the test vessel, that UV-irradiation at ambient intensities causes adverse effects at 0.2 mg $nTiO_2$ L^{-1}. Based on this effect concentration a predicted no effect concentration (PNEC) of 0.2 µg $nTiO_2$ L^{-1} will be obtained, if calculated according to established procedures on European chemical risk assessment (European Chemicals Bureau, 2003), including an assessment factor of 1000. As the predicted concentration of $nTiO_2$ in surface waters is about 0.021 µg L^{-1} (Gottschalk et al., 2009), it remains unlikely that environmental $nTiO_2$ levels will exceed this PNEC. Nevertheless, the present study showed that nanoparticles' interactions e.g. with environmental factors need to be investigated also from an ecotoxicological viewpoint. Finally, both main conclusions drawn from the present study, that take the nanoparticles' inherent properties into account, should carefully be considered during the environmental risk assessment of engineered nanoparticles.

Acknowledgements

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SUPPLEMENTARY MATERIAL

of Appendix A.3

EFFECTS OF NANO-TIO$_2$ IN COMBINATION WITH AMBIENT UV-IRRADIATION ON A LEAF SHREDDING AMPHIPOD

Mirco Bundschuh, Jochen Zubrod, Dominic Englert, Frank Seitz, Ricki R. Rosenfeldt, Ralf Schulz
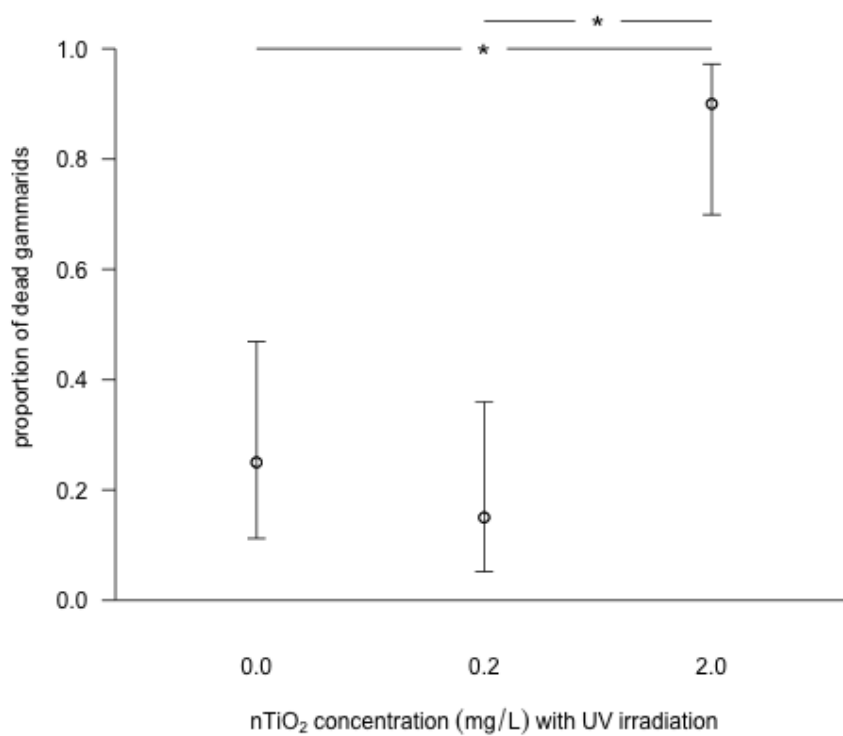
Figure SD1: Proportion (with 95% CI) of dead gammarids exposed to different $nTiO_2$ concentrations in combination with UV-irradiation. Asterisks denote significant differences between treatments.

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Appendix A.4

EFFECTS OF SILVER NANOPARTICLE PROPERTIES, MEDIA pH AND DISSOLVED ORGANIC MATTER ON TOXICITY TO *DAPHNIA MAGNA*

Frank Seitz, Ricki R. Rosenfeldt, Katharina Storm, George Metreveli, Gabriele E. Schaumann, Ralf Schulz, Mirco Bundschuh

Graphical abstract

high   bioavailability of Ag⁺   low

LOW

low
pH and/or DOM
level

EC₅₀

high
pH and/or DOM
level

HIGH

Ag⁺ Ag⁺
Ag⁺

high   toxicity   low
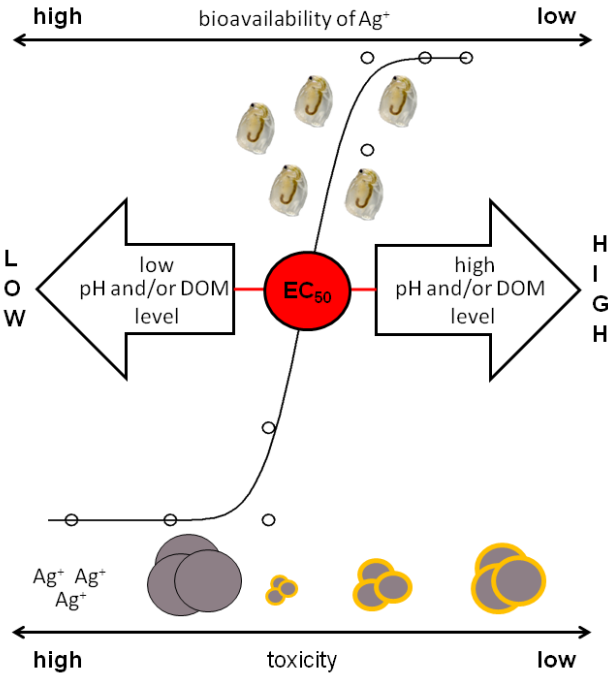
Abstract

Studies assessing the acute and chronic toxicity of silver nanoparticle (nAg) materials rarely consider potential implications of environmental variables. In order to increase our understanding in this respect, we investigated the acute and chronic effects of various nAg materials on *Daphnia magna*. Thereby, different nanoparticle size classes with a citrate coating (20-, ~30-, 60- as well as 100-nm nAg) and one size class without any coating (140 nm) were tested, considering at the same time two pH levels (6.5 and 8.0) as well as the absence or presence of dissolved organic matter (DOM; <0.1 or 8.0 mg total organic carbon/L). Results display a reduced toxicity of nAg in media with higher pH and the presence of DOM as well as increasing initial particle size, if similarly coated. This suggests that the associated fraction of Ag species <2 nm (including Ag^+) is driving the nAg toxicity. This hypothesis is supported by normalizing the 48-h EC_{50}-values to Ag species <2 nm, which displays comparable toxicity estimates for the majority of the nAg materials assessed. It may therefore be concluded that a combination of both the particle characteristics, i.e. its initial size and surface coating, and environmental factors trigger the toxicity of ion-releasing nanoparticles.

Keywords: nanomaterial, silver, acute toxicity, crustacean, environmental conditions

Introduction

Silver nanoparticles (nAg) are, amongst others driven by their antimicrobial properties (Morones et al., 2005), frequently used for e.g. textile and consumer products (Benn and Westerhoff, 2008). The increasing demand for nAg (Scheringer, 2008) may result in their unintentional release into aquatic environments potentially posing a significant threat to aquatic communities, although physical and chemical processes, such as sulfidation, might significantly lower its toxicity (Levard et al., 2012). With the purpose of characterizing potential environmental risks, several studies investigated the acute and chronic toxicity of different nAg materials to aquatic organisms mostly focusing on the standard test organism *Daphnia magna* (e.g. Asghari et al., 2012; Kennedy et al., 2010; Zhao and Wang, 2010). The outcome of such acute studies displayed highly variable 48-h median effective concentrations (48-h EC_{50}) for nAg ranging from 0.75 to 187 µg/L (Asghari et al., 2012; Lee et al., 2012). Also chronic experiments investigating effects on the fecundity and growth of daphnids revealed comparable differences among studies showing adverse effects at nAg concentrations equal to or higher than 50 µg nAg/L (Blinova et al., 2012; Zhao and Wang, 2010). This highly variable toxicity may be attributed to specific nAg characteristics such as, initial particle size or surface coatings (e.g. Hoheisel et al., 2012; Ma et al., 2011; Zhao and Wang, 2011) which are known to meaningfully influence the release of Ag ions (Ag^+) from nAg (Hoheisel et al., 2012). These Ag^+ are hypothesized as the driver for nAg toxicity (Kennedy et al., 2010). The fate and toxicity of such ions is in turn also determined by environmental parameters such as pH, the amount of organic matter or the presence of complexing agents such as chloride or thiosulfate (Erickson et al., 1998; Ratte, 1999).

Some studies investigated the ion release kinetics of different nAg materials under

varying environmental conditions and observed notable differences (e.g. Dobias and Bernier-Latmani, 2013; Levard et al., 2012; Tejamaya et al., 2011; Thio et al., 2011). Recently Levard et al. (2012) pointed out that a systematic investigation, assessing the implications of nAg characteristics and environmental parameters on aquatic species is needed. This seems particularly important as this gap of knowledge may have unintentionally caused an over- or underestimation of the potential risks associated with the incorporation of nAg in our daily used products and their subsequent release into the aquatic environment.

In this context, the present study investigated the acute and chronic toxicity of differently initially sized coated and uncoated nAg materials as well as silver nitrate (AgNO$_3$) to $D. magna$ using the respective standard test protocols (OECD, 2004 and 2008). By doing so, two pH levels (6.5 and 8.0) as well as two DOM (<0.1 and 8.0 mg TOC/L) levels, which represent environmentally realistic concentrations (Ryan et al., 2009), were considered. Potential effects of silver nitrate (AgNO$_3$), a positive control for Ag$^+$ toxicity, uncoated (~140 nm) and citrate coated silver nanoparticles (Cit nAg; 20, 60, 100 nm) on $D. magna$ were investigated during 48-h acute exposure periods. Subsequently, 21-d chronic experiments with the same test organism were conducted using ~30 nm laboratory synthesized Cit nAg, representing a citrate coated nAg material suggested to be rather toxic, that is also frequently used for toxicity testing (Kennedy et al., 2010; Pokhrel et al., 2013; Römer et al., 2013).

Material and Methods

Material preparation and characterization

Stock solutions of AgNO$_3$ (American Chemical Society reagent, ≥99.0%, Sigma-Aldrich) were prepared in Milli-Q water and similar to all other materials diluted to the desired test concentration in the respective test medium (varying in pH and DOM). The uncoated silver nanoparticle (140 nm uncoated nAg) dispersion was prepared by ultrasonication (amplitude set to 20% at 18°C; Sonopuls, Bandelin, Germany) of 200 mL Milli-Q water amended with 10.0 mg of 35 nm sized nAg powder (99.5% purity; Ionic-Liquids-Technology) for 20 minutes. Subsequently the dispersion was filtered over a nitrocellulose membrane (0.22 µm pore size; Sigma). Exclusively the filtrate was used in the present study. The particles of the filtrate consisted of 90% (mass per mass) aggregates (50-160 nm) as described and characterized in detail by Abraham et al. (2013). The citrate coated silver nanoparticles (Cit nAg; Sigma-Aldrich) with a primary particle size of 20, 60 and 100 nm were purchased as 20 mg/L dispersions. The preparation of the laboratory synthesized ~30 nm Cit nAg followed in general Turkevich et al., (1951) while it was adapted to silver. This procedure resulted in an initial concentration of 94.5 mg Ag/L.

Prior to each experiment or water exchange, any nanoparticle dispersion was analyzed for its initial particle size distribution via dynamic light scattering (Delsa^{TM} Nano C, Beckman Coulter; Tab. 1), whereas for the laboratory synthesized ~30 nm Cit nAg stock solution, additionally transmission electron microscope analyses were performed once to verify the primary particle size (Fig. A.1). Moreover, the average particle size of each nAg material was monitored daily in the test medium during all acute tests (Tab. 1) and once over 3 days representing the time between two water

exchanges during the chronic tests (Tab. 1). To exclude any bias caused by algal food or excretions on the measurements, additional test vessels were used. Samples were taken 2 cm beneath the water surface, which is considered as appropriate for pelagic species such as *Daphnia*, of the respective highest test concentration (which ensured a sufficient intensity) and analyzed immediately.

Table 1: Mean particle size (±SD; n=3) and the 10th (D 10%) and 90th (D 90%) percentile of their particle size distribution for different nAg materials measured in the stock dispersions and test medium exhibiting different pH (6.5 and 8.0) and DOM levels (<0.1 and 8.0 mg TOC/L), respectively. The particle size was assessed over the entire study duration of either 48 h during the acute toxicity tests or representative for the time between a water exchange (over 72 h) during all chronic investigations (~30-nm-Cit nAg).

| | | pH 6.5 | | | | | | | | | | | | | | | |
| | | - DOM | | | | | | | | | + DOM | | | | | | |
silver material	initial particle size [PdI]^a	D 10%	D 90%	t_{0h}	D 10%	D 90%	t_{48h}	D 10%	D 90%	PdI^a	t_{0h}	D 10%	D 90%	t_{48h}	D 10%	D 90%	PdI^a
140-nm-bare nAg	144 (± 25) [0.227]	68 (± 1)	258 (± 51)	317 (± 12)	153 (± 8)	666 (± 13)	307 (± 8)	139 (± 11)	721 (± 26)	0.240 – 0.281	236 (± 11)	109 (± 19)	674 (± 215)	389 (± 163)	79 (± 6)	793 (± 19)	0.287 – 0.333
20-nm-Cit nAg	26 (± 1) [0.309]	12 (± 1)	64 (± 7)	67 (± 2)	24 (± 2)	299 (± 9)	258 (± 10)	54 (± 11)	292 (± 41)	0.137 – 0.349	36 (± 1)	7 (± 3)	185 (± 38)	34 (± 1)	6 (± 3)	148 (± 24)	0.495 – 0.654
60-nm-Cit nAg	68 (± 7) [0.149]	35 (± 7)	122 (± 43)	103 (± 38)	33 (± 2)	176 (± 50)	1010 (± 305)	67 (± 14)	27270 (± 10152)	0.083 – 0.615	NA	NA	NA	NA	NA	NA	NA
100-nm-Cit nAg	106 (± 2) [0.213]	61 (± 2)	156 (± 12)	206 (± 20)	76 (± 9)	149 (± 16)	315 (± 122)	75 (± 5)	551 (± 69)	0.107 – 0.231	NA	NA	NA	NA	NA	NA	NA
				t_{0h}			t_{72h}			PdI^a	t_{0h}			t_{72h}			PdI^a
~30-nm-Cit nAg	35 (± 8) [0.405]	8 (± 3)	204 (± 62)	332 (± 121)	37 (± 12)	390 (± 263)	1460 (± 300)	143 (± 70)	16263 (± 12272)	0.133 – 0.796	266 (± 186)	12 (± 1)	143 (± 42)	NA	NA	NA	0.130 – 0.344
		pH 8.0															
140-nm-bare nAg	144 (± 25) [0.227]	68 (± 1)	258 (± 51)	283 (± 31)	112 (± 16)	1110 (± 10)	266 (± 28)	124 (± 30)	865 (± 221)	0.260 – 0.401	242 (± 27)	67 (± 21)	648 (± 279)	252 (± 38)	121 (± 1)	1171 (± 221)	0.146 – 0.618
20-nm-Cit nAg	26 (± 1) [0.309]	12 (± 1)	64 (± 7)	301 (± 43)	110 (± 12)	1756 (± 308)	1847 (± 220)	242 (± 42)	1029 (± 29)	0.177 – 0.842	92 (± 2)	46 (± 1)	175 (± 11)	827 (± 203)	141 (± 6)	16337 (± 6000)	0.244 – 0.456
60-nm-Cit nAg	68 (± 7) [0.149]	35 (± 7)	122 (± 43)	281 (± 22)	66 (± 2)	319 (± 15)	1013 (± 219)	91 (± 12)	7059 (± 1776)	0.138 – 0.555	NA	NA	NA	NA	NA	NA	NA
100-nm-Cit nAg	106 (± 2) [0.094]	61 (± 2)	156 (± 12)	405 (± 35)	93 (± 2)	2903 (± 396)	560 (± 11)	78 (± 18)	3822 (± 1795)	0.204 – 0.281	NA	NA	NA	NA	NA	NA	NA
				t_{0h}			t_{72h}			PdI^a	t_{0h}			t_{72h}			PdI^a
~30-nm-Cit nAg	35 (± 8) [0.405]	8 (± 3)	204 (± 62)	394 (± 231)	46 (± 10)	515 (± 186)	1045 (± 435)	132 (± 67)	20939 (± 16995)	0.130 – 0.749	320 (± 225)	16 (± 6)	181 (± 60)	257 (± 276)	25 (± 2)	253 (± 19)	0.157 – 0.394

a
Polydispersity Index

For quantification of Ag, one water sample (finally resulting in three measurement replicates) originating from the median concentration tested, but separated by the nAg characteristics and combination of environmental factors (pH and DOM), was taken at the start ($t_{0\,h}$) as well as at the end ($t_{48\,h}$) of each acute toxicity test. Samples of $t_{0\,h}$ were analyzed immediately, while samples of $t_{48\,h}$ were split in two subsamples. One subsample was directly analyzed while the other was subject to ultracentrifugation (t=35 min; 546883 x g, Sorvall WX Ultra Series WX 90; Thermo Fisher Scientific) allowing for a separation of nanoparticles with a size ≥2 nm from Ag^+ and nAg with a particle size <2 nm (Kennedy et al., 2010; Tab. 2). The 2 nm cut-off was calculated considering the centrifugation duration, particle density, rotor radius and rotor speed. With this method and due to the low density of DOM (~ 1.5 g/cm³) if compared to the nanoparticles (~ 10.5 g/cm³), DOM molecules or associated Ag^+ complexes will remain in the supernatant.

Table 2: Mean (±SE; n=3) Ag concentrations (µg/L) for each silver material and environmental scenario (pH and DOM level) investigated. Measurements were performed at different time intervals during the acute and the semi-static experiments by inductively coupled plasma mass spectrometry (Seitz et al., 2013). All samples of the acute toxicity tests were also subjected to an ultracentrifugation process to analyze a respective Ag^+ release after 48 h. NA: data not evaluated.

acute toxicity test

| | | - DOM | | | | | | + DOM | | | | | | |
| | | pH 6.5 | | | pH 8.0 | | | | pH 6.5 | | | pH 8.0 | | |
silver material	nominal	0 h	48 h	48 h[a]	0 h	48 h	48 h[a]	nominal	0 h	48 h	48 h[a]	0 h	48 h	48 h[a]
AgNO$_3$	32.4	27.0 (± 0.1)	27.7 (± 0.2)	27.5 (± 0.1)	31.3 (± 0.1)	24.6 (± 0.1)	22.1 (± 0.1)	32.4	27.3 (± 0.1)	24.6 (± 0.1)	18.0 (± 0.1)	30.0 (± 0.1)	24.8 (± 0.14)	32.0 (± 0.2)
140 nm bare nAg	62.5	42.8 (±0.8)	39.5 (±0.6)	5.5 (±0.1)	38.1 (±0.4)	29.5 (±0.5)	6.0 (± 2.5)	62.5	70.2 (± 0.5)	58.9 (± 0.4)	3.7 (± 0.1)	64.1 (± 0.1)	40.7 (± 0.7)	3.1 (± 0.1)
20 nm Cit nAg	80.0	56.1 (± 0.6)	39.5 (±0.4)	3.4 (± 0.9)	50.8 (± 0.6)	37.1 (± 0.6)	5.0 (± 0.1)	80	62.2 (± 0.3)	67.0 (± 1.1)	2.0 (± 1.2)	67.5 (± 0.4)	62.0 (± 0.7)	0.8 (± 0.8)
60 nm Cit nAg	93.8	27.0 (± 0.7)	22.0 (± 0.6)	0.3 (± 0.1)	26.0 (± 0.5)	21.8 (± 0.5)	1.8 (± 0.1)	NA	NA	NA	NA	NA	NA	NA
100 nm Cit nAg	75.0	41.3 (± 0.8)	33.7 (± 0.8)	2.4 (± 0.1)	36.6 (± 0.7)	21.9 (± 0.6)	1.9 (± 0.1)	NA	NA	NA	NA	NA	NA	NA

chronic toxicity test

	0 h	72 h	0 h	72 h	0 h	72 h	0 h	72 h
~30 nm Cit nAg	73.2 (± 0.1)	49.2 (± 0.1)	73.2 (± 0.1)	51.3 (± 0.1)	78.4 (± 1.5)	51.7 (± 1.3)	75.7 (±0.1)	69.5 (± 0.1)

[a] Following centrifugation

During each chronic test one sample was taken at $t_{0 h}$ and $t_{72 h}$, which reflects the time between two water exchanges over the course of the experiment, exclusively from the highest test concentration applied and processed as detailed above (Tab. 2). Although different time periods were used we assumed a transferability in terms of dissociation of ions from nAg between the acute and chronic experiments, and hence the fraction of Ag^+ and nAg with a particle size <2 nm was not quantified again. The final analytical quantification of Ag (mass 107) was carried out using a Quadrupole ICP-MS (XSeries2, Thermo Fischer Scientific, Germany) equipped with a FAST autosampler (ESI, Thermo Fischer Scientific, Germany), a peek spray chamber (Thermo Fischer Scientific, Germany) as well as a robust Mira Mist peek nebulizer (Burgener, United Kingdom). For the ultracentrifuged samples, speciation of Ag^+ was calculated (Tab. A.1) based on the assumption that the particle size fraction <2 nm solely represents Ag^+ species. The calculation was performed for each silver material and environmental scenario (pH and DOM level) considering the composition of the test medium at the highest test concentration, using Visual MINTEQ software version 3.0 (Gustafsson, 2011; Tab. A.1). Formation of complexes between metal cations and DOM molecules was calculated using the Stockholm Humic Model (SHM; Gustafsson, 2001) assuming that all DOM is comprised of fulvic acid (FA) containing 50% carbon. This assumption seems reasonable as seaweed extract is mainly composed of alginates with high amounts of carboxylic functional groups (Fourest and Volesky, 1996; Fourest and Volesky, 1997), which is in its properties comparable to fulvic acid. The calculations were performed for a CO_2 equilibrium between air and aqueous phase.

Test organism

Daphnia magna were cultured under standardized procedures in a climate controlled chamber (Weiss Environmental Technology, Germany) at 20±1°C with a 16:8 h (light:dark) photoperiod. Therefore, modified ASTM reconstituted hard freshwater (ASTM, 2007) amended with additions of selenium, vitamins and seaweed extract (Seitz et al., 2013) was used as culture medium. Daphnids were daily fed with the green algae *Desmodesmus* sp. (200 µg C/organism).

General test design

ASTM reconstituted hard freshwater (Bundschuh et al., 2012) containing selenium and vitamins (test medium) was used for all experiments. In order to account for varying pH levels the test medium was additionally buffered with 3-*N*-Morpholino-propanesulfonic acid (De Schamphelaere and Janssen, 2004) and finally adjusted to a pH of either 6.5 and 8.0 using 2 molar hydrochloric acid and sodium hydroxide, respectively. Moreover, with respect to the wide presence of DOM in natural surface waters (Ryan et al., 2009), two levels of commercially available seaweed extract (Marinure[®], Glenside), namely <0.1 (= absence of DOM) or 8.0 mg TOC/L (= presence of DOM), were established in the test medium. All experiments were conducted under temperature and light conditions, described in section 3.2. Dissolved oxygen and pH were measured using a WTW Multi 340i set (WTW Inc.; Germany) and met the water quality requirements (Tab. A.2) of the respective test guidelines (OECD 2004 and 2008).

48-h acute toxicity tests

The 48-h acute toxicity tests were conducted according to the OECD guideline 202 (OECD, 2004): Twenty juveniles (age <24 h) per treatment were placed in groups of five in 50 mL test medium, previously adjusted to one set of environmental conditions described in section 3.3. Each (n)Ag material tested in the present study was used in an acute toxicity test at pH levels of either 6.5 or 8.0 in the absence of DOM. In the presence of DOM at both pH levels only the most toxic materials were tested, namely $AgNO_3$, 140 nm uncoated nAg and 20 nm Cit nAg. Based on the expected acute toxicity (relying on results of own preliminary tests; data not shown), the chosen test concentrations differed among all Ag materials applied and thus ranged from 0.4 to 32.4 µg/L for $AgNO_3$, from 0.1 to 62.5 µg/L for 140 nm uncoated nAg, from 10.0 to 160.0 µg/L for 20 nm Cit nAg, from 47.9 to 750.0 µg/L for 60 nm Cit nAg and, from 75.0 to 1200.0 µg/L for 100 nm Cit nAg. Mobility of daphnids was checked every 24 hours. As our chemical analysis revealed significant differences between the nominal and measured initial concentrations for most of the silver materials applied in the present study (Tab. 2), the following results refer to the measured silver concentrations.

21-d chronic toxicity tests

The chronic reproduction experiments were performed using ~30 nm sized laboratory synthesized Cit nAg. This nAg material is (although it exhibits an approximately three-fold lower ecotoxicological potential relative to 20 nm Cit nAg) still considered as comparably toxic to daphnids (Tab. A.3). Each of the four semi-static reproduction tests (pH 6.5 or 8.0 with either a DOM level of <0.1 or 8.0 mg TOC/L) followed the

OECD guideline 211 (OECD, 2008), and fulfilled (irrespective of the environmental scenario applied) the validity criteria. At the initiation of each 21-d experiment, 10 juvenile (age <24 h) daphnids were placed individually in 50 mL test medium amended with the respective nAg concentration (n=10). The test medium was renewed three times a week, including a careful transfer of adult daphnids to the new test medium by using disposable Pasteur pipettes. For each reproduction test, the test organisms were exposed to several concentrations of ~30 nm Cit nAg, i.e. 0.0, 1.0, 3.0, 9.0, 26.0 to 78.0 µg/L. Daphnids were fed daily in an age dependent manner with *Desmodesmus* sp. (50–100 µg C/organism). Mortality as well as the number of released offspring was checked every 24 h.

Statistical analyses

Acute toxicity data was adjusted for control mortality with Abbott's formula, if necessary (e.g. if exceeding 0% but no more than 20%), and analyzed for respective 48-h effective median concentration (48-h EC_{50}) by fitting adequate dose-response models. Model selection was based on Akaike information criterion and expert judgment. Gained EC_{50} values were compared among treatments via confidence interval testing to test for statistical significant differences (Wheeler et al., 2006). Similarly, confidence interval testing was applied to judge statistical significance among different exposure conditions during the chronic experiments, while the basis for these calculations was the mean differences in cumulative reproduction between the respective control and the lowest observed effect concentration (LOEC; in either case 78.0 µg Cit nAg/L; Altman et al., 2000). In addition a comparative time to event (death) analysis was performed by separately applying the Kaplan-Meier estimator for the data of each environmental scenario at 78.0 µg Cit nAg/L, as this was the only

concentration causing mortality during each chronic toxicity test. Statistical analyses and respective figures were accomplished with the statistical software environment R for Windows (Version 2.15.3; 2013) and corresponding packages (Hothorn et al., 2008; Lemon, 2010; Ritz and Streibig, 2005; Therneau, 2013).

Results and Discussion

Nanoparticle characteristics affect their ecotoxicity

The acute toxicity of AgNO$_3$ and the role of silver ions

Independently of the composition of the test medium, AgNO$_3$ always displayed the highest toxicity of all silver materials investigated in the present study. AgNO$_3$ revealed 48-h EC$_{50}$ ranging from ~1.7 (at lower levels of pH and DOM; Fig. 1A-B; Tab. A.3 and A.4) to ~3.0 µg Ag/L (at higher levels of pH and DOM; Fig. 1A-B; Tab. A.3 and A.4). These results are in accordance with literature data for *D. magna* reporting 48-h EC$_{50}$ values of up to 2.5 µg/L (Zhao and Wang, 2010, 2011) and other studies (e.g. a review by Ratte, 1999) that indicate environmental parameters such as pH and DOM influencing heavy metal related toxicity by controlling the bioavailability of their toxic ions. In detail, AgNO$_3$ toxicity has been attributed to free Ag$^+$, which can induce ion regulatory disturbances in the gill system of *Daphnia* by mimicking endogenous ions (Bianchini et al., 2002; Völker et al., 2013). In contrast, the mechanism of nAg related toxicity is not yet fully understood. Völker et al. (2013) suggested that adverse effects (e.g. oxidative stress, damage of proteins, etc.) can frequently be explained by a combination of Ag$^+$ released from the nanoparticle into the test medium and the silver nanoparticles themselves, as both have the potential i)

to induce reactive oxygen species (ROS), ii) to interact with cellular enzymes and iii) to mimic endogenous ions. Irrespective of the underlying mechanisms, the acute toxicity tests of the present study suggest an approximately 6 and 40 times higher ecotoxicological potential of $AgNO_3$ relative to uncoated nAg and 20 nm Cit nAg, respectively (Fig. 1A-B; Tab. A.4).
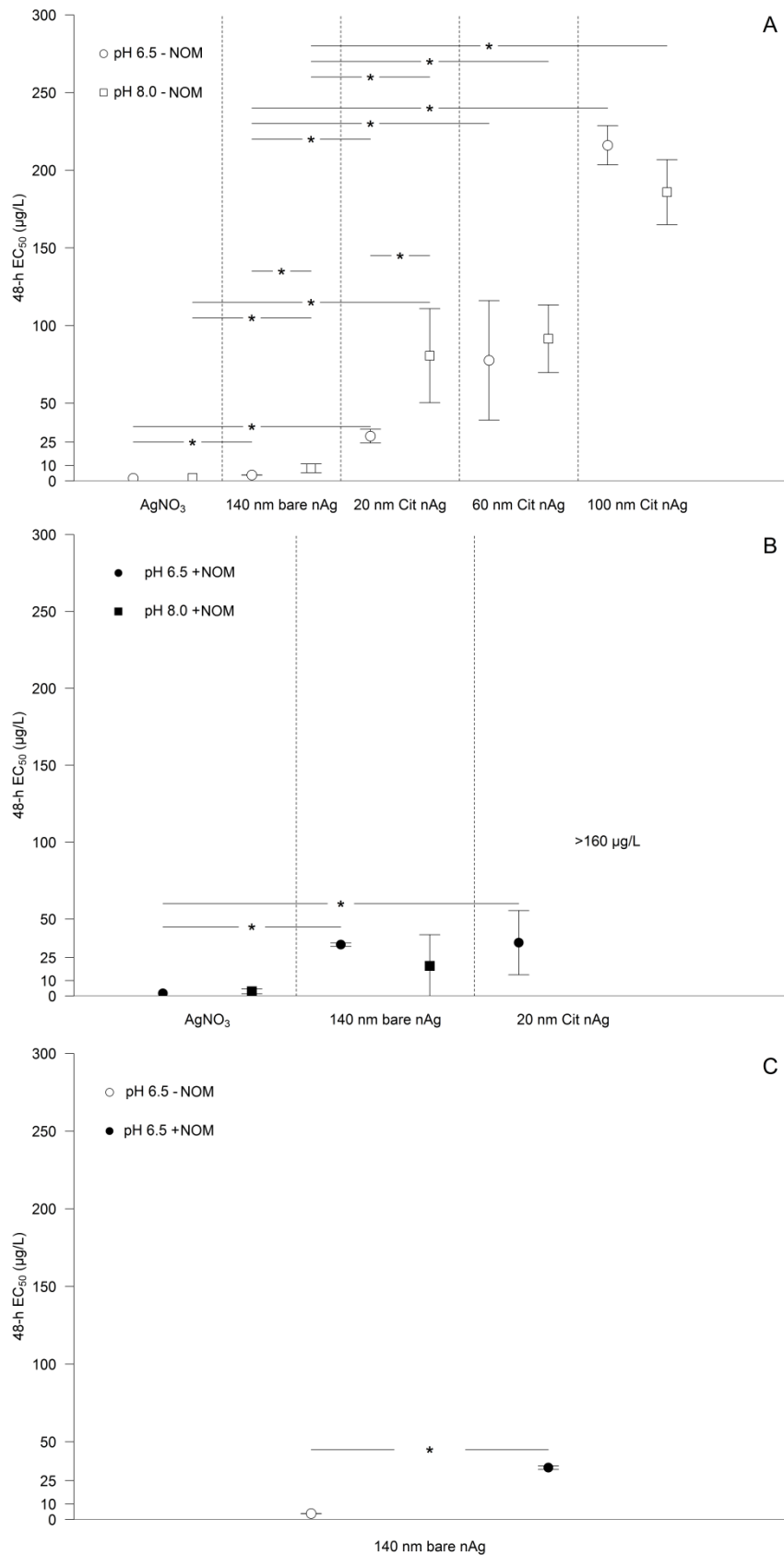
Figure **1A-C**: 48-h EC_{50} values (with 95 % CIs) of different silver materials at varying pH levels 6.5 and 8.0 in the (+) presence and (-) absence of dissolved organic matter (DOM; <0.1 and 8.0 mg TOC/L). Asterisks (*) denote statistically significant differences between 48-h EC_{50} values.

The role of particle coating and initial particle size for the acute toxicity of nAg

The 140 nm uncoated nAg delivered (irrespective of the environmental conditions assessed) 48-h EC_{50} values (3.9 - 33.4 µg/L; Fig. 1A-C; Tab. A.3 and A.4) in agreement with literature data on nAg in general (0.75 to 187 µg/L; cf. Allen et al., 2010; Asghari et al., 2012; Gaiser et al., 2011; Lee et al., 2012). The broad range of EC_{50} values among published studies can be explained by differing particle characteristics, e.g. particle coating and initial particle size (Allen et al., 2010), triggering agglomeration state and finally nanoparticles' surface-to-volume ratio (Hoheisel et al., 2012). The latter in turn has been related directly to the Ag^+ release from the nAg into the test medium (Hoheisel et al., 2012; Kennedy et al., 2010; Zhao and Wang, 2011), which may trigger nAg related effects.

This assumption is further underpinned by the generally lower toxicity of citrate-coated nAg relative to uncoated nAg. The 20 nm Cit nAg-treatment, which represented the most toxic citrate coated nAg, exhibited an up to ~10-fold lower toxicity relatively to the 140 nm uncoated nAg (Fig. 1A-B; Tab. A.4). Moreover, larger citrate-coated nAg, i.e. 60 and 100 nm Cit nAg, were again up to a factor of approximately 7.5 less toxic than their smaller counterpart, namely 20 nm Cit nAg (Fig. 1A; Tab. A.4). This phenomenon was observed for a majority of Cit nAg except for 60 and 100 nm Cit nAg at a pH of 6.5 in the absence of DOM, which we attribute to methodological shortcomings of the nAg analysis as discussed further below. This relation distinctly underpins the importance of determining the particle size at the test initiation, especially as the particle size and the respective polydispersity index of all Cit nAg products increased over time (Tab. 1). The nAg initial size inversely correlates with their capability to release Ag^+ (Hoheisel et al., 2012), and thus explains to some extent the induced toxicity of nAg since the type of coating was the

same for these Ag materials. Furthermore, besides citrate other types of coating are available, that may alter the ecotoxicological potential of nAg by their efficiency of limiting Ag^+ release (Liu et al., 2010).

In addition, our Ag analysis, which displayed a higher fraction of Ag^+ and nAg <2 nm in the test medium for 140 nm uncoated nAg (Ag^+ and nAg <2 nm fraction: ~ 16% at pH 8.0 in absence of DOM) and for smaller initial size classes of citrate-coated nAg (up to 10% for 20 nm Cit nAg) compared to the other nAg materials investigated in the present study (i.e. 60 and 100 nm Cit nAg at pH 8.0 in the absence of DOM; Tab. 2; Tab. A.4) support this hypothesis. Moreover, as the majority of 48-h EC_{50} values normalized to the Ag concentration with a particle size <2 nm, composed of Ag^+ and very small nAg, were (independent of the environmental conditions) at a similar level as $AgNO_3$, especially uncoated and 20 nm citrate coated nAg (Fig. A.2), this hypothesis is further facilitated. However, also considerable deviations among the normalized 48-h EC_{50} values (e.g. 20 or 60 nm Cit nAg at pH 8.0 in the absence of DOM) were observed (Fig. A.2). This may be explained by the analytical method applied (cf. Kennedy et al., 2010) which may have overestimated the Ag^+ concentration, and hence underestimated the observed toxicity, as no additional filtering step prior to the analysis was involved potentially including fractions of nAg slightly larger than 2 nm (e.g. Allen et al., 2010). Nonetheless, the results of the present study suggest that a large proportion of nAg related effects are attributed to the concentrations of Ag^+ and nAg <2 nm.

Effects on the acute nAg toxicity

As both the Ag^+ and the nanoparticle itself are supposed to contribute to the observed ecotoxicity of nAg, environmental conditions in the test medium, which trigger the fate and release of metal ions from e.g. nAg (Levard et al., 2012), might go along with alterations in toxicity, as reported for silver and copper (e.g. De Schamphelaere and Janssen, 2004; Ratte, 1999). Indeed, the present study uncovered that higher levels of pH and DOM meaningfully reduced the acute but also chronic ecotoxicity of Ag materials (Fig. 1A-C and 2A-B; Tab. A.4), underpinning the importance of environmental conditions and their interaction for the ecotoxicological potential of both $AgNO_3$ and more importantly nAg.

In detail, at a pH of 8.0, the acute toxicity of $AgNO_3$ was approximately 1.5-fold lower in the presence of DOM (48-h EC_{50}: 3.02 µg Ag/L) relative to all other exposure scenarios (i.e. pH of 6.5 in presence or absence of DOM; pH of 8.0 in absence of DOM; Fig. 1B, Tab. A.3), which may be explained by a reduced bioavailability of free Ag^+ (e.g. review by Ratte, 1999). This is, however, not supported by our ICP-MS analysis, which uncovered no meaningful reduction (when directly related to the observed toxicity) of the Ag^+ and nAg < 2 nm fraction in the medium in presence of DOM (Tab. 2). As this method measures Ag^+, irrespective of whether the ions are bioavailable or complexed by DOM, the observations do not contradict each other. Nevertheless, Ag species modeling suggests a maximal percentage of Ag^+ complexed and electrostatically (weakly) bound to DOM of 5% and 0.05% respectively (Tab. A.1). Thus, the observed reduction in toxicity cannot solely be explained by a decreased availability of Ag^+ due to the formation of Ag-DOM

complexes. However, DOM was hypothesized to reduce the interaction of free Ag$^+$ with the sodium uptake pathway in fish (i.e. *Oncorhynchus mykiss*) and respective toxicity (Janes and Playle, 1995). As likewise mechanisms are suggested to be the driving factor for the acute toxicity of AgNO$_3$ towards *Daphnia* (Bianchini and Wood, 2003) similar explanations may hold also true for the present study, while this effect was not observable at the low pH-level.

In contrast to this observation, the approximately 9-fold reduced toxicity of uncoated nAg in the presence compared to the absence of DOM (Fig. 1C; Tab. A.4) comes along with a reduction of the Ag$^+$ and nAg <2 nm by 33% (pH 6.5; Tab. 2). This effect may thus be explained by the relevance of Ag$^+$ for the toxicity of nAg (Tab. 2). Dissolved organic matter may have coated the initially uncoated nAg and thereby blocked oxidation sites finally reducing a further Ag$^+$ release from the material (Liu and Hurt, 2010). This explanation seems reasonable on the basis of the chemical analysis (Tab. 2). A similar process may have occurred for most of the Cit nAg materials investigated in the present study further reducing the release of Ag$^+$ (cf. Fabrega et al., 2009; Newton et al., 2013) and subsequently also toxicity in the presence of DOM as well as at higher pH-levels (Fig. 1A-C; Tab. A.4), which is widely underpinned by our analytical results (Tab. 2).

Effects on the chronic nAg toxicity

The previously stated hypothesis is further supported by our chronic investigations: Concentrations of ~30 nm Cit nAg as high as 26.0 µg/L (no observed effect concentration) did not adversely affect the reproductive output of *D. magna* (Tab. A.5). However, at the highest test concentration, which is equivalent to the LOEC (pairwise Wilcoxon rank sum tests: $p<0.05$), lower levels of pH and DOM significantly

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decreased the number of offspring released (Fig. 2A). Additionally, the time to death analysis showed a similar tendency (Fig. 2B): A pH of 6.5 caused 90% (without DOM) or 40% (with DOM) mortality of adult daphnids (Fig. 2B) after 21 d exposure to 78.0 µg ~30 nm Cit nAg/L. A higher pH of 8.0 at the same concentration displayed mortalities ranging from 20% (with DOM) to 30% (without DOM).
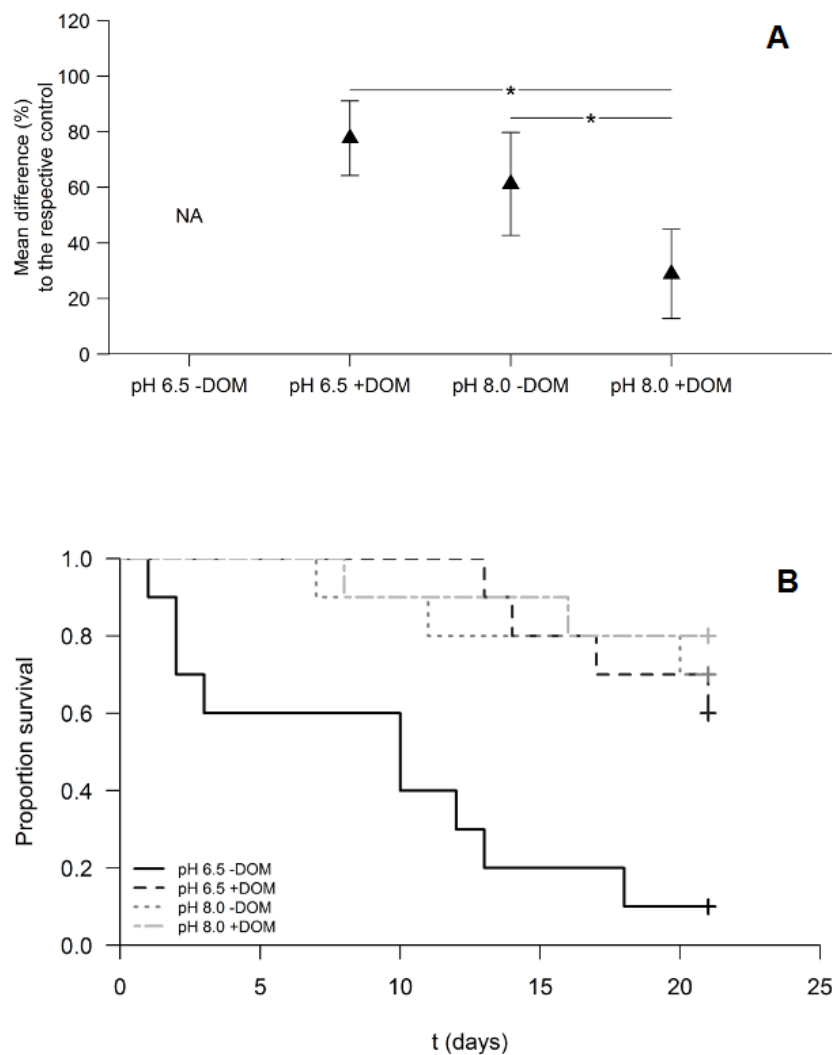
Figure 2:   (A) Mean difference in the reproduction of *Daphnia* (±95 % CIs; n=10) to the respective control, when exposed to 78.0 µg/L (●) 30-nm-Cit nAg under differing pH (6.5 or 8.0) and DOM (<0.1 or 8.0 mg TOC/L) levels after 21 days of exposure. Asterisks (*) denote statistically significant differences between respective environmental scenarios.(B) Proportion survival distribution of *Daphnia* in the time course of a 21-d exposure to 78.0 µg/L 30-nm-Cit nAg under different environmental scenarios exhibiting pH levels of either 6.5 or 8.0 in the absence (-) or presence (+) of DOM. Censored individuals that survived beyond the end of the experiment are indicated by +.

These data suggest that the presence of DOM can reduce toxicity of Cit nAg up to 50% (Fig. 2B), which is in accordance with an earlier study by Blinova et al. (2012): These authors observed a decreasing toxicity of two differently coated nAg with increasing concentrations of DOM (5-35 mg C/L) in different natural surface waters, while not considering among others the factor pH. Those observations may be explained, in addition to the factors detailed above, by the utilization of DOM by *Daphnia* as energy source potentially resulting in a higher tolerance of the test specimen (Bergman Filho et al., 2011).

Conclusion

The present study clearly showed how particle characteristics (i.e. the presence or absence of particle surface coating and initial particle size) as well as varying environmental conditions (i.e. pH and DOM) considerably influence the ecotoxicological potential particularly of silver nanoparticles. As a consequence, future investigations are urged considering the diversity of nanoparticle characteristics, their fate and ecotoxicological potential under varying, field relevant environmental conditions, which are by no means limited to pH and DOM. This would result in a scientifically sound basis allowing for a more precise, but also reasonable risk prediction under near natural conditions.

Appendix A. Supplementary data

The supplementary data contains further details about the initial particle size of laboratory sysnthesized ~30 nm Cit nAg (Fig. A.1), the results of the species calculation for ultracentrifuged samples (Tab. A.1), the applied statistical analyses, measured environmental parameters (Tab. A.2), acute toxicity data of all Ag materials (Tab. A.3), an overview of their statistical comparisons (Tab. A.4) as well as Ag^+/nAg <2 nm normalized EC_{50} values (Fig. A.2). Furthermore respective data for the chronic toxicity tests with 30 nm Cit nAg are provided in Tab. A.5.

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SUPPLEMENTARY MATERIAL

of Appendix A.4

EFFECTS OF SILVER NANOPARTICLE PROPERTIES, MEDIA PH AND DISSOLVED ORGANIC MATTER ON TOXICITY TO *DAPHNIA MAGNA*

Frank Seitz, Ricki R. Rosenfeldt, Katharina Storm, George Metreveli, Gabriele E. Schaumann, Ralf Schulz, Mirco Bundschuh

1. Sample preparation

As described in the manuscript, one subsample was directly analyzed for Ag content while the other was subject to ultracentrifugation (t=35 min; 80000 rpm, Sorvall WX Ultra Series WX 90; Thermo Fisher Scientific). The latter step allowed for a separation of nanoparticles with a size ≥2 nm from Ag^+ and nAg with a particle size <2 nm (Kennedy et al., 2010; Table 2). In order to exclude loss of any (n)Ag in terms of adsorption to e.g. the wall of the test vessel or analysis container, each subsample was prior to its analysis two-fold diluted using acidified (HNO_3) MILI-Q water.

Reference:

Kennedy, A.J., Hull, M.S., Bednar, A.J., Goss, J.D., Gunter, J.C., Bouldin, J.L., Vikesland, P.J., Steevens, J.A., 2010. Fractionating nanosilver: Importance for determining toxicity to aquatic test organisms. Environ. Sci. Technol. 44, 9571-9577.

100 nm
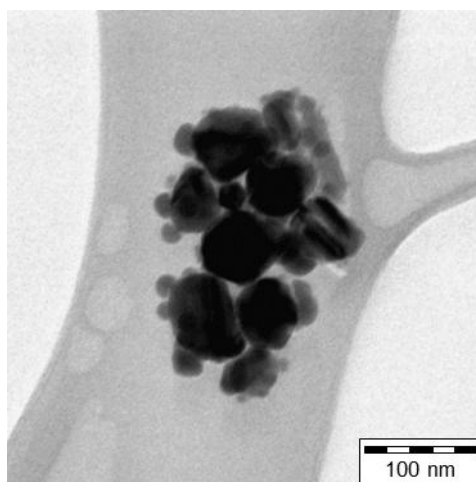
Figure A.1: Transmission electron microscopy image of the laboratory synthesized 30 nm Cit nAg stock solution (LEO 922 OMEGA, Germany). For respective analysis samples were placed onto carbon coated copper grid using applying ultrasonic nebulisation.

2. Statistical analyses

Reproduction data was initially assessed for normal distribution and variance of homogeneity using the Shapiro-Wilk- and Bartlett-Test, respectively. If requirements for parametric testing were met, one-way ANOVAs and subsequent Dunnett's post hoc tests were accomplished to determine statistically significant differences ($p < 0.05$) among all tested treatments. In case the requirements were not met nonparametric alternatives, namely Kruskal-Wallis tests and pairwise Wilcoxon rank sum tests, were performed.

Table A.1: Speciation of silver (in % of total Ag^+ species concentration) for each silver material and environmental scenario (pH and DOM level). Calculations were performed based on the assumption that the ultracentrifuged samples (<2 nm) solely contain Ag^+ species.

silver material			Ag $<2nm^a$	Ag^+	AgCl(aq)	$AgSO_4^-$	$AgCl_2^-$	$AgCl_3^{2-}$	AgOH(aq)	$Ag(OH)_2^-$	$AgSeO_3^-$	$Ag(SeO_3)_2^{3-}$	Ag-FA^b	Ag-FA(el)^c
AgNO₃	pH 6.5	- DOM	28	82.03	16.20	1.62	0.15	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	0	0
		+ DOM	18	77.86	15.38	1.54	0.15	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	5.04	0.04
	pH 8.0	- DOM	22	82.08	16.15	1.61	0.15	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	0	0
		+ DOM	32	78.04	15.35	1.54	0.15	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	4.87	0.05
140 nm uncoated nAg	pH 6.5	- DOM	5	82.02	16.20	1.62	0.15	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	0	0
		+ DOM	4	77.82	15.37	1.54	0.15	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	5.08	0.04
	pH 8.0	- DOM	6	82.07	16.15	1.61	0.15	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	0	0
		+ DOM	3	77.97	15.35	1.54	0.15	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	4.94	0.05
20 nm Cit nAg	pH 6.5	- DOM	3	82.02	16.20	1.62	0.15	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	0	0
		+ DOM	2	77.81	15.37	1.54	0.15	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	5.09	0.04
	pH 8.0	- DOM	5	82.07	16.15	1.61	0.15	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	0	0
		+ DOM	1	77.97	15.35	1.54	0.15	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	4.95	0.05
60 nm Cit nAg	pH 6.5	- DOM	0.3	81.97	16.19	1.62	0.15	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	0	0
		+ DOM	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
	pH 8.0	- DOM	2	82.08	16.15	1.61	0.15	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	0	0
		+ DOM	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
100 nm Cit nAg	pH 6.5	- DOM	2	82.02	16.20	1.62	0.15	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	0	0
		+ DOM	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
	pH 8.0	- DOM	2	82.06	16.15	1.61	0.15	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	0	0
		+ DOM	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA

^{a}Ag concentrations in µg/L (48 h) after centrifugation (values from Tab. 2); ^{b}Ag complexed with fulvic acid; ^{c} electrostatically bound silver to fulvic acid; NA = not assessed

Table A.2: Environmental quality parameters under different testing conditions, adhering to pH levels of either 6.5 or 8.0 in the absence (-) or presence (+) of dissolved organic matter (DOM; <0.1 or 8 mg TOC/L). Measurements were made at the beginning of the bioassay ($t_{0\,h}$) as well as after 48 h of exposure ($t_{48\,h}$) to the respective silver product. NA = not assessed.

	nominal pH 6.5							
	-DOM				+DOM			
	$t_{0\,h}$			$t_{48\,h}$	$t_{0\,h}$			$t_{48\,h}$
silver material	O_2 (mg/L)	Temp. (°C)	pH	pH	O_2 (mg/L)	Temp. (°C)	pH	pH
AgNO$_3$	7.2	19.4	6.5	6.6	7.1	19.4	6.5	6.5
140 nm uncoated nAg	6.5	19.5	6.5	6.7	6.9	19.5	6.3	6.6
20 nm Cit nAg	8.4	19.7	6.5	6.7	8.2	20.1	6.4	6.6
60 nm Cit nAg	7.3	20.0	6.4	6.7	NA	NA	NA	NA
100 nm Cit nAg	7.9	20.0	6.4	6.7	NA	NA	NA	NA
30 nm Cit nAg	7.9	20.0	6.4	6.7	8.0	20.1	6.4	6.7
	nominal pH 8.0							
AgNO$_3$	7.1	19.4	8.0	8.0	7.0	19.6	7.9	8.0
140 nm uncoated nAg	6.6	19.5	8.0	7.9	7.0	20.3	8.0	7.9
20 nm Cit nAg	8.2	20.1	8.0	8.0	8.2	20.6	8.0	8.0
60 nm Cit nAg	7.1	19.9	8.0	8.1	NA	NA	NA	NA
100 nm Cit nAg	7.9	19.7	8.0	8.2	NA	NA	NA	NA
30 nm Cit nAg	7.9	20.0	8.0	8.1	8.0	20.1	8.0	8.0

Table A.3: 48-h EC_{50} values (and respective 95% CI; µg/L) of each nAg material tested under varying environmental conditions, exhibiting pH levels of either 6.5 or 8.0 in the absence (-) or presence (+) of dissolved organic matter (DOM; <0.1 or 8 mg TOC/L).

silver material	48-h EC_{50} (95% CI)			
	pH 6.5		pH 8.0	
	-DOM	+DOM	-DOM	+DOM
$AgNO_3$	1.7 (1.7 -1.8)	1.7 (1.7 - 1.8)	1.9 (1.9 - 2.0)	3.0 (1.5 - 4.6)
140 nm bare nAg	3.9 (3.8 - 3.9)	33.4 (32.2 - 34.6)	8.1 (5.3 - 10.9)	19 (0.0 - 39.9)
20 nm Cit nAg	28.9 (24.4 -33.3)	34.7 (13.8 - 55.6)	80.6 (50.3 - 110.9)	NA
30 nm Cit nAg	125.8 (66.3 - 185.4)	105.3 (35.5 – 175.0)	374.2 (213.8 - 534.7)	NA
60 nm Cit nAg	77.6 (39.1 - 116.06)	NA	91.6 (69.78 - 113.32)	NA
100 nm Cit nAg	216.1 (203.4 - 228.7)	NA	185.9 (165.0 - 206.8)	NA

NA = not assessed

Table A.4: Comparison between 48-h EC_{50} values of each silver material at varying environmental conditions. Thereby, **X** indicates a statistical significant difference between 48-h EC_{50} values of those products and environmental conditions listed in the respective column and row. **NS** represents a non statistical significant difference and **NA** a non computable comparison. Remaining empty fields represent not assessed comparisons.

silver material (environmental conndition)	AgNO₃ (pH 6.5; -DOM)	AgNO₃ (pH 6.5; +DOM)	AgNO₃ (pH 8.0; -DOM)	AgNO₃ (pH 8.0; +DOM)	140 nm bare nAg (pH 6.5; -DOM)	140 nm bare nAg (pH 6.5; +DOM)	140 nm bare nAg (pH 8.0; -DOM)	140 nm bare nAg (pH 8.0; +DOM)	20 nm Cit nAg (pH 6.5; -DOM)	20 nm Cit nAg (pH 6.5; +DOM)	20 nm Cit nAg (pH 8.0; -DOM)	20 nm Cit nAg (pH 8.0; +DOM)	60 nm Cit nAg (pH 6.5; -DOM)	60 nm Cit nAg (pH 6.5; +DOM)	60 nm Cit nAg (pH 8.0; -DOM)	60 nm Cit nAg (pH 8.0; +DOM)	100 nm Cit nAg (pH 6.5; -DOM)	100 nm Cit nAg (pH 6.5; +DOM)	100 nm Cit nAg (pH 8.0; -DOM)	100 nm Cit nAg (pH 8.0; +DOM)
AgNO₃ (pH 6.5; -DOM)					X				X											
AgNO₃ (pH 6.5; +DOM)						X				X										
AgNO₃ (pH 8.0; -DOM)							X				X									
AgNO₃ (pH 8.0; +DOM)								NS				NA								
140 nm bare nAg (pH 6.5; -DOM)	X					X	X		X				X				X			
140 nm bare nAg (pH 6.5; +DOM)		X			X					NS										
140 nm bare nAg (pH 8.0; -DOM)			X		X						X				X				X	
140 nm bare nAg (pH 8.0; +DOM)				NS								NA								
20 nm Cit nAg (pH 6.5; -DOM)	X				X						X									
20 nm Cit nAg (pH 6.5; +DOM)		X				NS														
20 nm Cit nAg (pH 8.0; -DOM)			X				X		X											
20 nm Cit nAg (pH 8.0; +DOM)				NA				NA												
60 nm Cit nAg (pH 6.5; -DOM)					X										NS					
60 nm Cit nAg (pH 6.5; +DOM)																				
60 nm Cit nAg (pH 8.0; -DOM)							X						NS							
60 nm Cit nAg (pH 8.0; +DOM)																				
100 nm Cit nAg (pH 6.5; -DOM)					X														NS	
100 nm Cit nAg (pH 6.5; +DOM)																				
100 nm Cit nAg (pH 8.0; -DOM)							X										NS			
100 nm Cit nAg (pH 8.0; +DOM)																				

Table A.5: Mean reproduction, expressed in percent relative to the respective control (±sd; %; initial n = 10) of *D. magna* after 21 d exposure to different 30 nm Cit nAg concentrations.

concen-tration (µg nAg/L)	0.00	1.00	3.00	9.00	26.00	100.00	0.00	1.00	3.00	9.00	26.00	100.00
product	pH 6											
	-DOM						+DOM					
30 nm Cit nAg	100 (±21.8)	102.2 (±33.2)	101.4 (±19.0)	108.0 (±12.9)	128.7 (±27.0)	NA	100 (±5.2)	107.3 (±6.7)	100.2 (±9.3)	103.8 (±6.2)	100.1 (±19.2)	22.3 (±19.0)
	pH 8											
	100 (±10.8)	101.3 (±13.1)	100.4 (±12.1)	92.8 (±7.2)	99.8 (±20.8)	38.7 (±24.6)	100 (±8.3)	96.7 (±8.7)	96.7 (±10.9)	96.0 (±7.9)	92.62 (±16.0)	71.1 (±22.2)

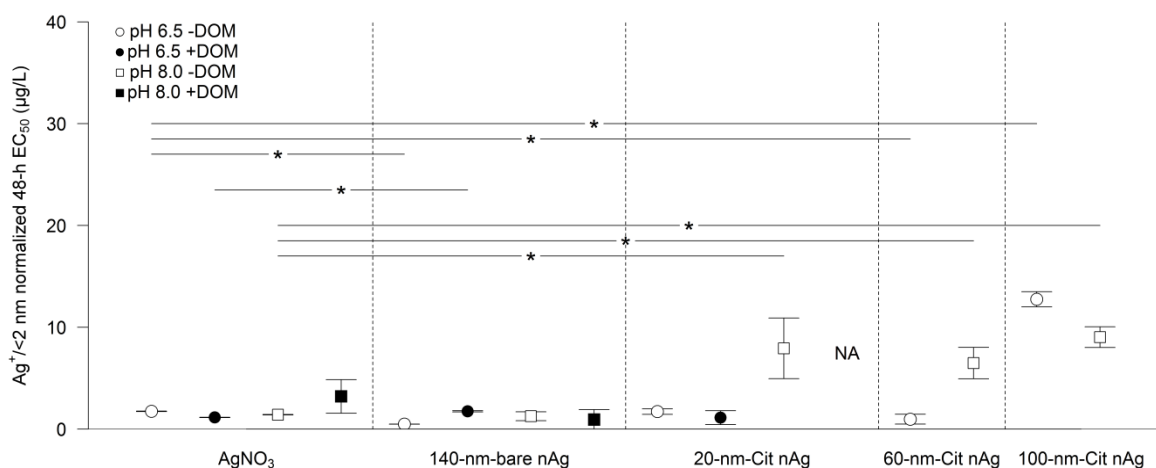NA= not calculable due to too low number of survivors

Figure A.2: 48-h EC_{50} values (with 95 % CIs) of *Daphnia magna* for each silver material as well as under varying environmental conditions which were assessed in the present study, i.e. pH levels of either 6.5 or 8.0 in the absence or presence of dissolved organic matter (DOM; <0.1 or 8 mg TOC/L). The 48-h EC_{50} values were normalized to the Ag concentration with a particle size below 2 nm, and are hence potentially composed of Ag^+ ions and very small nAg.

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of

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School

1995-2004:	Friedrich-Magnus-Schwerd-Gymnasiums, Speyer, Germany
2004:	Abitur

Basic military service

2004-2005:	III. Luftwaffenausbildungsregiment, Germersheim, Germany

Education and Career

2005:	Environmental sciences at the University of Koblenz-Landau, Campus Landau, Germany (diploma program)
2008:	Intermediate diploma in Environmental sciences
2009:	Change to the Master program Ecotoxicology at the University of Koblenz-Landau, Campus Landau, Germany
2010:	B.Sc. Environmental sciences (final grade: 2.2)
2011:	M.Sc. Ecotoxicology (final grade: 1.3)
2012:	PhD scholarship at the University of Koblenz-Landau, Campus Landau, Germany
2015:	Employee at the University of Koblenz-Landau, Campus Landau, Germany
	Co-founder of nEcoTox Consult (Schifferstadt, Germany). A spin-off company from the University of Koblenz-Landau.

Internship

2008:   Three-month internship at the BASF, Limburgerhof, Germany

- Terrestrial Ecotoxicology
- Accomplishment of standard- and nonstandard toxicity tests with honey bees (*Apis mellifera*), metallic wood-boring beetle (*Poecilus cupreus*) and spider mites (*Typhlodromus pyri*)

2009:   Three-month internship at the Virginia Institute of Marine Science, Gloucester Point, VA, USA

- Department of Environmental and Aquatic Animal Health
- Working group of Prof. Dr. Michael C. Newman
- Participating in the "Southriver mercury project" (Accomplishment of mercury quantification in fish, sampling and editing for GIS-data)
- Establishment of a test design allowing to evaluate potential effects of contaminated sediments on *Hyallela azteca*
- Tutorial for the use of the statistic software R

Student research assistant

2008-2011:   Student research assistant at the University of Koblenz-Landau, Campus Landau, Germany

- Aquatic ecotoxicology
- Investigations with titanium dioxide nanoparticles and *Daphnia magna*
- Supporting assistant in the project "MicroPoll"; Evaluation of ozonation as part of an extended wastewater treatment process
- Tutorial for the use of the statistic software R

Memberships

since 2010:   Society of Environmental Toxicology and Chemistry (SETAC)

since 2013:   European Cooperation in Science and Technology (COST) - The transfer of engineered nanomaterials from wastewater treatment & stormwater to rivers (ENTER)

Publications

19 peer-reviewed articles exhibiting 84 citations (excluding self-citations; Web of Science, 3rd of August 2015); h-index: 7.

- Rosenfeldt, R.R., **Seitz, F.**, Zubrod, J.P., Feckler, A., Merkel, T., Lüderwald, S., Bundschuh, R., Schulz, R., Bundschuh, M., 2015. Does the presence of titanium dioxide nanoparticles reduce copper toxicity? A factorial approach with the benthic amphipod Gammarus fossarum. Aquat. Toxicol. 165, 154-159.

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