COMBINED TOXICITY OF NANOPARTICULATE TITANIUM DIOXIDE PRODUCTS AND HEAVY METALS CONSIDERING VARIOUS ENVIRONMENTAL PARAMETERS

by

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DECLERATION

I hereby declare that I autonomously conducted the work shown in this PhD thesis entitled “Combined toxicity of nanoparticulate titanium dioxide products and heavy metals considering various environmental parameters”. All used assistances and involved contributors are clearly declared. They are co-authors of, or are acknowledged in, the respective publication. In all cases I designed and planed the studies, conducted the ecotoxicological tests (with assistance of the named persons), performed the required analyses, evaluated the data and wrote the resulting publication (with the help of my coauthors).

This thesis has never been submitted for an exam, as 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.

Place, Date Ricki Rosenfeldt

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ZUSAMMENFASSUNG

Der zunehmende Einsatz von Titandioxid-Nanopartikeln ($nTiO_2$) birgt das Risiko eines erhöhten Eintrags in Oberflächengewässer, wo diese mit weiteren anthropogenen Stressoren (z.B. Schwermetalle) vorkommen können. Das gemeinsame Auftreten ermöglicht die Adsorption von Schwermetallen an $nTiO_2$, welche aufgrund ihrer Agglomeration häufig aussedimentieren. Somit können Nanopartikel mit den adsorbierten Metallionen potentiell für pelagische aber auch benthische Organismen ein Risiko darstellen. Die kombinierte Toxizität von $nTiO_2$ und Schwermetallen wird vermutlich durch die Eigenschaften der Stressoren, aber auch durch verschiedene Umweltparameter (z.B. organisches Material, pH, Ionenstärke) bestimmt. Allerdings wurde der Einfluss dieser Faktoren bisher nicht systematisch untersucht. Daher zielte diese Arbeit darauf ab den Effekt von verschiedenen $nTiO_2$-Produkten, welche sich in der Zusammensetzung der kristallinen Struktur unterschieden, auf die Toxizität von Kupfer (als Stellvertreter für Schwermetalle) für den pelagischen Testorganismus *Daphnia magna* in Gegenwart von verschiedenen Formen und Konzentrationen organischen Materials zu untersuchen. Es ist anzunehmen, dass die Dauer der Interaktion (=Alterung) die kombinierte Toxizität der Stressoren beeinflusst. Ergänzend wurde deshalb der Einfluss von $nTiO_2$ auf die Kupfer-Toxizität nach einer Alterung unter dem Einfluss verschiedener Umweltparameter (nämlich organisches Material, pH, Ionenstärke) untersucht. Des Weiteren wurde die Übertragbarkeit der wesentlichen Ergebnisse auf benthisch lebende Organismen mit *Gammarus fossarum* geprüft. Die vorliegende Arbeit zeigte für alle untersuchten Szenarien eine reduzierte Kupfer-Toxizität in Gegenwart von $nTiO_2$, unabhängig von deren kristallinen Struktur. Dieser Effekt ließ sich auch auf benthische Lebewesen übertragen, obwohl die Exposition durch die Anwesenheit von $nTiO_2$ aufgrund der Sedimentation mit dem adsorbierten Kupfer potenziell erhöht war. Die erzielten Beobachtungen legen eine Verwendung von $nTiO_2$ zur Aufreinigung von beispielsweise Abwasser nahe. Allerdings sollten potentielle Nebeneffekte (z.B. chronische Toxizität, Bildung von Sauerstoffradikalen) zunächst gründlich untersucht werden. Darüber hinaus sind die Übertragbarkeit auf andere Stressoren (z.B. andere Schwermetalle, organische Chemikalien) und der Verbleib von Schwermetallen in aquatischen Ökosystemen nach einer Sorption an $nTiO_2$ ungewiss und bedürfen weiterer Forschung.

ABSTRACT

The increasing application of titanium dioxide nanoparticles ($nTiO_2$) entails an increased risk regarding their release to surface water bodies, where they likely co-occur with other anthropogenic stressors, such as heavy metals. Their co-occurrence may lead to an adsorption of the metal ions onto the particles. These nanoparticles often sediment, due to their agglomeration, and thus pose a risk for pelagic or benthic species. The combined toxicity of $nTiO_2$ and heavy metals is likely influenced by the properties of both stressors (since they may alter their interaction) and by environmental parameters (e.g., organic matter, pH, ionic strength) affecting their fate. These issues were not yet systematically examined by the recent literature. Therefore, this thesis investigated the influence of $nTiO_2$-products with differing crystalline phase composition on the toxicity of copper (as representative for heavy metals) in presence of different organic matters using the pelagic test organism *Daphnia magna*. Moreover, the duration of the stressors' interaction (=aging) likely modulates the combined toxicity. Hence, the influence of $nTiO_2$ on copper toxicity after aging as a function of environmental parameters (i.e., organic matter, pH, ionic strength) was additionally investigated. Finally, the transferability of the major findings to benthic species was examined using *Gammarus fossarum*. The present thesis discovered a reduction of the copper toxicity facilitated by $nTiO_2$ for all assessed scenarios, while its magnitude was determined by the surface area and structure of $nTiO_2$, the quantity and quality of organic matter as well as the aging of both stressors. The general copper toxicity reduction by $nTiO_2$ was also transferable to benthic species, despite their potentially increased exposure due to the sedimentation of $nTiO_2$ with adsorbed copper. These observations suggest the application of $nTiO_2$ as remediation agent, but potential side effects (e.g., chronic toxicity, reactive oxygen species formation) require further investigations. Moreover, questions regarding the transferability to other stressors (e.g., different heavy metals, organic chemicals) and the fate of stressors adsorbed to $nTiO_2$ in aquatic ecosystems remain open.

1. INTRODUCTION

1.1. Background

1.1.1. Nanoparticles

Engineered nanoparticles can be defined as materials purposefully produced by human activity, with at least one dimension between 1 and 100 nm (Lead and Smith, 2009). Today more than 1600 manufacturer-identified nanotechnology-based products are commercially available including amongst others cosmetics, paints, catalysts, clothing, medical and health cares, food and food packaging, electronics and batteries (Nanotechnologies, 2013). The nanoparticles can be roughly distinguished by their chemistry: Carbon-based (e.g. nanotubes, fullerenes), metal-based (e.g., silver, gold) and metal oxides (e.g. titanium dioxide, zinc oxide). Titanium dioxide nanoparticles ($nTiO_2$) offer a broad spectrum of application possibilities within the manufacturing of coatings and paints (Kaegi et al., 2008), but also textiles (Windler et al., 2012), food (Weir et al., 2012) and other consumer products, such as sunscreens. Therefore, the production of $nTiO_2$ is continuously increasing nowadays (Robichaud et al., 2009; Hendren et al., 2011; Hendren et al., 2013). The $nTiO_2$ can consist of different crystalline phase compositions (usually anatase, rutile or a mixture of both), each of them shows different beneficial properties (Macwan et al., 2011), but also differing toxicity (Seitz et al., 2014). Pure anatase is, due to its excellent photocatalytic activity, often utilized for decontamination or self-cleaning effects of construction materials, while rutile frequently functions as pigment in paints, food and personal care products (Macwan et al., 2011). As a result of their frequent application the risk of an unintentional release into surface waters is increasing (Mueller and Nowack, 2008; Robichaud et al., 2009; Gottschalk et al., 2015). Major sources of such a release are (un)treated wastewater and urban runoff (Sun et al., 2014). The concentration of $nTiO_2$ in wastewater effluents was expected to reach 110 µg/L (85^{th} percentile), while the concentration of TiO_2 measured in surfaces water was often in the low microgram per liter range as reviewed by Sun et al. (2014).

1.1.2. Heavy metals

Heavy metals are an anthropogenic stressor of high concern, since they can occur at high concentrations in surface waters. For example, in European rivers heavy metal concentrations (depending on the respective element) range from a few micrograms up to several milligrams per liter (Vink et al., 1999). One major source of heavy metal pollution (especially arsenic, chromium, copper, manganese and nickel) in aquatic ecosystems is domestic wastewater effluents (Nriagu and Pacyna, 1988). Although some heavy metals are essential micronutrients (e.g., copper which is part of many enzymes, functioning for example as dioxygen carrier), they are highly toxic towards aquatic organisms (Martin and Holdich, 1986). Copper is a ubiquitous representative of this subset of elements (Flemming and Trevors, 1989), which is toxic to aquatic life at low concentrations (depending on the test organism between 0.005 and 2.5 mg/L; Biesinger and Christensen, 1972; Baudouin and Scoppa, 1974). At the same time, the copper concentrations detected in wastewater effluents often reach 20 µg/L (Shafer et al., 1998; Oliveira et al., 2007), while in agriculturally influenced streams (particularly near orchards and viticulture) copper concentrations of 200 µg/L were measured due to the application of copper as fungicide (Süß et al., 2006; Bereswill et al., 2012). Depending on the environmental conditions, copper can exist in the form of several species (e.g., Cu^{2+}, CuOH, $Cu(CO_3)_2$) with varying toxicity, while Cu^{2+} is the most toxic species. In terms of simplification all copper species are subsumed under the term “copper” throughout the present thesis.

1.1.3. The interaction

Once $nTiO_2$ reach surface water bodies they likely co-occur with other (anthropogenic) stressors, such as heavy metals. The co-occurrence of $nTiO_2$ and a heavy metal often leads to an adsorption of the heavy metal to the $nTiO_2$, due to the large surface area and specific affinity of the metal oxide (Flemming and Trevors, 1989). The adsorption capability of $nTiO_2$ differs between products, on the basis of differing crystalline phase compositions (Giammar et al., 2006). More specifically, the adsorption of heavy metal ions is facilitated via unsaturated oxygen/deprotonated surface hydroxyls (Xie and Gao, 2009), while their availability is determined by the total surface area, which is in turn influenced by the crystalline phase composition and the particle size (Bourikas et al., 2014). The adsorption of the heavy metal ions lowers their presence in the water phase and potentially reduces the bioavailability of free ions, which could entail a reduced toxicity (Flemming and Trevors, 1989; Rosenfeldt et al., 2014). However, the heavy metal ultimately accumulates at the surface of $nTiO_2$, thus the ingestion of nanoparticles with high loads of heavy metal potentially increases the total uptake and finally the joint toxicity (=carrier effect) as for example observed by Fan et al. (2011). Especially filter-feeding organisms, such as the pelagic *Daphnia*, might be prone to this exposure type (Tan et al., 2012), since $nTiO_2$ and their agglomerates (range: 100-5000 nm) are easily retained by the organisms filtration apparatus and thus ingested (Gophen and Geller, 1984). Therefore, toxicity and bioaccumulation caused by an interaction of $nTiO_2$ and heavy metals was investigated with several pelagic standard test organisms, such as algae, water flea and fish (Sun et al., 2007; Zhang et al., 2007; Hartmann et al., 2010; Fan et al., 2011; Wang et al., 2011; Hartmann et al., 2012; Tan et al., 2012; Tan and Wang, 2014). However, these studies used different heavy metals, different types of $nTiO_2$ and different environmental conditions during the toxicity tests. Each alteration in any of those factors has the potential to impact the combined toxicity. For example, the identity of the heavy metal and the charge of its most toxic ion influence $nTiO_2$'s effect on the joint toxicity (Rosenfeldt et al., 2014). Moreover, the crystalline phase composition of the $nTiO_2$ is suspected to affect the adsorption of heavy metal ions, due to varying surface properties (Rashidi et al., 2010). Thus, the combined toxicity of $nTiO_2$ and heavy metals might be modulated accordingly. Finally, the alteration of these factors among studies potentially induced dissension in the recent literature regarding the direction of $nTiO_2$'s effect on heavy metal toxicity.

In addition to that, environmental conditions influence, besides the adsorption of the heavy metal to $nTiO_2$ (discussed in the following), the agglomeration of $nTiO_2$ with the adsorbed heavy metal. The agglomeration and sedimentation of $nTiO_2$ most likely lower the hazard for pelagic organisms. However, the sedimentation of heavy metals adsorbed to nanoparticles might ultimately increase the exposure of benthic organisms, such as *Gammarus*, which has not been taken into account by the recent literature.

1.1.4. The role of environmental parameters

Environmental parameters likely affecting the interaction of $nTiO_2$ and heavy metals include amongst others organic matter, pH and ionic strength. Ubiquitous in anthropogenically influenced and natural surface waters, organic matter is potentially the most important of these parameters. On the one hand organic matter can coat $nTiO_2$ and hence hinders the particles' agglomeration via electrostatic repulsion and steric hindrance, stabilizing them in the water column (Loosli et al., 2013, 2014). This process increases the particles' dwell time, but in most of the cases also lower their toxicity (Hall et al., 2009; Seitz et al., 2012b; Seitz et al., 2015). The efficiency of the coating by organic matter regarding stabilization is driven by its hydrophobicity and aromaticity (Lee et al., 2011; Pakarinen et al., 2013). On the other hand, some organic matters have the ability to reduce the toxicity of heavy metals, due to complexation of the toxic ions (Ma et al., 1999) utilizing their electron donor groups (i.e., O, N and S; Flemming and Trevors, 1989). However, organic matter can additionally influence the interaction of $nTiO_2$ and heavy metals, since the adsorption of organic matter to $nTiO_2$ impacts the particles' surface functionality (potentially increasing their adsorption capacity) as well as the organic matter's degree of protonation (Saito et al., 2005; Chen et al., 2012).

Another important environmental parameter is the pH of the surrounding water, since it could change the surface charge of the particles and hence influence their stability. For $nTiO_2$ the point of zero charge is often around pH 7 (Xie and Gao, 2009; Bekkouche et al., 2012), this means at a neutral pH the particles likely agglomerate due to the absence of electrostatic repulsion (cf. Derjaguin-Landau-Verwey-Overbeek theory), while at a more acidic or basic pH the particles are more stable and agglomerate less (French et al., 2009). The pH plays also an important role regarding the speciation of heavy metals, such as copper. At an acidic pH more metal ions are free (not complexed) and therefore exhibit a higher toxicity

(Flemming and Trevors, 1989), while they are complexed and less toxic at a basic pH. Both processes help the pH modulating the interaction of $nTiO_2$ and heavy metals, regarding the adsorption of ions to the nanoparticle surfaces (Vassileva et al., 1996; Rashidi et al., 2010; Bekkouche et al., 2012), and in turn potentially influence the uptake by organisms and the joint toxicity.

Furthermore, the ionic strength of the water affects the toxicity and interaction of the assessed stressors. A high ionic strength leads to a rapid agglomeration of $nTiO_2$, since the cations of the water lower the electrostatic repulsion of the nanoparticles (French et al., 2009). The speciation of heavy metals is additionally influenced by the ionic strength, thus a high ionic strength (especially the presence of anions) decrease the abundance of free ions, while soluble and insoluble complexes are formed (Flemming and Trevors, 1989). These processes rarely occur at low ionic strength. Moreover the adsorption of the heavy metal to $nTiO_2$ is potentially negatively influenced by the ionic strength, due to competitive sorption of cations (Vassileva et al., 1996; Yang et al., 2006).

An additional factor likely influencing the joint toxicity of $nTiO_2$ and heavy metals is the duration of their interaction prior the spiking of the ecotoxicity test (=aging), since the aging triggers both the amount of ions adsorbed onto $nTiO_2$ and the agglomeration state of the particles themselves (French et al., 2009; Rashidi et al., 2010). Moreover, the environmental parameters of the surrounding medium (discussed above) modulate the interaction between $nTiO_2$ and the heavy metal during aging, and thus complicate any prediction of the resulting combined toxicity.

1.2. Objective

1.2.1. Main objective

The objective of the present thesis was the comprehensive examination of the combined toxicity of $nTiO_2$ and copper (as a representative for heavy metals) to aquatic invertebrates. The combined toxicity is likely modulated by various factors, such as the crystalline phase composition of $nTiO_2$ and the presence of organic matter, but they were not investigated systematically yet. Furthermore, the combined toxicity is supposed to be affected by the aging of $nTiO_2$ and copper as a function of environmental conditions, what was waived by the current literature, too. Environmental parameters (e.g., organic matter, ionic strength, pH) are assumed to influence the fate of $nTiO_2$ (i.e., agglomeration and sedimentation) and copper (i.e., complexation and adsorption), determining their interaction. Moreover, the fate of both stressors directs the exposure towards distinct habitats (pelagic vs. benthic). However, this potential exposure shift also received no attention in the recent literature.

To fill these knowledge gaps the present thesis examined the interaction of $nTiO_2$ and copper based on the following assumptions: It was hypothesized that (I) $nTiO_2$ with larger surface area and a higher density of binding sites (both determined by the crystalline phase composition of the $nTiO_2$) adsorb more copper and hence lead to an increased uptake by filter-feeding organisms, which results in a higher toxicity. It was also hypothesized that (II) organic matter stabilizes $nTiO_2$ and thus increases their dwell time in the water column, which in turn increase their uptake by pelagic filter-feeders. Further, it was hypothesized that (III) the aging of $nTiO_2$ and copper increases the amount of adsorbed copper, depending on the environmental conditions, and thus also induces an increased uptake of copper. Finally, it was hypothesized that, in absence of stabilizing conditions, (IV) the agglomeration and sedimentation of $nTiO_2$ with the adsorbed copper might reduce the toxicity to pelagic organisms, but increases the toxicity for benthic species.

These hypotheses were tackled by the following tasks (Figure 1.1) as continuation of an earlier study (Rosenfeldt et al., 2014):

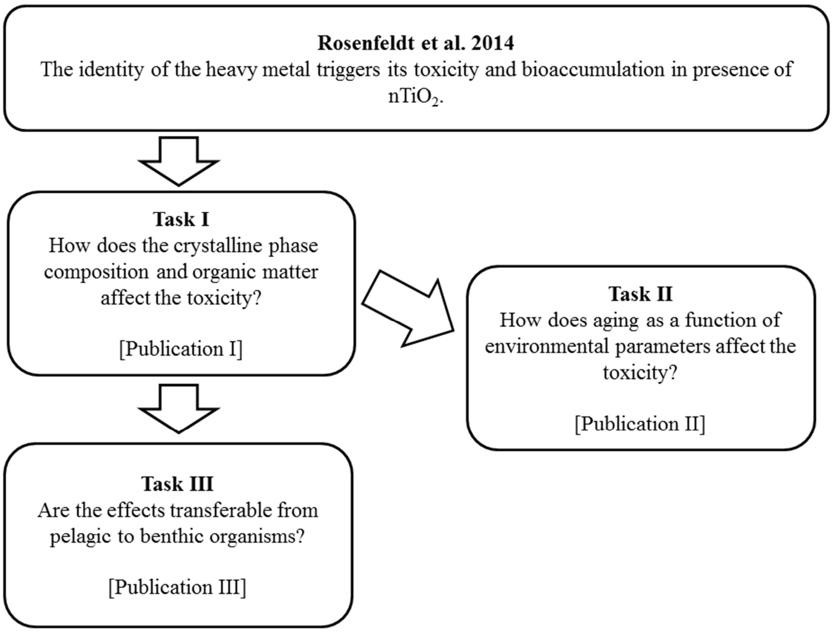

Figure 1.1: **Tasks of this thesis**. The flowchart displays the subdivision of the present thesis into three parts (Task I-III), while each of them was examined by an individual study [**Publication I-III**], basing on an earlier publication (Rosenfeldt et al., 2014).

1.2.2. Task I

Following hypothesis I, the influence of $nTiO_2$'s intrinsic properties (i.e., surface properties) on the direction of their effect on the copper toxicity was investigated using three $nTiO_2$-products with differing crystalline phase composition including pure anatase, pure rutile and a mixture of both [**Publication I**].

Moreover, to test hypothesis II, the influence of organic matter on the combined toxicity of $nTiO_2$ and copper, through modification of the nanoparticles dwell time, was investigated based on the organic matters seaweed extract, Suwannee River natural organic matter and sucralose [**Publication I**]. Their effect was assumed to be determined by their quantity (i.e., concentration) and quality (i.e., properties).

1.2.3. Task II

Moreover, shifts of the joint toxicity, caused by aging as a function of environmental parameters, were examined by the assessment of combinations containing $nTiO_2$ and copper [**Publication II**]. The environmental parameters ionic strength, pH and organic matter were suspected to determine the adsorption of copper and thus the direction of $nTiO_2$'s effect on the toxicity after aging (cf. hypothesis III).

1.2.4. Task III

For pelagic organisms the presence of $nTiO_2$ could diminish the uptake and toxicity of copper following agglomeration and sedimentation of $nTiO_2$ with the adsorbed metal. This process, in turn, could increase the exposure of benthic organisms, questioning the transferability of this observation to benthic species, which was investigated using *Gammarus fossarum* [**Publication III**] to test hypothesis IV.

1.3. Study layout and method overview

1.3.1. Method overview of task I

The influence of $nTiO_2$'s intrinsic properties and organic matter on the combined toxicity of $nTiO_2$ and copper was examined within one approach. This approach included nine 2×3×7 factorial acute *Daphnia* tests assessing all combination of $nTiO_2$ and organic matter, while the effect of $nTiO_2$'s intrinsic properties was assessed by the application of three different $nTiO_2$-products, namely A-100 (Crenox, Germany; mainly anatase), R050P (Mknano, Canada; mainly rutile) and P25 (Evonik, Germany; mixture of anatase and rutile), at concentrations equal to their 96-h EC_{50} (i.e., A-100: 1 mg/L, P25: 2 mg/L and R050P: 4 mg/L; Bundschuh et al., 2012; Seitz et al., 2014) [**Publication I**]. The products showed, due to their differing crystalline phase composition different surface properties, while the particle size distributions of the stock suspensions were kept at the same level (Table 1.1). Additionally, the influence of organic matter on the interaction of $nTiO_2$ and heavy metals was investigated on basis of three organic matters, namely sucralose, seaweed extract and Suwannee River natural organic matter. These organic matters represent a range of properties reflecting their diversity in natural and anthropogenically influenced waters (Shon et al., 2006) and were applied at three different environmentally relevant levels corresponding to 0, 2 and 8 mg total organic carbon/L (Ryan et al., 2009). Moreover, these tests involved, beside the absence and presence of $nTiO_2$ and the three levels of organic matter, seven concentrations of copper ($Cu(NO_3)_2 \times 3H_2O$; Carl Roth GmbH + Co. KG, Germany) as representative for heavy metals. Reconstituted hard water (ASTM, 2007) amended with selenium and vitamins (biotin, thiamine, cyanocobalamine, according to the OECD guideline 202 (OECD, 2004)) served as test medium. The applied concentrations allowed the proper modelling of dose-response curves required for the calculation of the respective EC_{50} (=median effective concentration; regarding immobility) values. All tests were performed according to the OECD test guideline 202 with *Daphnia magna* (age<24 h; clone V, Eurofins-GAB laboratories, Germany) and five replicates per treatment. Although the test duration was elongated to 96 h, as recommended by earlier studies (Lazorchak et al., 2009; Dabrunz et al., 2011), primarily the 48-h EC_{50} values of copper in absence and presence of $nTiO_2$ were determined for each treatment, to limit masking effects of direct $nTiO_2$ toxicity. The EC_{50} values were statistically compared using confidence interval testing (Wheeler et al., 2006). The copper concentration in presence and absence of $nTiO_2$ was determined utilizing inductively coupled plasma equipped with a quadrupole mass spectrometer (ICP-MS; XSeries 2; Thermo Fisher Scientific, Germany) as described earlier (Rosenfeldt et al., 2014). The adsorption behavior of copper in presence of $nTiO_2$ and organic matter was examined with ICP measurements in additional samples after centrifugation.

Table 1.1: Properties of the applied $nTiO_2$-products (A-100, P25, R050P) [**Publication I**].

properties	method	A-100	AEROXIDE® TiO_2 P 25	MKN-TiO_2-R050P
abbreviation	-	A-100	P25	R050P
producer	-	Crenox, Germany	Evonik, Germany	Mknano, Canada
crystalline composition	-	anatase	anatase/rutile	rutile
primary particle size (nm)	advertised[a]	6	21	50
surface area (m²/g)	BET/advertised[a]	~230	50±15	~30
ave. diameter in suspension[b] (nm)	DLS	87.3±2.4	86.0±2.1	90.0±0.7
PI in suspension[b]	DLS	0.132-0.189	0.118-0.164	0.180-0.189
surface area in suspension[b] (m²/g)	NTA	32.5±9.9	28.1±2.2	51.8±8.2
ave. diameter in suspension[b] (nm)	NTA	109.0±11.8	95.2±5.7	140.4±20.8

[a] information given by the producer

[b] suspended in deionized water, stabilized by a low pH

1.3.2.Method overview of task II

The shifts in combined toxicity, caused by an interaction prior the spiking of the ecotoxicity test, were investigated taking the following factors into account: time, ionic strength, pH and organic matter [**Publication II**]. The tests comprised the moderately toxic $nTiO_2$ product P25 as model substance (representing the crystalline phases anatase and rutile) and copper (exemplifying heavy metals). The approach involved seven copper concentrations, chosen to enable the proper determination of EC_{50} values, in absence and presence of $nTiO_2$ (2 mg/L; equal to the 96-h EC_{50}, only negligibly influencing the test organism after 48 h). The concentrations of both substances during aging were 100 times the concentrations of the actual ecotoxicity test. The aging was performed in 50-mL centrifuge tubes containing deionized water or test medium (see section 1.3.1), which were placed for 0-168 h (depending on the assessed scenario) on an overhead shaker. In parallel to these setups, unaged treatments were assessed. After an additional thorough mixing they were applied to the toxicity test with *D. magna*, which were conducted and statistically evaluated as described above.

In a second step, the aging of $nTiO_2$ and copper, was performed in test medium with a pH adjusted to either 6.5 or 8.0 using the buffer MOPS (3-(N-morpholino)propansulfonic acid; Carl Roth GmbH + Co.

KG, Germany) – a non-toxic substance (Rendal et al., 2012). The ecotoxicological tests were afterwards carried out in (I) test medium buffered to pH 6.5, (II) unbuffered test medium and (III) test medium buffered to pH 8.0, considering pH-dependent desorption of copper during the ecotoxicological assessment (Hu and Shipley, 2012).

In a third step, the combinations of $nTiO_2$ and copper were aged in deionized water amended with organic matter (8 mg total organic carbon/L; namely sucralose, seaweed extract and Suwannee River natural organic matter).

1.3.3. Method overview of task III

The transferability of the major observations, namely the reduction of copper toxicity and bioaccumulation due to an adsorption of the copper ions by $nTiO_2$, to benthic organisms was investigated using *G. fossarum* and the model substances P25 and copper [**Publication III**]. Therefore, a 24-d feeding assay was conducted, taking advantage of a 2×2 factorial design, involving absence and presence of 2 mg $nTiO_2$/L (which has shown to trigger no sublethal effects in previous experiments (Seitz et al., 2014)) and 40 µg copper/L (expected to cause sublethal effects in gammarids according to Zubrod et al. (2014)). Besides the endpoint mortality, sublethal endpoints related to the energy processing including leaf consumption, feces production and assimilation as well as endpoints related to the physiological fitness including weight and lipid content were assessed. All replicates (n=45) consisted of a beaker filled with SAM-5S medium (Borgmann, 1996) reflecting the respective treatment, an individual male gammarid with a cephalothorax diameter between 1.6 and 2.0 mm (corresponding to an average dry mass of 5.7±0.7 mg, mean±SD, n=50) and two leaf discs. The medium of each treatment was renewed every third day to ensure a continuous exposure. After the careful transfer of the gammarid as well as the leaf material to fresh medium, the old medium was filtered individually for each beaker over a pre-weighed glass fiber filter (pore size: 1-3 µm; GF 6, Whatman GmbH, Germany). The filter was dried and weighed to quantify feces production. Furthermore, during every second water exchange leaf discs were replaced, while the removed leaf material was dried and weighed to determine leaf consumption. All endpoints, except mortality, were statistically evaluated by two-factor ANOVAs. Mortality was evaluated by pairwise comparisons for proportions using chi-square tests with Bonferroni-Holm correction.

In addition to the determination of $nTiO_2$ and copper water concentrations (via ICP-MS), measurements of gammarids' total TiO_2 body burden were achieved by coupling electro thermal vaporization (ETV; ETV 4000; Spectral Systems, Germany) to the ICP-MS, in compliance with Duester et al. (2010) and modified as described in an earlier publication (Rosenfeldt et al., 2014). The total copper body burden of the respective organisms was measured after an acid digestion (aqua regia) as liquid sample using ICP-MS [**Publication III**].

2. RESULTS

2.1. The role of crystalline phase composition and organic matter

Titanium dioxide nanoparticles reduced the copper toxicity, while the effect size was depending on the product applied (A-100, P25, R050P; 30-200% higher 48-h EC_{50}; Figure 2.1). The respective toxicity reduction coincided with a lowered copper concentration measured in the water column. In the presence of organic materials (i.e., seaweed extract (SW), Suwannee River natural organic matter (SR)) and $nTiO_2$, the copper toxicity was further reduced (up to sevenfold higher 48-h EC_{50}). However, this effect was not observed in presence of sucralose (SL; Figure 2.1).

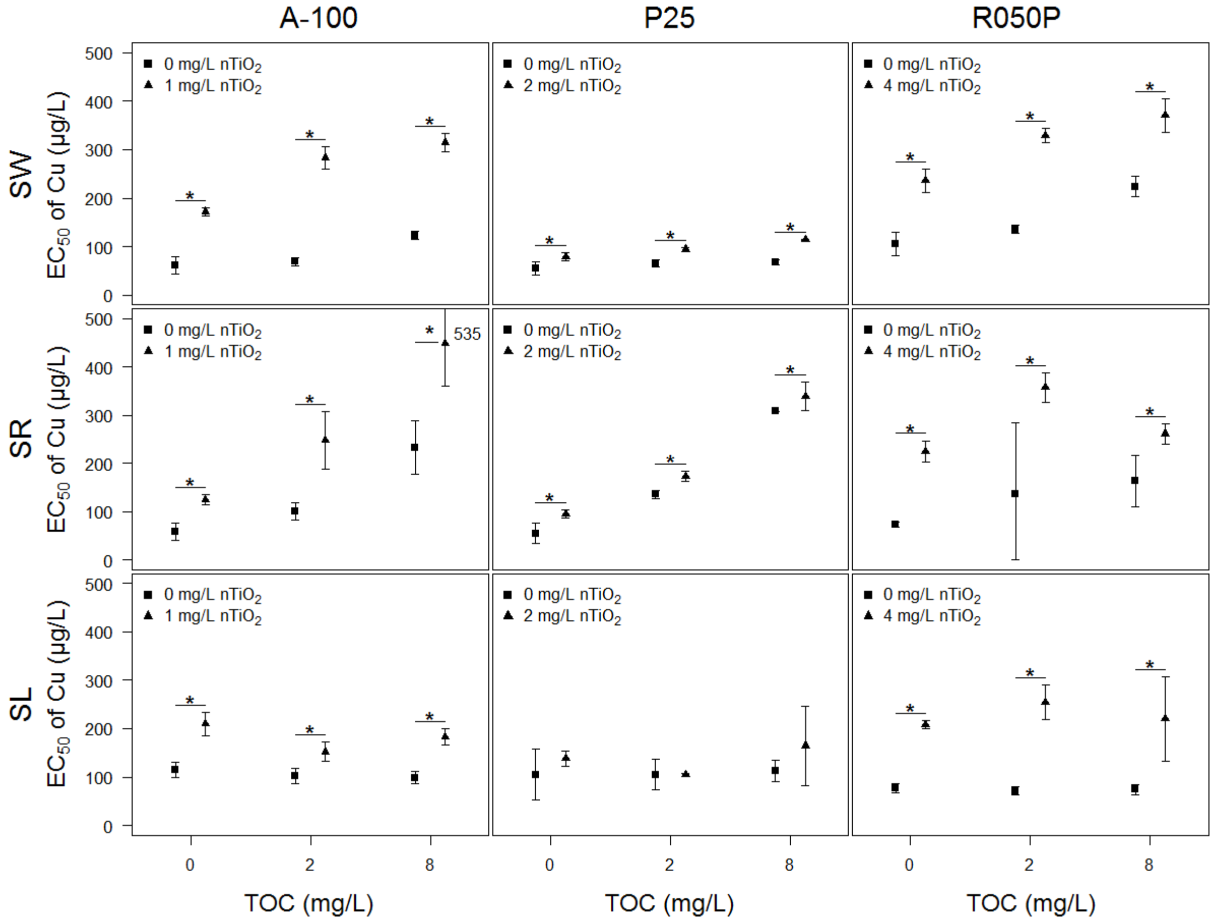

Figure 2.1: **EC$_{50}$ values of copper after 48 h test duration**. The 48-h EC$_{50}$ values (±95% confidence intervals) were determined at different levels (0, 2 and 8 mg TOC/L) and types of organic matter (seaweed extract (SW), Suwannee River natural organic matter (SR) and sucralose (SL)) and in absence (■) and presence (▲) of titanium dioxide nanoparticles (nTiO$_2$; i.e., A-100, P25 and R050P). Asterisks denote statistically significant differences between the absence and presence of nTiO$_2$ [**Publication I**].

2.2. Effect of aging as a function of environmental parameters

At the first glance, an aging in test medium in presence of nTiO$_2$ increased the toxicity of copper (Figure 2.2). However, this phenomenon was caused by an enhanced transfer of copper adsorbed to nTiO$_2$ from the aging vessels to the actual ecotoxicological assessment, while in absence of nTiO$_2$ copper remained partly adsorbed to the aging vessels. This was verified by the analytical measurements in the test beakers at initiation of the ecotoxicological test (=exposure of the organisms). The EC$_{50}$ values based on the measured concentration displayed, in contrast to the EC$_{50}$ values based on the nominal concentration, a reduced toxicity in presence of $nTiO_2$, as observed for unaged treatments. Furthermore, the aging in deionized water led to the same toxicity reduction as determined for the unaged treatments (Figures 2.1 and 2.2).

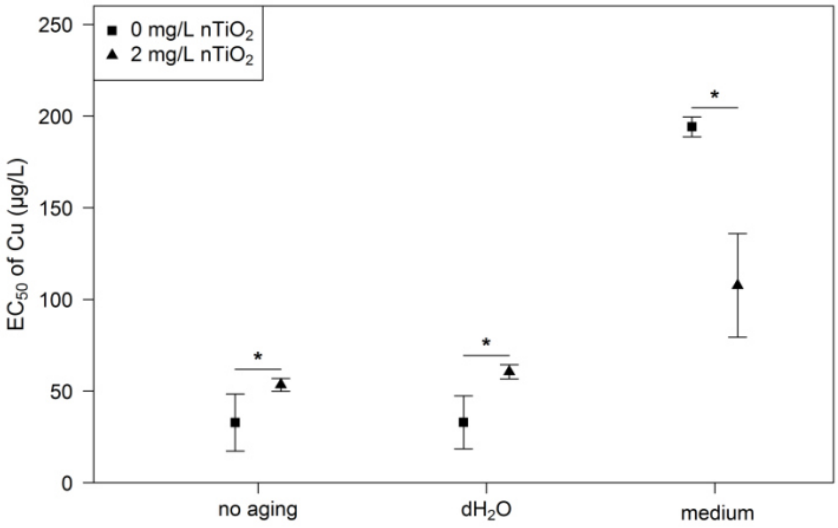

Figure 2.2: **The effect of ionic strength**. The 48-h EC_{50} values (±95% confidence intervals) of copper in absence (■) and presence (▲) of titanium dioxide nanoparticles ($nTiO_2$) without previous aging (=no aging) and after an aging for 72 h in deionized water (dH_2O) and test medium. Asterisks denote statistically significant differences between the absence and presence of $nTiO_2$ [**Publication II**].

Moreover, regardless of the pH in the ecotoxicity test, the copper toxicity was significantly reduced after 72 h of aging at pH 6.5 in presence of $nTiO_2$ compared to their absence (Figure 2.3A). The same pattern was observed after aging at pH 8.0 (Figure 2.3B). In addition, the treatments aged in presence of $nTiO_2$ at pH 6.5 displayed an on average 39% (range: 10-65%) higher EC_{50} compared to their counterparts aged at pH 8.0 (Figure 2.3A and B). However, for the treatments aged in absence of $nTiO_2$ these differences were negligible.

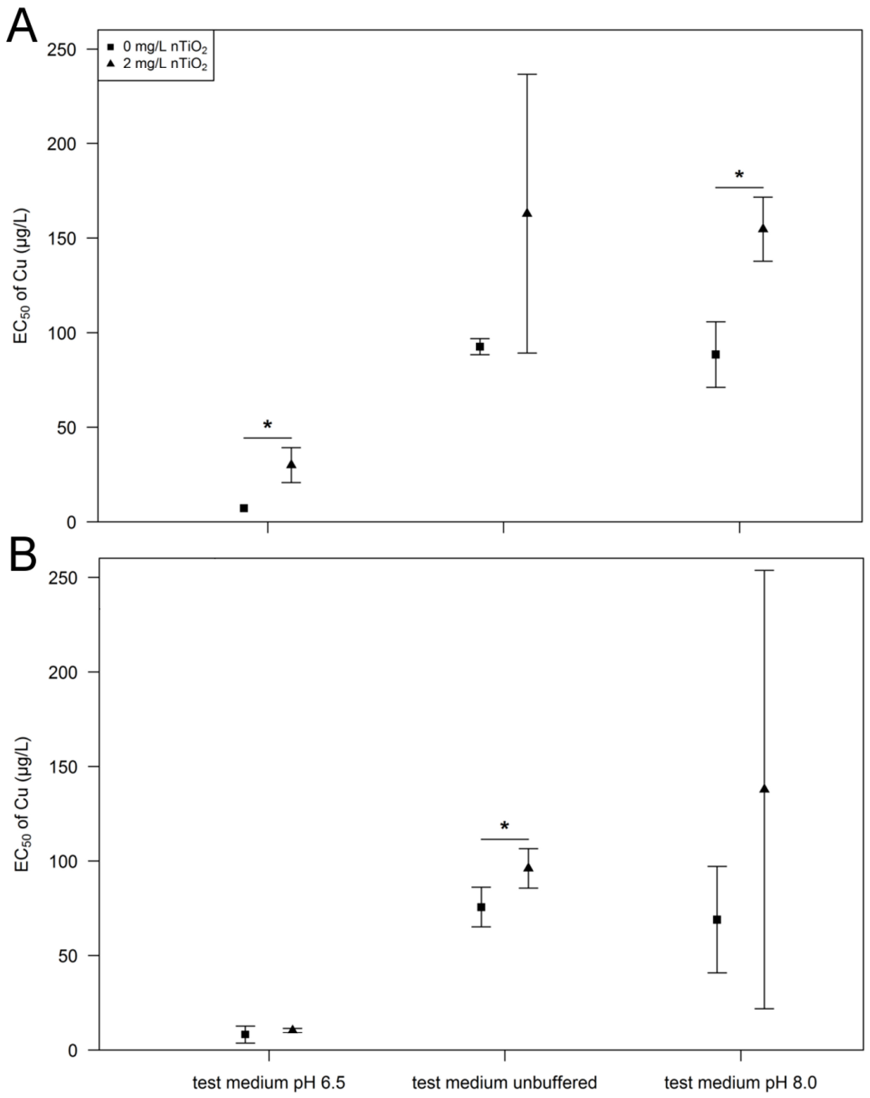

Figure 2.3: **The effect of pH**. The 48-h EC_{50} values (±95% confidence intervals) of copper in absence (■) and presence (▲) of titanium dioxide nanoparticles ($nTiO_2$) after an aging for 72 h in test medium buffered to either pH (A) 6.5 or (B) 8.0, assessed in test medium buffered to pH 6.5, unbuffered test medium and test medium buffered to pH 8.0, respectively. Asterisks denote statistically significant differences between the absence and presence of $nTiO_2$ [**Publication II**].

The aging in presence of organic matter led to a reduced toxicity in presence of $nTiO_2$ compared to their absence, irrespective of organic matters' quality. Finally, these results indicated that the presence of $nTiO_2$ reduced under all assessed aging scenarios, regardless of the environmental conditions, the copper-induced toxicity for daphnids – although with varying effect sizes [**Publication II**].

2.3. The transferability from pelagic to benthic organisms

Titanium dioxide nanoparticles alone did not affect mortality and leaf consumption of *G. fossarum*, whereas copper alone caused high mortality (>70%), reduced leaf consumption (25%; two-factor ANOVA: *p*-value=0.037) and feces production (30%; two-factor ANOVA: *p*-value=0.076) relative to the control (Figure 2.4). In presence of $nTiO_2$, copper-induced toxicity was largely eliminated. This reduction of copper toxicity coincided with a reduced total body burden of copper in presence of $nTiO_2$, compared to their absence, measured after test termination. However, independent of copper, $nTiO_2$ decreased the gammarids' assimilation and weight (Table 2.1).

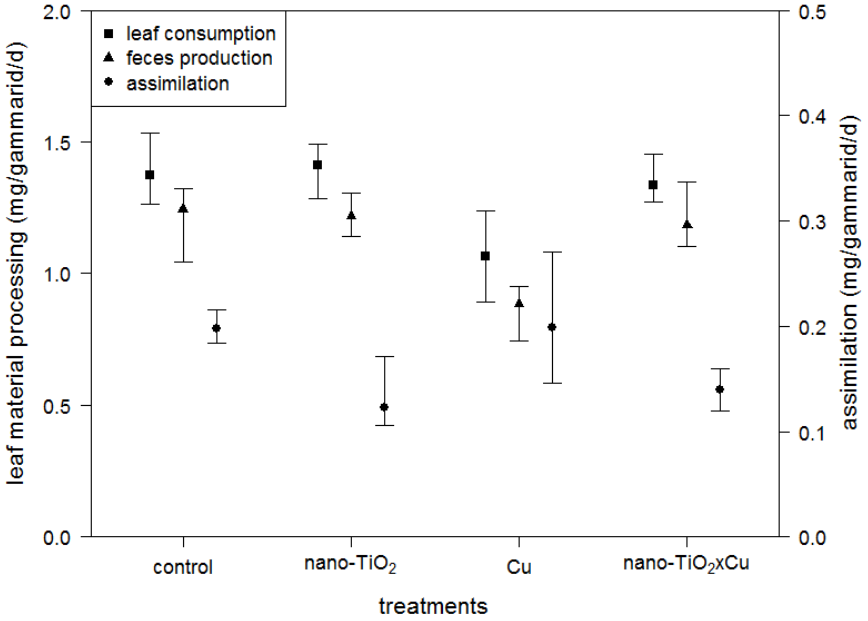

Figure 2.4: **Leaf material processing and assimilation.** Medians (with 95% confidence intervals) of the endpoints related to the processing of leaf material (i.e., leaf consumption ■, feces production ▲) and assimilation ●; all in mg/gammarid/d) of *G. fossarum* exposed to the control, 2 mg $nTiO_2$/L, 40 µg copper/L as well as the combination of titanium dioxide nanoparticles ($nTiO_2$) and copper over the entire study duration of 24-d [**Publication III**]. The statistical significance of the variables and their interaction can be extracted from Table 2.1.

Table 2.1: Output of two-factor ANOVAs for leaf consumption, feces production, assimilation, weight and lipids [**Publication III**].

	Df	Sum Sq	Mean Sq	F value	*p*-value
leaf consumption					
$\mathrm{nTiO_2}$	1	0.033	0.034	0.370	0.544
Cu	1	0.401	0.401	4.425	0.037
$\mathrm{nTiO_2}$×Cu	1	0.441	0.441	4.867	0.029
residuals	130	11.781	0.091		
feces production					
$\mathrm{nTiO_2}$	1	0.343	0.343	3.862	0.052
Cu	1	0.285	0.285	3.206	0.076
$\mathrm{nTiO_2}$×Cu	1	0.583	0.583	6.563	0.012
residuals	130	11.541	0.089		
assimilation					
$\mathrm{nTiO_2}$	1	16865	16865	11.949	<0.001
Cu	1	150	150	0.106	0.745
$\mathrm{nTiO_2}$×Cu	1	2	2	0.002	0.967
residuals	130	183480	1411		
weight					
$\mathrm{nTiO_2}$	1	1.950	1.951	3.214	0.075
Cu	1	6.210	6.215	10.238	0.002
$\mathrm{nTiO_2}$×Cu	1	1.010	1.010	1.664	0.199
residuals	131	79.520	0.607		
lipids					
$\mathrm{nTiO_2}$	1	3883	3883	6.786	0.019
Cu	1	8	8	0.015	0.906
$\mathrm{nTiO_2}$×Cu	1	140	140	0.244	0.628
residuals	16	9154	572		

Df, degrees of freedom
Sum Sq, sum of squares
Mean Sq, mean squares

3. CONCLUSION AND SYNTHESIS

3.1. Nanosized titanium dioxide reduces copper toxicity

The presence of $nTiO_2$ reduced the toxicity of copper throughout this thesis, irrespective of the scenario assessed [see **Publications I, II** and **III**]. This observation could be explained by the adsorption of copper to $nTiO_2$ leading to a reduced concentration of free ions (Bekkouche et al., 2012). Furthermore, the agglomeration and sedimentation of $nTiO_2$ ultimately lowered the presence of copper in the water column [**Publication I**]. Although, an ingestion of the sedimented nanoparticles together with adsorbed copper by daphnids grazing at the bottom of the test beaker is conceivable (Horton et al., 1979), the toxicity reduction facilitated by $nTiO_2$ resulted mainly from a decreased uptake (Rosenfeldt et al., 2014). Furthermore, one fifth of the metal ions was likely irreversibly adsorbed by $nTiO_2$ (Xie and Gao, 2009).

The remaining toxicity observed in presence of $nTiO_2$ may be caused by ions (not adsorbed or) desorbed in high amounts from $nTiO_2$ within the gastrointestinal tract of *D. magna*. Even though the pH in the gastrointestinal tract of daphnids is neutral (Pennak, 1953), their enzymes are hypothesized by Weltens et al. (2000) to promote the release of metals adsorbed onto ingested organic and inorganic particles (e.g., clay, sand, and peat).

The adsorption capability of $nTiO_2$, and thus the reduction efficiency of copper toxicity, differed between the applied products in the following order: A-100 > R050P > P25 (standardized to the applied concentration) [**Publication I**]. This order was assumed to result from the varying surface energy and abundance of binding sites, namely unsaturated oxygen (Xie and Gao, 2009), which is determined by the surface roughness and hence the total surface area, which in turn depends on the crystalline phase composition of the products. However, none of the $nTiO_2$-products increased the toxicity of copper at the applied concentrations.

3.2. Environmental parameters determining the interaction

The observed copper toxicity reduction in presence of $nTiO_2$ was in sharp contrast to some other studies assessing the combined toxicity of $nTiO_2$ and heavy metals (e.g., Fan et al., 2011). During an earlier study (Rosenfeldt et al., 2014), this discrepancy was attributed to the following factors: Crystalline phase composition of $nTiO_2$, preparation of the nanoparticle suspension, quality of organic matter present in the test medium, and other water quality parameters (e.g., pH, ionic strength). Therefore, the present thesis took these factors into account [**Publications I** and **II**], but was not able to detect an increased copper toxicity in presence of $nTiO_2$, rendering these factors as unimportant for the direction of $nTiO_2$'s effect on heavy metal toxicity.

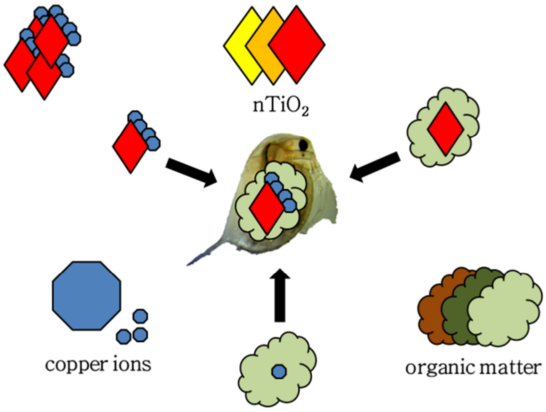

Figure 3.1: **Assumed interactions**. Potentially occurring processes within a combination of different crystalline phase compositions of nanosized titanium dioxide ($nTiO_2$) and copper in the presence of different qualities of organic matter [**Publication I**].

Moreover, organic matter, as one of these factors, was found to further decrease the combined toxicity of $nTiO_2$ and heavy metals, despite the stabilization of $nTiO_2$ by a coating utilizing the organic material and the resulting increase of their dwell time with the adsorbed metal (Figure 3.1). A priori, this process was assumed to increase the uptake (cf. hypothesis II), since *D. magna* is a non-selective filter-feeding organism (Kirk, 1991). However, it was discovered that the toxicity reduction was facilitated via a simultaneous adsorption and complexation of copper by $nTiO_2$ and organic matter, respectively, with the following order regarding their efficiency: Suwannee River natural organic matter > seaweed extract > sucralose [**Publication I**]. Apparently, this toxicity reduction by organic matter originated form the quantity and quality of their humic components. Thus, sucralose only marginally influenced the interaction of $nTiO_2$ and copper, but it also did not stabilize the nanoparticles. Concluding that a potential increase in copper toxicity, facilitated by $nTiO_2$, is still conceivable, if the present organic material is stabilizing $nTiO_2$, while not complexing the heavy metal ions.

Furthermore, it was assumed, that an increased time of interaction prior to exposure of the test organisms also increases the toxicity, since more copper could be adsorbed to $nTiO_2$ (cf. hypothesis III). Indeed, the $nTiO_2$ increased the transfer of copper from the aging vessels to the ecotoxicological test, which was underpinned by the ICP-MS measurements [**Publication II**]. Nevertheless, the comparison of the EC_{50} values based on the measured copper concentration revealed an $nTiO_2$-induced toxicity reduction. The enhanced transport of copper in presence of $nTiO_2$ compared to their absence was only observed after an aging in test medium (high ionic strength and basic pH), while deionized water delayed the interaction of $nTiO_2$ and copper to the initiation of the ecotoxicological test (i.e., the mixture with anions and cations and hence a higher ionic strength). Therefore, the measured copper concentration and toxicity was comparable to unaged combinations and, regardless of the aging duration, matching the nominal concentration. Besides the ionic strength, the pH was assumed being an important factor triggering the adsorption of copper and the resulting toxicity, which was assessed in further experiments performing the aging in test medium buffered to either pH 6.5 or 8.0. At pH 8.0, the presence of $nTiO_2$ during aging of copper caused a higher toxicity, compared to an aging at pH 6.5, while in absence of $nTiO_2$ the pH only negligibly influenced the toxicity. This observation substantiate hypothesis III, namely the influence of environmental parameters (here pH) during aging on the $nTiO_2$-induced change of copper toxicity. The slightly increased copper toxicity after the aging at pH 8.0 in presence of $nTiO_2$ compared to the aging at pH 6.5, was most likely caused by an higher adsorption of copper under basic conditions, compared to acidic (Vassileva et al., 1996; Bekkouche et al., 2012). However, the toxicity of copper was, irrespective of the pH during aging and during the actual ecotoxicological assessment, in presence of $nTiO_2$ reduced, when compared to their absence. Thus, regardless of the pH, $nTiO_2$ did not increase the copper-induced toxicity, but the applied buffer might have affected the adsorption of copper. Moreover, the aging in presence of organic matter, led to the same toxicity reduction pattern as observed for unaged mixtures, assuming that all forms of organic material inhibited the adsorption of copper to $nTiO_2$, irrespective of their quality. This might be explained, for the buffer and the organic matter, by a coating of $nTiO_2$ and the aging vessel altering the respective surface properties.

It can be concluded, that $nTiO_2$ reduce the copper toxicity also after aging, while the magnitude of this effect is likely determined by the prevalent environmental conditions, although an adequate assessment of pH was not possible.

3.3. Transferability to other invertebrates

Contrary to the observations with pelagic organisms, it was hypothesized, that benthic organisms display an increased copper toxicity in presence of $nTiO_2$ compared to their absence, since agglomeration and sedimentation of $nTiO_2$ with adsorbed copper elevated the exposure of benthic habitats (cf. hypothesis IV).

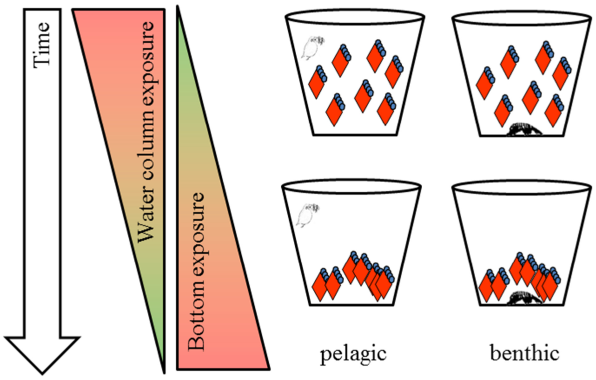

Figure 3.2: **Exposure shift**. Titanium dioxide nanoparticles (red) agglomerate and settle with the adsorbed copper ions (blue), which increases the bottom exposure and decreases the water exposure with time.

Although, the exposure was increased for benthic organisms (Figure 3.2) and copper adsorbed to particulates might induce toxic effects (Hook et al., 2014), a clear reduction of the copper toxicity, on the lethal and sublethal level, was observed in presence of $nTiO_2$ compared to their absence [**Publication III**]. Moreover, the total body burden of copper was lower in presence of $nTiO_2$, as observed for the pelagic daphnids (Rosenfeldt et al., 2014). It remains, however, unclear whether the processes responsible for the reduced total body burden are the same for pelagic and benthic organisms. For the pelagic organisms, the sedimentation of $nTiO_2$ with the adsorbed copper might be the most important process, since the risk of ingestion is reduced. For benthic organisms the adsorption of copper to $nTiO_2$ might simplify the excretion of the heavy metal, since a sequestration as solid metallic sulfur granules might not be necessary for excretion. Potentially, gammarids were able to easily excrete copper if adsorbed to $nTiO_2$ prior the release of ions during gut passage. However, some of the copper-induced effects on the sublethal level were not eliminated by the presence of $nTiO_2$ (i.e., reduced weight), and in addition negative effects of the $nTiO_2$ themselves were observed for benthic (e.g., decreased assimilation, unknown lipid availability), but also for pelagic organisms (after 72 and 96 h test duration).

In conclusion, the general toxicity reduction observed for pelagic freshwater species is transferable to benthic freshwater species. Presumably, this reduction is also transferable to other aquatic invertebrates, since the underlying processes seem unspecific, but this assumption needs further investigations. Furthermore, potential long-term effects of $nTiO_2$ should not be waived during future examinations. Moreover, the factors triggering an increased toxicity of heavy metals in presence of $nTiO_2$, observed by some other researchers (e.g., Fan et al., 2011; Tan and Wang, 2014), remain unclear.

4. PERSPECTIVE

4.1. Potential applications of $nTiO_2$ during water treatment

Various metal oxides including nanoparticles, such as $nTiO_2$, are due to their high surface area and specific affinity for heavy metals and other substances, suggested as adsorbent for anthropogenic stressors, during waste- and drinking water treatment (Hristovski et al., 2007; Mohan and Pittman, 2007; Luo et al., 2010; Rashidi et al., 2010; Pirilä et al., 2011; Recillas et al., 2011; Hua et al., 2012). Moreover, this thesis displayed, in all investigated cases, a reduction of the copper toxicity in presence of $nTiO_2$ due to the absorbance of the metal ions. The application of $nTiO_2$ – in the same manner as activated carbon – may be conceivable (Mohan and Pittman, 2007), for example in fixed bed columns (Hristovski et al., 2007), but also the slurry contact adsorption might be a possible technique.

However, there are several issues which should be reconsidered prior to the application of $nTiO_2$ as adsorbent or remediation agent. For instance, a lot of environmental parameters, which were not yet sufficiently assessed, including temperature (Bekkouche et al., 2012), might change the expected reduction of heavy metal abundance and/or bioavailability and hence toxicity. In addition, $nTiO_2$ are relatively expensive, compared to other materials (Bailey et al., 1999; Babel and Kurniawan, 2003) examined for such applications (e.g., industrial or agricultural by-products; Mohan and Pittman, 2007), if not recovered with additional efforts. Potentially, cheaper materials with comparable adsorption capabilities may be considered as substitute (Mohan and Pittman, 2007).

4.2. Transferability to other stressors

Although most of the observed effects are likely not limited to copper, the properties among elements, assigned to the group "heavy metals" are quite diverse. For example the charge of the most toxic species, which is an important factor regarding interaction via adsorption, differs enormously: Silver ions carry a single positive charge (Ag^+), and the most toxic copper species carry two positive charges (Cu^{2+}), whereas the most toxic species of arsenic, for example, has two negative charges ($HAsO_4^{2-}$). This aspect was found to impact the direction of $nTiO_2$'s effect on heavy metal toxicity during an earlier study (Rosenfeldt et al., 2014). Therefore, assumptions beyond two-fold positively charged metal ions should be made with caution. Further, it should be noted, that although all two-fold positively charged metal ions assessed in the recent literature were adsorbed by $nTiO_2$, the magnitude of this effect is variable (Vassileva et al., 1996; Bekkouche et al., 2012; Hua et al., 2012), thus screening test examining $nTiO_2$'s efficiency might be required in each case. In addition, and in contrast to the fate of heavy metals in presence of $nTiO_2$, it should also be noted, that some studies found an increased heavy metal toxicity in presence of $nTiO_2$ (e.g., Fan et al., 2011; Tan and Wang, 2014).

Furthermore, besides heavy metals a broad range of other anthropogenic stressors might interact with $nTiO_2$. This interaction could decrease the toxicity of such stressors for example via photocatalytic degradation, as observed by Seitz et al. (2012a) for pirimicarb and Bundschuh et al. (2011) for wastewater in general, but also via reduced bioavailability as observed by Farkas et al. (2015) for benzo(a)pyrene. Photocatalytic detoxification of organic substances by $nTiO_2$ was observed for a lot of further model compounds (Herrmann et al., 1998; Lawton et al., 1999; Konstantinou and Albanis, 2003; Aguedach et al., 2005; Kim et al., 2006; Arques et al., 2007). However, photocatalytic degradation can be a two-edged sword, since intermediates might be more toxic than the original substance (Muneer et al., 1999). Moreover, $nTiO_2$ could probably also increase the toxicity of co-occurring anthropogenic stressors, depending on the stressor and the environmental conditions, as observed by Zheng et al. (2012), where $nTiO_2$ functioned as carrier of bisphenol A. Shi et al. (2010) also found synergistic toxicity of $nTiO_2$ and DDT, while in this case the toxicity was not attributed to a carrier effect. Thus, the release of $nTiO_2$ into surface water bodies should be carefully considered and where possible avoided. Even though the particles themselves display a rather low acute toxicity, their interaction with other components of waste- and surface waters might synergistically increase the combined toxicity.

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APPENDIX

Publication I:
Nanosized titanium dioxide reduces copper toxicity – the role of organic material and the crystalline phase

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Abstract

Titanium dioxide nanoparticles ($nTiO_2$) are expected to interact with natural substances and other chemicals in the environment, however little is known about their combined effects. Therefore, this study assessed the toxicity of copper (Cu) in combination with varying crystalline phases (anatase, rutile, and the mixture) of $nTiO_2$ and differing organic materials on *Daphnia magna*. The nanoparticles reduced the Cu-toxicity depending on the product (30-200% higher 48-h EC_{50}). This decrease in toxicity coincided with a lowered Cu-concentration in the water column, which was driven by the adsorption of Cu to $nTiO_2$ – depending on surface area and structure – and their subsequent sedimentation. In the presence of organic material and $nTiO_2$, the Cu-toxicity was further reduced (up to sevenfold higher 48-h EC_{50}). This observation can be explained by a reduced Cu-bioavailability as a result of complexation and adsorption by the organic material and $nTiO_2$, respectively. Thus, the crystalline phase composition, which determining the surface area and structure of $nTiO_2$, seems to be of major importance for the toxicity reduction of heavy metals, while the influence of the organic materials was mainly driven by the quantity and quality of humic substances.

Keywords: Mixture toxicity – Interaction – Factorial approach – Ecotoxicology – Remediation

Introduction

The growing production of titanium dioxide nanoparticles (nTiO_2) is due to their broad and ever-increasing application range.[1, 2] In this context, two crystalline phases, namely anatase and rutile, are commonly used for industrial and commercial products, while their selection is driven by the function these products provide.[3] Anatase, due to its massive potential for the generation of reactive oxygen species, is frequently applied in decontaminating and self-cleaning products, while rutile is used as a pigment in paints, food and personal care products.[3] This widespread application – primarily in urban areas – may result in an unintentional release of nTiO_2 into aquatic ecosystems (i.e. surface water) especially via wastewater treatment plant (WWTP) effluents. These effluents' concentrations are predicted to reach up to 110 µg/L (85th percentile).[4] There, nTiO_2 co-occur with organic material (OM) potentially coating their surfaces.[5-7] This coating with OM could stabilize nTiO_2 via electrostatic repulsion and steric hindrance increasing the dwell time of the particles in the water column.[7] In this regard, OM's ability to stabilize nTiO2 is highly dependent on its characteristics. A higher hydrophobicity and/or aromaticity elevate OMs' stabilization potential.[8, 9] In addition, the adsorption of OM might change both: the surface properties of the nTiO_2, and the degree of protonation of the OM. Therefore these changes may alter the adsorption and complexation of co-occurring pollutants, e.g. heavy metals.[10, 11] Among heavy metals, copper (Cu) is ubiquitously present in urban areas and has been measured at concentrations of up to 20 µg/L in WWTP effluents,[12] while Cu in streams from other sources (e.g. agriculture, particularly orchards and viticulture) may reach concentrations above 200 µg/L.[13]

Thus Cu, nTiO_2 and OM undoubtedly co-occur and interact in surface water bodies, and during wastewater treatment processes, which can affect the bioavailability as well as ecotoxicity of the heavy metal.[14, 15] Due to the substantial surface area and the selective affinity of nTiO_2,[16] Cu may be adsorbed (e.g. via unsaturated oxygen/deprotonated surface hydroxyls) finally reducing its ecotoxicological potential.[17-19] Besides leading to a reduced concentration of Cu ions (i.e. Cu^{2+})[16,] in the water column, the adsorption of Cu also results in a high concentration of this element at the particles' surface. These particles may remain in the water column where they can become ingested by pelagic, filter-feeding organisms, such as *Daphnia magna*, potentially causing an increased Cu body burden and toxicity.[14] However, sedimentation of $nTiO_2$ together with adsorbed metal ions may limit Cu-toxicity for such pelagic organisms.[15] This interaction and the subsequent fate of both Cu and $nTiO_2$ may be substantially influenced by the presence of OM – either during wastewater treatment or in surface water bodies. Titanium dioxide nanoparticles can be stabilized by OM together with the adsorbed Cu. In addition, OM may form complexes with Cu directly utilizing their electron donor groups (i.e., O, N, and S).[16] Thus, both processes might influence Cu-toxicity, and while the complexation with OM reduces the Cu-toxicity, the effect of an OM-coating on $nTiO_2$, which have adsorbed Cu, has yet to be investigated systematically.[16] This knowledge, however, would considerably facilitate the extrapolation and prediction of potential impacts in the field. In this context, this study used several 2×3×7 factorial *Daphnia* acute toxicity tests, assessing two levels of $nTiO_2$, three levels of OM and seven Cu concentrations. All tests were accompanied by chemical analyses of the Cu concentration in the water phase. In addition, the share of Cu^{2+} in the presence and absence of $nTiO_2$ or OM was estimated. The factorial test design was carried out for three $nTiO_2$-products with differing $nTiO_2$ crystalline phase compositions (hence varying surface properties such as surface area and charge), namely anatase (A-100), rutile (R050P) and a mixture of both (P25). Each used three different types of OM exhibiting a wide range of properties (e.g. different aromaticity and hydrophobicity). These types of OM consisted of: seaweed extract (SW), Suwannee River natural organic matter (SR) and sucralose (SL). Suwannee River natural organic matter was – due to its detailed characterization – used as a reference substance. Seaweed extract is not as well-characterized, but is recommended for reproduction tests and long-term *D. magna* cultures by the OECD guideline 211.[21] Sucralose is an artificial organic material, whose molecules due to their charging status, and in contrast to SR and SW, should not complex Cu but may coat $nTiO_2$.[22] Thus, these materials represent a range of properties, which may be characteristic for the OM composition in WWTP effluents.[23]

Material and methods

Test substances

The Cu stock solution was prepared, immediately prior to each application by mixing $Cu(NO_3)_2 \times 3H_2O$ salt (purity ≥99%; Roth, Germany) with one liter of deionized water in a polypropylene volumetric flask. Subsequently, a serial dilution was carried out to achieve the nominal test concentrations of 0, 12, 24, 48, 96, 192 and 384 µg Cu/L in the acute toxicity tests. These concentrations were chosen to obtain a complete dose-response curve (case by case all concentrations were increased by a factor of two), which improves accuracy of the estimated median effective concentrations (EC_{50} values) using immobility as an endpoint.[24] For simplification purposes, the resulting Cu species (88.1% $CuCO_3$(aq), 4.9% $CuOH^+$, 2.0% Cu^{2+}; determined via visual MINTEQ 3.0;[25] cp. Table S1) in the test medium (see section *Test organism*) are henceforth summarized as Cu.

The $nTiO_2$ suspensions – with intensity weighted average particle sizes <100 nm (measured using the DelsaNano C Particle Analyzer; Beckman Coulter, Germany; Table 1) – were prepared additive-free at the Institute for Particle Technology (TU Braunschweig, Germany) by stirred media milling (PML 2, Bühler AG, Switzerland) of the purchased powders, i.e. A-100, P25 and R050P, in deionized water. These $nTiO_2$-products were chosen due to their differing properties, in terms of their crystalline phase composition, surface area and surface charge (Table 1), which are supposed to influence their fate and their effect on Cu-toxicity.[26] The stock suspensions are stabilized by a low pH and showed monodisperse size distributions (polydispersity index <0.2; cp. Table 1). Prior to each test initiation the respective stock suspension (2 g/L) was ultrasonicated for ten minutes to ensure a uniform particle distribution within the suspension. Further, the surface-weighted particle size as well as the initial surface area in diluted stock suspensions (800 µg/L; this concentration was required by the device) were determined for all three $nTiO_2$-products using nanoparticle tracking analysis (NTA 2.3; Malvern Instruments GmbH, Germany; Table 1). Although NTA measurements are based on several simplifying assumptions (e.g. spherical particles, smooth surface), which brings their accuracy into question, this tool is to the best of our knowledge the sole possibility to estimate the surface area in suspension.[27] Therefore, these data should be interpreted with some caution.

Table 1. Properties of the applied $nTiO_2$-products (A-100, P25, R050P).

properties	method	A-100	AEROXIDE® TiO_2 P 25	MKN-TiO_2-R050P
abbreviation	-	A-100	P25	R050P
producer	-	Crenox, Germany	Evonik, Germany	Mknano, Canada
crystalline composition	-	anatase	anatase/rutile	rutile
primary particle size (nm)	advertised[a]	6	21	50
surface area (m²/g)	BET/advertised[a]	~230	50±15	~30
ave. diameter in suspension[b] (nm)	DLS	87.3±2.4	86.0±2.1	90.0±0.7
PI in suspension[b]	DLS	0.132-0.189	0.118-0.164	0.180-0.189
surface area in suspension[b] (m²/g)	NTA	32.5±9.9	28.1±2.2	51.8±8.2
ave. diameter in suspension[b] (nm)	NTA	109.0±11.8	95.2±5.7	140.4±20.8

[a] information given by the producer

[b] suspended in deionized water, stabilized by a low pH

Test organism

The test organism *D. magna* (clone V, Eurofins-GAB laboratories, Germany) was cultured in ASTM reconstituted hard water amended with selenium and vitamins (biotin, thiamine, cyanocobalamine; according to the OECD guideline 202)[24] as well as 20 µL SW/L.[28] The medium was changed three times a week and the organisms were fed with the green algae *Desmodesmus* sp. equivalent to approximately 200 µg carbon per organism on a daily basis. The culture was kept at 20±1°C and a light:dark rhythm of 18:6 h (800-1000 lux; OSRAM L 58W/21-840 ECO, Germany) in a climate-controlled chamber (Weiss Environmental Technology Inc., Germany).

Test design

The tests (2×3×7 factorial design) were conducted in accordance to the OECD guideline 202 (water quality parameters in Table S2) using ASTM reconstituted hard water amended with selenium and vitamins as test medium (see section *Test organism*). The first factor of the test design was the presence or absence of the $nTiO_2$-product, in a concentration reflecting the respective 96-h EC_{50} with an endpoint of immobilization i.e. A-100: 1 mg/L, P25: 2 mg/L and R050P: 4 mg/L (data not shown).[29, 30] The second factor was the level of total organic carbon (TOC; 0, 2 and 8 mg/L; constituting environmentally relevant concentrations) of the applied OM (i.e., SW, SR and SL) which are considered non-toxic to *D. magna*, [31] while the seven Cu concentrations, ranging from 0 to 384 or to 768 µg/L, represent the third factor. Each treatment consisted of five independent replicates while the immobility of the test organisms was assessed as a measure of toxicity every 24 h; focusing on the immobility after 48 – longer exposure durations showed $nTiO_2$-induced toxicity, partly masking the interactions.[32] The experiments were run in 225-mL polystyrene beakers (Duni GmbH, Germany) filled with 50 mL of the test suspension. The test vessels were kept under controlled environmental conditions (see section *Test organism*).

Particle characterization in test medium

The actual particle size distributions of the three applied products were characterized over the entire course of each of the individual experiments, in the presence of three levels (0, 2 and 8 mg TOC/L) of the respective OM (i.e. SW, SR and SL; described below), using the DelsaNano C Particle Analyzer. Therefore 2.5-mL samples were taken from the test vessels (approximately 1 cm under the water surface) at the start of the experiment and every 24 h (Table S3). The actual zeta potentials of the tested $nTiO_2$-products at each OM type and concentration investigated were also measured. These measurements were conducted in the test medium at pH 8.2±0.1 (cp. Table 4). Additionally the DelsaNano C Particle Analyzer was used to assess the nanoparticles electrophoretic mobility. Therefore, all products were mixed with the respective test medium achieving a final concentration of 100 mg $nTiO_2$/L, a concentration enabling valid measurements.

Characterization of the applied organic material

Two of the OMs, i.e. SW and SR, were characterized by the DOC-Labor (Germany) applying SEC-OCD-OND (size-exclusion chromatography - organic carbon detection - organic nitrogen detection): The proportion of dissolved organic carbon (DOC) relative to TOC was 16% for SW, while it was 76% for SR (Table 2; the DOC was summarily determined in the bypass of the chromatographic column after passing a 0.45-µm in-line membrane filter). Furthermore, SR bares slightly higher amounts of hydrophobic components (HOC; 0.45 mg/L) than SW, which had only 0.36 mg HOC/L. The SW had relatively high shares of BIO-polymers (0.1 mg/L), but only 0.2 mg/L of humic substances (HS). Suwanee River natural OM on the other hand, had 4.1 mg/L of HS and 0.1 mg/L of dissolved organic nitrogen (cp. Table 2). Furthermore, the aromaticity was higher for SR than for SW. Other differences between both OMs are detailed in Table S4. Sucralose was not characterized, since its chemical structure/composition (1,6-Dichloro-1,6-dideoxy-β-D-fructofuranosyl-4-chloro-4-deoxy-α-D-galactopyranoside, $C_{12}H_{19}Cl_3O_8$, M=397.63 g/mol; purity >98%; Sigma-Aldrich, USA) as well as its hydrophilicity is well known.[33] Moreover, the Cu concentrations in all OMs were below the limit of quantification (LOQ=0.4 µg/L; see section *Chemical analysis*).

Table 2. Relative composition (%) of the applied OMs (SW and SR) based on the measured DOC.

OM	DOC							
	total	HOC[a]	CDOC[b]	major CDOC constituents				
			total	BIO-polymers	HS	building blocks	LMW[c] neutrals	LMW[c] acids
SW	16.0	28.6	71.4	5.5	15.3	22.3	24.4	3.8
SR	76.4	7.4	92.6	0.1	66.7	12.6	12.6	0.5

[a] based on the measured DOC.

[b] CDOC, chromomorphic dissolved organic carbon

[c] LMW, low molecular weight

Chemical analysis

Samples for the determination of Cu in the water column were taken: at the initiation and termination of each acute toxicity test, from the control, and from an intermediate Cu concentration (near the EC_{50} of Cu; 48 µg/L) of each combination of OM and $nTiO_2$. The samples were taken approximately 1 cm below the water surface. Following acidification with HNO_3, samples were stored at 4°C. The total Cu (including suspended insoluble species) of each individual sample was then measured for ^{65}Cu with inductively coupled plasma and a quadrupole mass spectrometer (ICP-MS; XSeries 2, Thermo Fisher Scientific, Germany) using a FAST autosampler (ESI, Thermo Fisher Scientific, Germany), a peek spray chamber (Thermo Fisher Scientific, Germany) and a robust Mira Mist peek nebulizer (Burgener, England) as described elsewhere.[15] The initial Cu concentrations deviated marginally from the nominal concentration, regardless of the applied type and amount of $nTiO_2$ and OM. Therefore, the nominal concentrations are used for all calculations in this study.

To investigate the Cu-adsorption behavior of the tested $nTiO_2$-products, the latter were mixed in an additional experiment at concentrations reflecting the respective 96-h EC_{50} i.e. A-100: 1 mg/L, P25: 2 mg/L and R050P: 4 mg/L with 0 and 8 mg TOC/L of the respective OMs and 100 µg/L Cu. After 24 h reaction time (equilibrium expected)[34-36] the samples were centrifuged (10015g for 1 h; forcing $nTiO_2$ >25 nm to settle) and the supernatants (5 of 15 mL) were quantified for Cu. This set up allowed the discrimination between Cu complexed by OM (as well as unbound Cu, i.e. neither adsorbed by $nTiO_2$, nor complexed by OM) and Cu adsorbed to $nTiO_2$ (and additionally OM), since only Cu not associated to $nTiO_2$ could be quantified in the supernatant.

In addition to the chemical analysis, the concentration of Cu^{2+} was modeled via visual MINTEQ (see supporting information for further details).[25]

Statistics

The statistical analyses were accomplished using the statistics program R version 3.0.1 for Windows and the extension package drc.[37, 38] The EC_{50} values of Cu after 48 and 72 h were determined by fitting adequate dose-response models (Figures S1-S9). The respective models were selected based on Akaike's information criterion and visual inspection. To assess the implication of the respective $nTiO_2$-product or type of OM, as well as their combination on Cu-toxicity, confidence interval (CI) testing between the respective EC_{50} values was applied.[39] If 95% CIs did not include zero, the difference was judged as statistically significant. This procedure avoided current shortcomings of null hypotheses significance testing such as ignoring the effect sizes and their precision.[40] The EC_{50} values following 24 and 96 h of exposure were not determined since the toxicity caused by Cu was either too low (especially in presence of OM) or already masked by effects of $nTiO_2$ to such an extent, that a reliable fitting of dose-response models was impossible.

Results and discussion

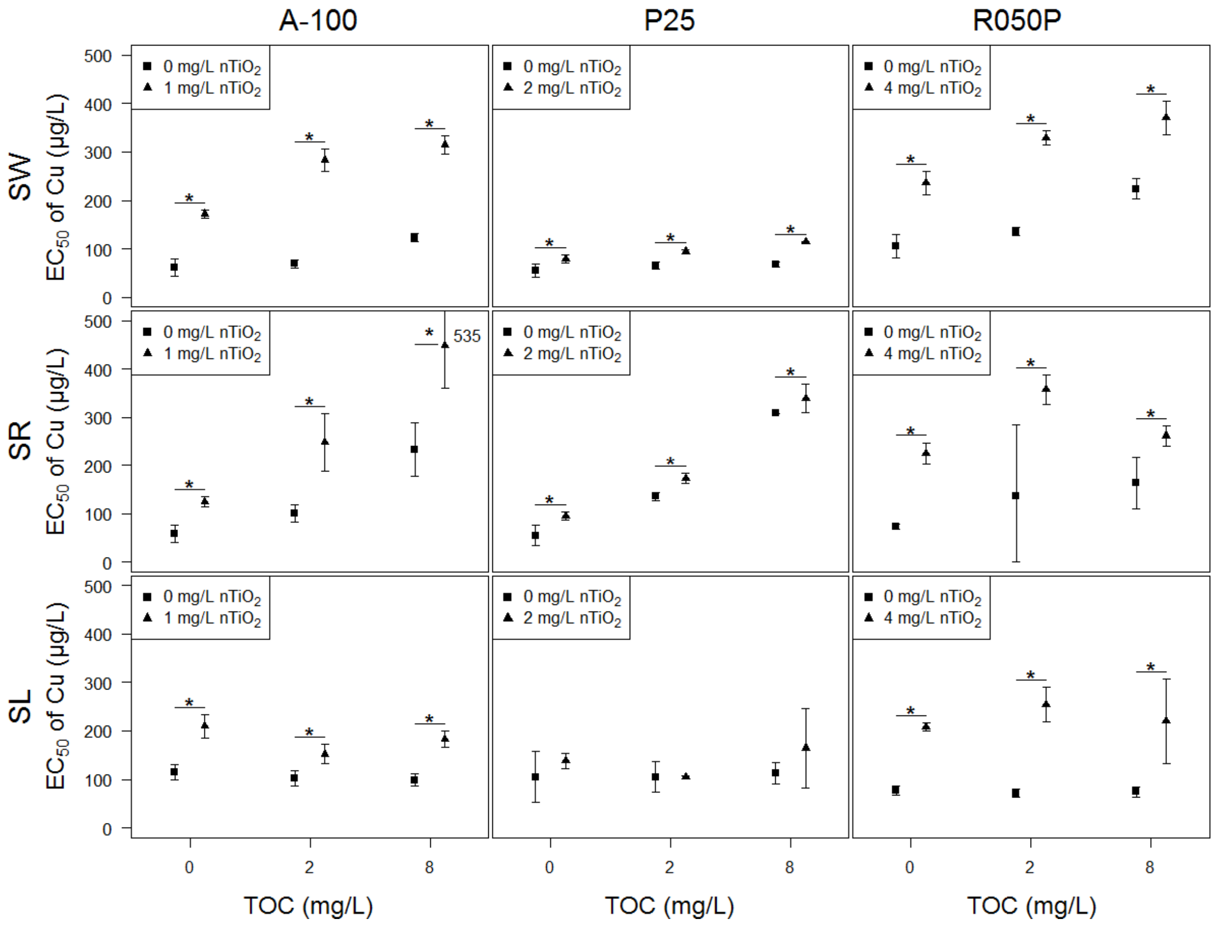

Figure 1: EC$_{50}$ values of Cu after 48 h test duration. The 48-h EC$_{50}$ values were determined at different levels (0, 2 and 8 mg TOC/L) and types of organic matter (seaweed extract (SW), Suwannee River natural organic matter (SR) and sucralose (SL)) and in absence (■) and presence (▲) of nTiO$_2$ (i.e. A-100, P25 and R050P). Asterisks denote statistically significant differences between the absence and presence of nTiO$_2$.

Copper toxicity reduction by nTiO$_2$

All applied nTiO$_2$-products reduced the toxicity of Cu after 48 h of exposure: R050P was – with a 165% (range: 124-203%; Figure 1) higher Cu 48-h EC$_{50}$ on average – the most efficient, while P25 (average: 50% increased Cu 48-h EC$_{50}$; range: 32-72%) was the least efficient. The differing efficiency of the three nTiO$_2$-products in reducing Cu-related toxicity may partly be explained by the different concentrations applied. By standardizing the reductions in Cu-toxicity to the nTiO$_2$ concentration it was uncovered that A-100 – with, on average, a 125% (range: 83-179%) higher Cu 48-h EC$_{50}$ compared to Cu solely – was the most efficient product. By further standardizing the Cu-toxicity reduction potential to the total NTA measured surface area of the nTiO$_2$-products (Table 1), each applied square meter of A-100 surface area reduced the Cu-toxicity by 4%, followed by R050P and P25 with approximately 1% each (Table S5). This supports the assumption that the crystalline structure is more important than the particle size, while anatase is supposed to be an intrinsically stronger sorbent than rutile.[41] This effectiveness of the A-100 surface in reducing Cu-toxicity might be explained by its surface structure (i.e. porosity), which is likely overlooked by NTA measurements although it would offer substantial binding sites (i.e. unsaturated oxygen/deprotonated surface hydroxyls).[26, 27]

The measured Cu residuals (total Cu) in the water column after centrifugation (Table 3) displayed comparable amounts of Cu adsorbed to nTiO$_2$ (approximately 80%) for A-100 and R050P, however, P25 adsorbed much less Cu (approximately 40%). This coincides well with the toxicity data and the nanoparticles' surface area. To achieve the measured Cu-removal and hence a comparably reduced toxicity of Cu, R050P had to be applied at four times the concentration of A-100. Moreover, this pattern can largely be explained by the modeled adsorption of Cu^{2+}, which was highest for A-100 followed by R050P and P25 throughout all nominal Cu concentrations (Table S1). Since the differences in Cu removal predicted by the model were smaller (at 48 µg Cu/L the concentration of Cu^{2+} was 42, 22 and 18% lower in the presence of A-100, R050P and P25 compared to the absence of nTiO$_2$, respectively) than the differences expected based on the responses of the test species, other mechanisms besides surface complexation, such as photodeposition (which is the reduction of metal ions (e.g., M$^+$→M^0) by electrons produced at the surface of the semiconducting $nTiO_2$ during irradiation), may have an effect.[42] Furthermore, the measured zeta potentials underpin the differences observed regarding Cu-toxicity reduction. R050P and A-100 displayed a more negative zeta potential in the test medium (pH 8.2±0.1; cp. Table S2) than P25, which is utilized as a proxy for the surface charge (Table 4). This observation suggests a higher amount of deprotonated surface hydroxyls and thus negative charges compared to P25, which in turn bear a higher potential for the adsorption of Cu^{2+}.

Table 3. Copper residuals (mean±SD in % relative to the Cu only treatment; total Cu) in the supernatant after 24 h reaction time followed by 1 h centrifugation (10015g) in presence of the three $nTiO_2$ products (i.e., A-100, P25 and R050P; 1, 2 and 4 mg $nTiO_2$/L, respectively) and OM types (i.e., SW, SR and SL; 0 and 8 mg TOC/L). In addition, the calculated percentage of Cu adsorbed to $nTiO_2$. The applied nominal Cu concentration was 100 µg/L. All values were corrected for a potential adsorption to the test vessels.

OM[a] type	Cu in the water column			Cu adsorbed to $nTiO_2$		
	A-100	P25	R050P	A-100	P25	R050P
-	21±4	58±1	22±5	79	42	78
SW	32±2	57±1	43±3	68	43	57
SR	64±1	81±1	73±2	36	19	27
SL	17±4	53±1	23±5	83	47	77

[a] 8 mg TOC/L

Table 4. Zeta potentials (mV) of $nTiO_2$ (i.e. P25, A-100 and R050P) in the test medium (pH 8.2±0.1) at different levels (i.e. 0, 2 and 8 mg TOC/L) of three types of OM (i.e. SW, SR and SL).

product	0 mg TOC/L	2 mg TOC/L			8 mg TOC/L		
		SW	SR	SL	SW	SR	SL
P25	-9.2±0.2	-19.5±0.2	-17.9±0.5	-7.5±0.1	-19.9±0.4	-23.4±0.3	-7.9±0.6
A-100	-11.7±0.3	-14.4±0.2	-13.9±0.2	-10.0±0.3	-14.3±0.3	-19.8±0.4	-10.8±0.5
R050P	-13.4±0.2	-21.4±0.4	-23.8±0.2	-6.3±0.6	-22.7±0.4	-23.0±0.3	-6.8±0.5

A similar toxicity pattern after 48 h was uncovered after 72 h of exposure (72-h EC_{50}), although the effect sizes between treatments decreased (data not shown). This phenomenon may be explained by an ingestion of sedimented $nTiO_2$ agglomerates together with adsorbed Cu – since *D. magna* also forages at the bottom when suspended food concentrations are low [43] – and a subsequent release of Cu ions during gut passage.

Although the pH in daphnids gut is neutral,[44] the release of ions could be promoted by the enzymatic activity.[45] The successful ingestion of $nTiO_2$ was observed and measured (including the uptake of adsorbed Cu) during earlier studies supporting this assumption.[30, 15] Moreover, the toxic effect attributed solely to $nTiO_2$, evident after 72 h of exposure, might contribute to this observation.[32] Irrespective of the decreasing effect size with increasing study duration, the responses of *Daphnia* are in agreement with the quantified Cu residuals (total Cu) in the water column at test termination (96 h; Table 5). The product P25 reduced the Cu concentration in the water column by only 60% (Table 5), while a more substantial reduction (approximately 85%) was observed in the presence of both A-100 and R050P individually (Table 5). Finally, the efficiency of $nTiO_2$-products to reduce Cu-toxicity may be explained by the combination of surface area, structure and ultimately the availability of binding sites.

Table 5. Copper residuals at test termination (%; mean±SD) relative to the nominal concentration at test initiation in the absence and presence of the three $nTiO_2$ products (i.e., A-100, P25 and R050P; 1, 2 and 4 mg $nTiO_2$/L, respectively) and OM types (i.e., SW, SR and SL; 0 and 8 mg TOC/L).

OM	OM	absence of	presence of		
(mg TOC/L)	type	$nTiO_2$	A-100	P25	R050P
0	-	92±5	14±4	37±4	13±1
	SW	85±7	50±1	37±0	19±0
2	SR	90±7	81±0	85±1	24±0
	SL	89±2	11±0	33±1	16±0
	SW	83±7	79±0	81±0	77±1
8	SR	92±6	107±0	98±1	89±1
	SL	90±2	12±0	33±0	15±0

Reduction of copper toxicity by OM

Besides $nTiO_2$, the presence of OM also reduced Cu-toxicity: SR at 8 mg TOC/L lowered the Cu-toxicity the most, showing a 48-h EC_{50} approximately 300% (range: 121-460%) higher than in the absence of OM (Figure 1) followed by SW with approximately 80% increase compared to OM's absence (range: 21-113%).[46] These effect sizes even slightly increase after 72 h of exposure. In contrast, SL (8 mg TOC/L) does not seem to influence Cu-toxicity (Figure 1), which may be explained by its neutral charge and the lack of good complexing groups.[16] Thus, SL is not able to complex metal ions, while SW and SR contain humic substances and hence carboxylic and phenolic groups, which can form chelate complexes and thereby reduce the bioavailability of Cu.[47]

Complex interactions of the OM properties may explain the higher efficiency of SR compared to SW in terms of Cu-complexation and hence toxicity reduction. In this context, the contribution of DOC in the SW and SR treatments, which was 16 and 76% (1.3 and 6.1 mg DOC/L) of the total applied OM, respectively, may be an important trigger. As a result, the modeled concentration of Cu^{2+} in presence of SR was lower compared to SW, however this difference decreases with increasing Cu concentration. This difference coincides with the four-fold difference in Cu-toxicity reduction between both OM types (Table 2). Furthermore, the content of humic substances, which was higher by a factor of 20 in SR than in SW, could be an accessory parameter indicating the potential of OMs to reduce Cu-toxicity.[47-51] Moreover, Croué et al.[52] discovered that the hydrophobic acid (mainly polyhydroxy and unsubstituted aromatics) and the transphilic neutral fraction (mainly proteins) of OMs can complex up to 75% of the Cu. Both fractions, indicated by the aromaticity as well as the nitrogen content (as a proxy for amino groups and hence proteins) are present to a larger extent in SR than in SW (Table S4) further explaining the observed differences.

Copper toxicity reduction by the combination of $nTiO_2$ and OM

The presence of 2 and 8 mg TOC/L of natural OMs, i.e. SR and SW, in combination with $nTiO_2$ further increased the 48-h EC_{50} values and hence decreased Cu-toxicity by an average factor of approximately two (range: 1.3-4.4) and four (range: 1.5-10.6) relative to their absence, respectively. More specifically, an amendment of the test medium with SR at 8 mg TOC/L resulted in a more pronounced decrease in Cu-toxicity (in average 5.8-fold, range: 1.5-10.6) compared to SW (in average 2.3-fold, range: 1.5-3.3). As detailed above, this difference between SR and SW might be attributed to the varying quantity as well as quality (e.g. amount of humic substances and aromaticity) of DOC.

The substantial increase of EC_{50} values in the presence of both natural OM and $nTiO_2$ may be explained by the combination of complexation and adsorption processes, which may have reduced the bioavailability of Cu jointly. But, in some cases the decrease in Cu-toxicity was higher as expected based on the individual effects of both factors. Such a synergistic activity can be on average 46% (range: 36-62%; after a deduction of the individual effects) of the in total observed reduction in Cu-toxicity. This observation was, however, only statistically significant for the combination of A-100 and 2 mg TOC/L. An explanation might be the adsorption of OM onto the surface of $nTiO_2$, which was indicated by an increased zeta-potential of the particles (average factor 1.7, range: 1.2-2.6; cp. Table 4).[53] Subsequently, the surface functionality of $nTiO_2$ may have been altered increasing its adsorption capacity as well as the OM's degree of protonation.[54, 10]

Although the responses of *Daphnia* indicated a massive reduction of Cu-toxicity, the Cu concentrations in the water column in presence of 8 mg TOC/L and $nTiO_2$ were at the termination of the experiment on average at 88% of the nominal concentration (range: 77-107%; Table 5). This discrepancy may be explained by the Cu-complexation, as well as the stabilization of $nTiO_2$ together with potentially adsorbed Cu in the water column by OM.[7] To distinguish between these two processes additional experiments quantified the Cu concentration in the water column after an interaction including all combinations of OMs (0 vs. 8 mg TOC/L) and $nTiO_2$-products for 24-h and subsequent centrifugation. The centrifugation forced $nTiO_2$ – together with potentially adsorbed Cu – above 25 nm in size to settle. Thus, at least 90% of the applied particles, regardless of a stabilizing coating with OM, were removed from the water column and hence mainly unbound and Cu complexed only by OM was quantified in the supernatant. This experiment indicated that the presence of OM resulted in high residual Cu concentrations in the supernatant (in average 51% of the nominal concentration; Table 3), which were much lower in the absence of OM (in average 21% of the nominal concentration) for A-100 and R050P. Furthermore, these results show that roughly 55% of the residual Cu concentration at the termination of the acute toxicity test with *Daphnia* in presence of both OM and $nTiO_2$ can be allocated to the complexation by OM and 45% to $nTiO_2$. Both processes, therefore, seem to be important in reducing the ecotoxicological potential of Cu.

In contrast to SW and SR, the presence of SL decreased rather than increased the 48-h EC_{50} in combination with $nTiO_2$-products by up to 93% (average: 50, range: -8-93). This lowered potential of $nTiO_2$ to reduce Cu-toxicity may be explained by the decreasing surface charge in the presence of SL (Table 4), which in turn may limit the adsorption of Cu. However, this reduced adsorption was not confirmed by the measurements of Cu in the water column after test termination or in the supernatant of the additional experiment. The supernatant measurements were as low as in the absence of SL (on average 20% of the nominal concentration; Table 5). These data finally suggest that either SL increases the toxicity of $nTiO_2$ or that the degradation products of SL are directly toxic for *Daphnia*, such processes might have masked the Cu-toxicity reduction of $nTiO_2$.[55, 56] The presence of degradation products – probably caused by hydroxyl radicals – was indirectly assessed via the quantification of SL, chloride and TOC in the presence and absence of $nTiO_2$ by the Water Technology Center (Germany; Table S6).[57] Indeed, in the presence of A-100 1 mg SL/L (more than 4%) vanished and the chloride concentration increased by 0.1 mg/L (approximately 3%) in comparison to the absence of A-100. This observation was most likely due to dechlorination or mineralization by radicals.[55, 57] Even though the identity of the degradation products was not revealed, the results suggest that they were formed and might have contributed to the effect on *Daphnids* survival. It may therefore be concluded that the identity of the organic molecules adsorbed to $nTiO_2$ influences the interaction of heavy metals and the nanoparticles dramatically.

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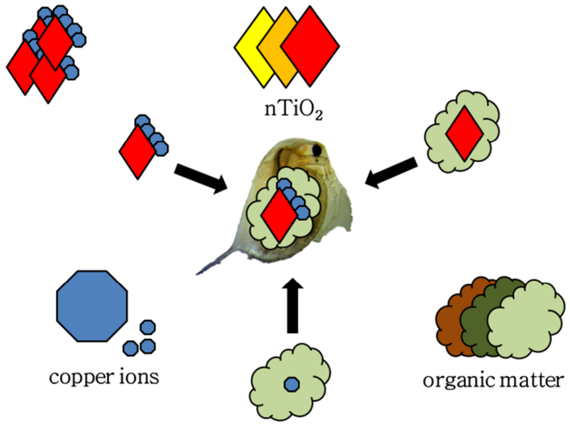

Supporting information

Copper species distribution

The Cu species distribution including the adsorption to $nTiO_2$ and the complexation by OMs was modeled for all factor combinations assessed during the present study with the software visual MINTEQ version 3.0.[1] For the model no interaction between $nTiO_2$ and OM was assumed. The modeling of a ternary system including the change in surface properties of $nTiO_2$ and the degree of protonation of the OMs as a result of their adsorption was not within the scope of this study.[2] For the assessment of complexation by OM (8 mg/L) the NICA-Donnan model for fulvic acids was utilized setting the DOC concentration within the model to the measured levels (Table S4). For reasons of simplification, properties beyond the determined fulvic acid-like character of SW and SR (such as the unusually high concentrations of building blocks and low molecular weight acids in case of SW) were not particularly considered during the calculations, potentially underestimating their complexation capability. The model is a combination of the non-ideal competitive adsorption (NICA) isotherm, which describes the binding to heterogeneous material, and a Donnan electrostatic sub-model covering the electrostatic interactions between ions and OM.[3-5]

The $nTiO_2$ surface complexation reactions, which trigger the adsorption of Cu to $nTiO_2$ were assessed by applying the multi site complexation model approach extended by the Pauling charge distribution concept (CD-MUSIC).[6] This approach is based on a standard three planes model assuming an inner capacitance of 1.25 F/m² for $nTiO_2$, which is the mean of the usually found values, grouping around 0.9 and 1.6 F/m², regardless of the crystalline phase. This variation is suggested to result from different surface roughness or surface protrusions as a consequence of the production process.[7] The outer capacitance was set to 5 F/m², a common value for metal oxides.[8, 9] The site density was set 7 and 8 nm^{-2} for A-100 and R050P, respectively, while we assumed a site density of 7.1 nm^{-2} for P25, since those nanoparticles' surfaces have a patch-wise structure consisting mainly of anatase and <10% rutile.[7] The protonation as well as the ion pair formation constants were taken from the literature.[7, 10-12] Furthermore, the surface area and particle diameter stated by the producer were used as basis for the calculation of surface complexation reactions.

Table S1. The modeled Cu^{2+} concentration (µg/L) in the test medium for the respective nominal total Cu concentrations and treatment.

treatment	nominal Cu concentration (µg/L)						
	12	24	48	96	192	384	768
Cu	0.2	0.5	0.9	1.9	3.8	8.0	17.6
A-100 x Cu	0.0	0.1	0.5	1.5	3.4	7.6	17.2
P25 x Cu	0.1	0.3	0.8	1.7	3.6	7.8	17.4
R050P x Cu	0.1	0.3	0.7	1.7	3.6	7.8	17.4
SW x Cu	0.0	0.1	0.4	1.2	3.3	8.0	18.9
SR x Cu	0.0	0.0	0.0	0.2	1.3	5.9	20.2
A-100 x SW x Cu	0.0	0.0	0.2	0.9	2.8	7.4	18.4
P25 x SW x Cu	0.0	0.0	0.3	1.1	3.1	7.7	18.7
R050P x SW x Cu	0.0	0.0	0.3	1.0	3.0	7.7	18.6
A-100 x SR x Cu	0.0	0.0	0.0	0.1	1.0	5.3	19.3
P25 x SR x Cu	0.0	0.0	0.0	0.2	1.2	5.7	19.8
R050P x SR x Cu	0.0	0.0	0.0	0.2	1.2	5.6	19.7

Table S2. Measured pH and conductivity (mean±SD) of the different treatments, containing different organic matter (OM) types (i.e. SW, SR, SL) and levels (i.e. 0, 2, 8 mg TOC/L). The measured oxygen concentration was above 8 mg/L and the temperature 20±1°C.

TOC (mg/L)	OM	pH			conductivity (µS/cm)		
0	-	8.2	±	0.1	610.6	±	43.5
	SW	8.2	±	0.0	598.0	±	14.1
2	SR	8.2	±	0.2	587.0	±	30.9
	SL	8.2	±	0.2	615.0	±	73.5
	SW	8.3	±	0.0	607.3	±	25.0
8	SR	8.1	±	0.1	578.7	±	29.3
	SL	8.1	±	0.2	612.7	±	79.9

Table S3. Agglomeration state of $nTiO_2$ (i.e. A-100, P25 and R050P) monitored throughout the test duration. Average particle diameter (mean in nm with (SD) and [PI]; n≥3) measured immediately and every 24 h following application in test medium amended with different levels (i.e. 0, 2 and 8 mg TOC/L) of OM (i.e. SW, SR and SL).

product	time	0	SW		SR		SL	
			2	8	2	8	2	8
A-100	0	1160.8	1234.6	564.9	720.1	480.9	910.4	781.0
		(91.5)	(99.2)	(59.1)	(32.8)	(25.4)	(71.1)	(40.7)
		[0.504-0.586]	[0.635-0.677]	[0.201-0.235]	[0.385-0.407]	[0.235-0.304]	[0.296-0.375]	[0.278-0.304]
	24	4250.3	888.8	553.7	798.2	484.1	4239.0	3899.2
		(362.4)	(32.4)	(21.5)	(115.9)	(17.0)	(67.9)	(209.5)
		[1.016-1.398]	[0.414-0.424]	[0.203-0.275]	[0.337-0.524]	[0.203-0.211]	[0.962-1.159]	[0.896-0.959]
	48	4316.0	850.8	480.8	686.0	471.6	NA	4128.9
		(432.0)	(48.2)	(37.5)	(32.9)	(25.9)	NA	(398.5)
		[1.045-1.408]	[0.380-0.409]	[0.197-0.265]	[0.327-0.380]	[0.192-0.284]	NA	[1.049-1.355]
	72	NA	848.8	480.3	680.2	455.1	NA	NA
		NA	(52.4)	(25.0)	(50.1)	(16.1)	NA	NA
		NA	[0.371-0.438]	[0.208-0.223]	[0.328-0.381]	[0.189-0.207]	NA	NA
	96	NA	881.1	488.4	907.2	446.7	NA	NA
		NA	(37.3)	(34.5)	(39.3)	(11.4)	NA	NA
		NA	[0.403-0.435]	[0.196-0.274]	[0.429-0.446]	[0.191-0.292]	NA	NA
P25	0	521.2	231.2	221.8	196.8	179.7	361.6	390.6
		(20.1)	(23.9)	(4.3)	(3.6)	(4.3)	(27.4)	(28.6)
		[0.224-0.261]	[0.149-0.256]	[0.185-0.234]	[0.212-0.236]	[0.185-0.226]	[0.222-0.269]	[0.231-0.262]
	24	2974.4	232.1	226.6	213.5	182.4	3214.9	3171.1
		(278.5)	(5.9)	(0.9)	(1.4)	(1.9)	(37.6)	(66.3)
		[0.802-0.988]	[0.211-0.219]	[0.242-0.253]	[0.203-0.249]	[0.203-0.224]	[0.920-0.938]	[0.821-0.959]
	48	3111.4	228.4	235.1	256.7	181.5	3992.5	4890.2
		(535.9)	(1.6)	(8.1)	(20.2)	(2.4)	(1041.6)	(789.3)
		[0.882-0.974]	[0.221-0.229]	[0.207-0.247]	[0.229-0.275]	[0.211-0.227]	[1.076-1.391]	[1.115-1.217]
	72	NA	265.6	236.5	506.1	179.4	NA	NA
		NA	(16.5)	(17.1)	(13.6)	(1.6)	NA	NA
		NA	[0.148-0.236]	[0.136-0.241]	[0.233-0.237]	[0.210-0.226]	NA	NA
	96	NA	336.3	238.5	1186.2	182.1	NA	NA
		NA	(43.1)	(10.0)	(57.7)	(2.1)	NA	NA
		NA	[0.178-0.217]	[0.154-0.251]	[0.427-0.477]	[0.187-0.227]	NA	NA
R050P	0	492.9	213.6	195.9	180.7	209.3	451.7	463.8
		(25.6)	(2.1)	(11.6)	(1.1)	(1.0)	(37.8)	(38.3)
		[0.265-0.271]	[0.234-0.250]	[0.146-0.239]	[0.208-0.213]	[0.222-0.250]	[0.242-0.248]	[0.257-0.270]
	24	3219.4	252.0	197.7	189.6	210.5	2890.7	2032.3
		(966.1)	(30.8)	(9.2)	(2.2)	(4.0)	(225.9)	(44.6)
		[0.737-1.132]	[0.150-0.243]	[0.216-0.235]	[0.201-0.225]	[0.216-0.248]	[0.792-0.917]	[0.661-0.669]
	48	5869.1	261.1	183.6	277.5	213.2	4969.6	3355.8

	(924.6) [1.365-2.001]	(9.0) [0.241-0.266]	(11.2) [0.214-0.243]	(24.1) [0.162-0.269]	(3.2) [0.224-0.236]	(631.1) [0.870-1.537]	(170.8) [0.814-0.869]
72	NA NA NA	364.6 (8.3) [0.182-0.199]	189.9 (3.6) [0.232-0.255]	629.7 (29.3) [0.264-0.284]	211.6 (1.7) [0.233-0.256]	NA NA NA	8540.8 (1281.7) [2.000-2.298]
96	NA NA NA	484.2 (10.1) [0.223-0.246]	196.2 (3.7) [0.233-0.239]	2063.0 (146.6) [0.696-0.775]	210.2 (4.3) [0.234-0.257]	NA NA NA	NA NA NA

Table S4. Composition of the applied OMs (SW and SR) in μg C/L if not stated otherwise.

OM	DOC											SUVA
	total	HOC	CDOC									
			total	BIO-polymers	HS				Building blocks	LMW neutrals	LMW acids	
					total	DON	Aromacity[a]	Mol-weight[b]				
SW	1276	366	911	71	196	10	4.96	954	285	312	48	2.13
SR	6113	455	5658	6	4080	59	5.5	808	772	770	31	4.65

[a] in L/(mg*m)

[b] in g/mol

OM, organic matter
DOC, dissolved organic carbon
HOC, hydrophobic organic carbon
CDOC, chromomorphic dissolved organic carbon
HS, humic substances
DON, dissolved organic nitrogen in μg N/L
LMW, low molecular weight
SUVA, specivic UV absorbance in L/(mg*m)

Table S5. Surface area standardized change in 48-h EC_{50} values (%/m²) for Cu in the presence and absence of $nTiO_2$ and/or organic matter (OM; i.e. 0, 2 and 8 mg TOC/L of SW, SR and SL) compared to the Cu only treatment.

OM (mg TOC/L)	OM type	A-100	P25	R050P
0	-	4	1	1
2	Sw	11	1	1
	SR	10	4	2
	SL	1	0	1
8	Sw	13	2	1
	SR	21	9	1
	SL	2	1	1

Table S6. The measured concentrations of SL (HPLC-MS/MS), TOC, and chloride (ion chromatography), in two samples containing: SL (8 mg TOC/L) in the presence, and in the absence of A-100. Both samples are composite samples of 20 beakers treated exactly like the beaker in the acute toxicity tests, including light conditions and temperature.

parameter	SL in absence of A-100	SL in presence of A-100
sucralose (mg/L)	23.0	22.0
TOC (mg/L)	8.8	9.1
chloride (mg/L)	3.7	3.8

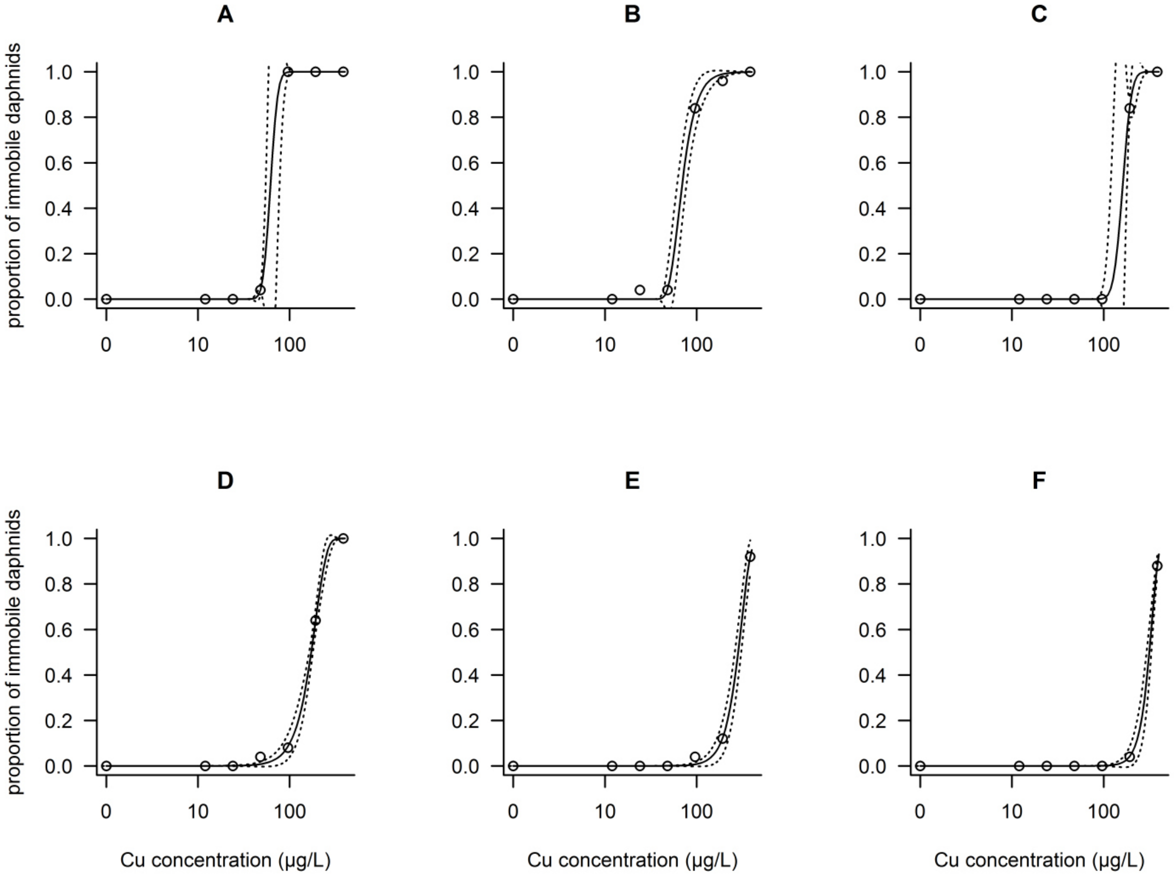

Figure S1. A-100 × Sw – Dose-response curves of Cu in absence (A-C) and presence (D-F) of $nTiO_2$ and the respective organic matter (OM) level (A+D: 0 mg TOC/L, B+E: 2 mg TOC/L, C+F: 8 mg/L) with 95% confidence intervals after 48 h.

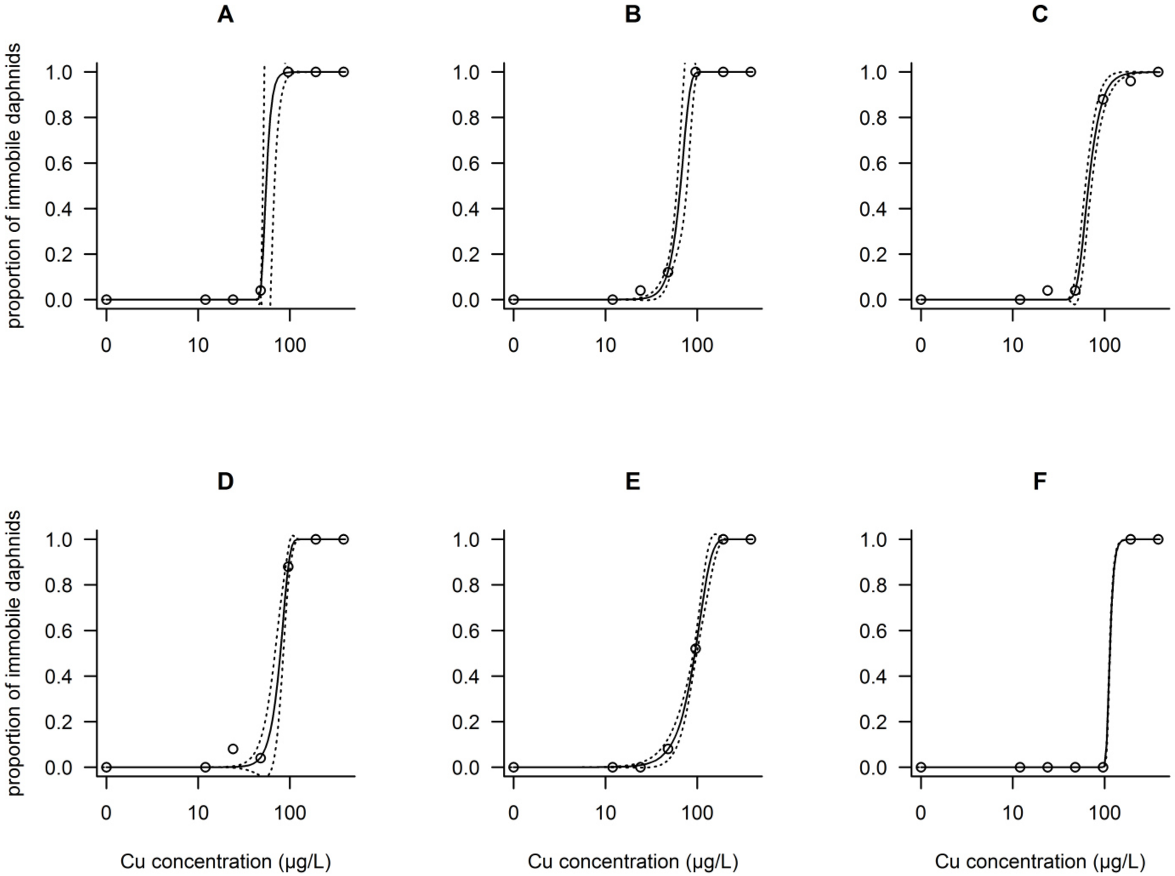

Figure S2. P25 × Sw – Dose-response curves of Cu in absence (A-C) and presence (D-F) of $nTiO_2$ and the respective organic matter (OM) level (A+D: 0 mg TOC/L, B+E: 2 mg TOC/L, C+F: 8 mg/L) with 95% confidence intervals after 48 h.

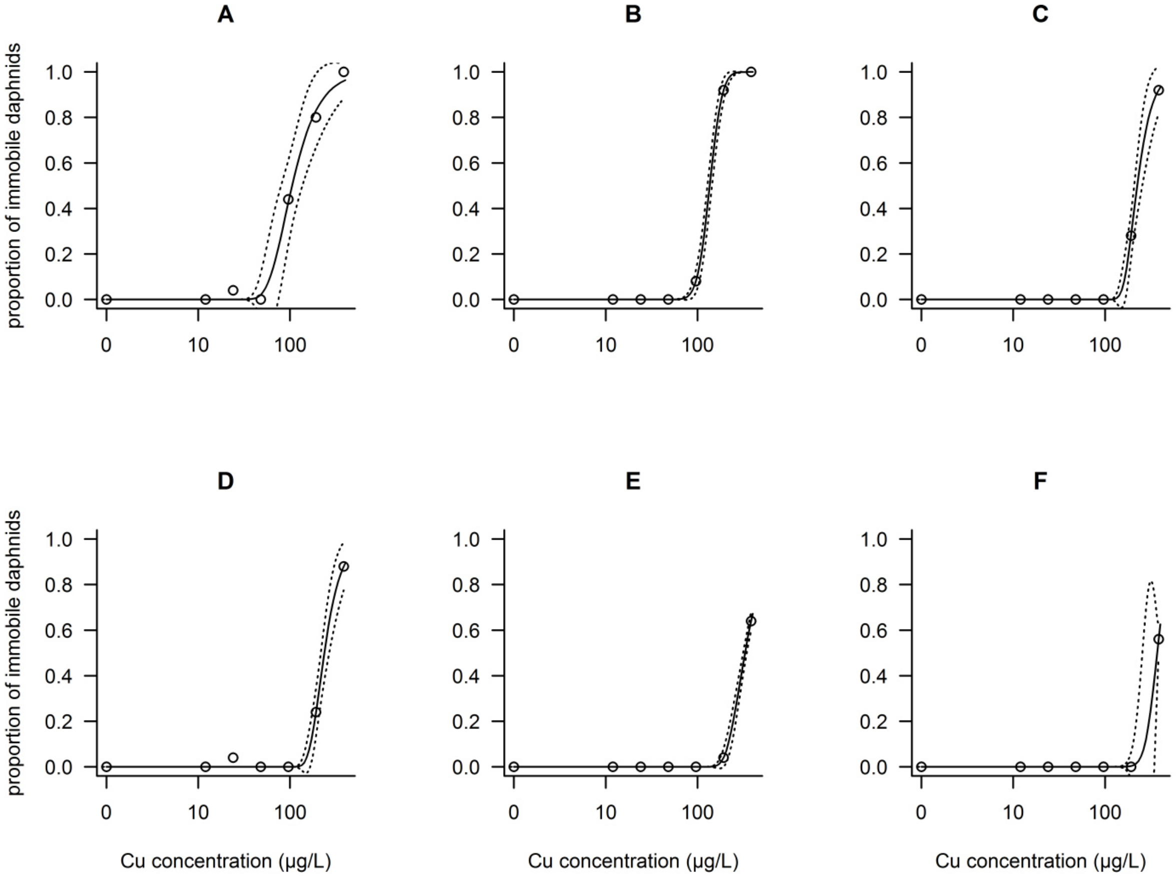

Figure S3. R050P × SW – Dose-response curves of Cu in absence (A-C) and presence (D-F) of $nTiO_2$ and the respective organic matter (OM) level (A+D: 0 mg TOC/L, B+E: 2 mg TOC/L, C+F: 8 mg/L) with 95% confidence intervals after 48 h.

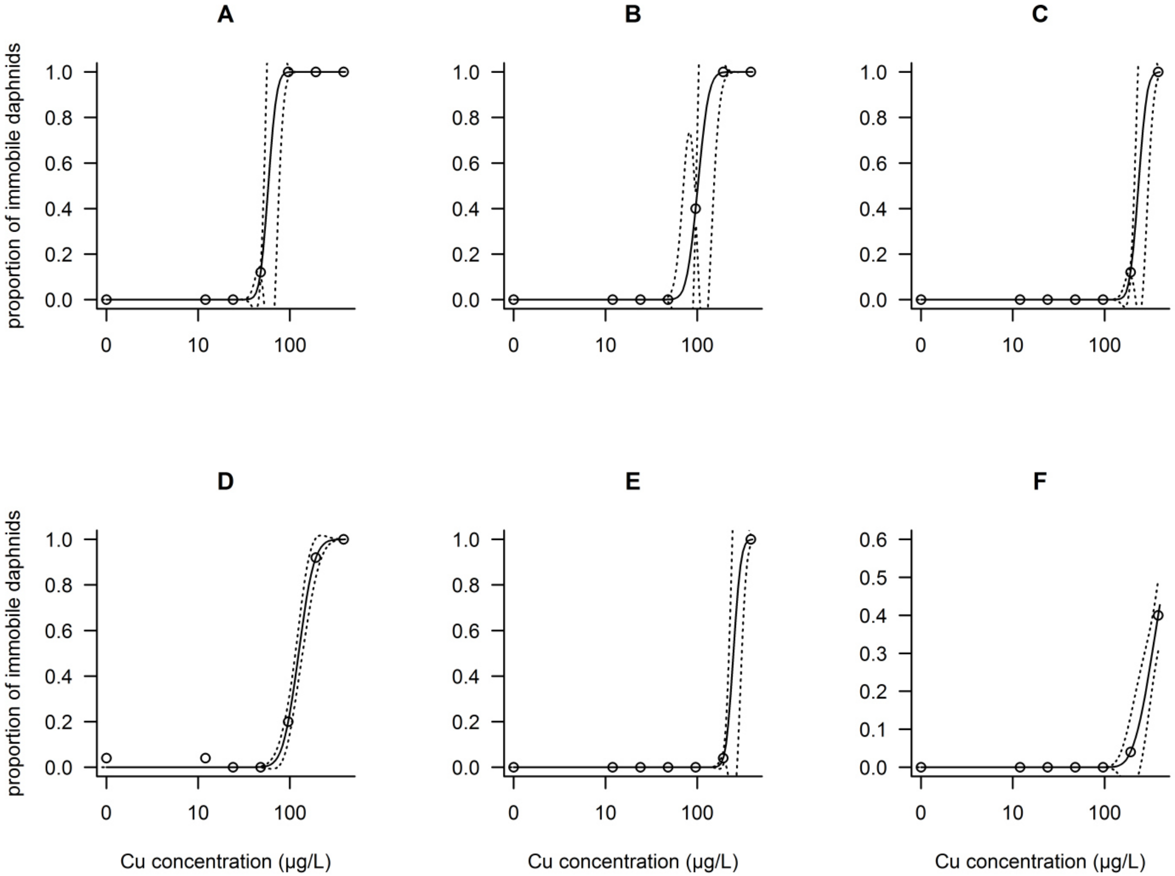

Figure S4. A-100 × SR – Dose-response curves of Cu in absence (A-C) and presence (D-F) of $nTiO_2$ and the respective organic matter (OM) level (A+D: 0 mg TOC/L, B+E: 2 mg TOC/L, C+F: 8 mg/L) with 95% confidence intervals after 48 h.

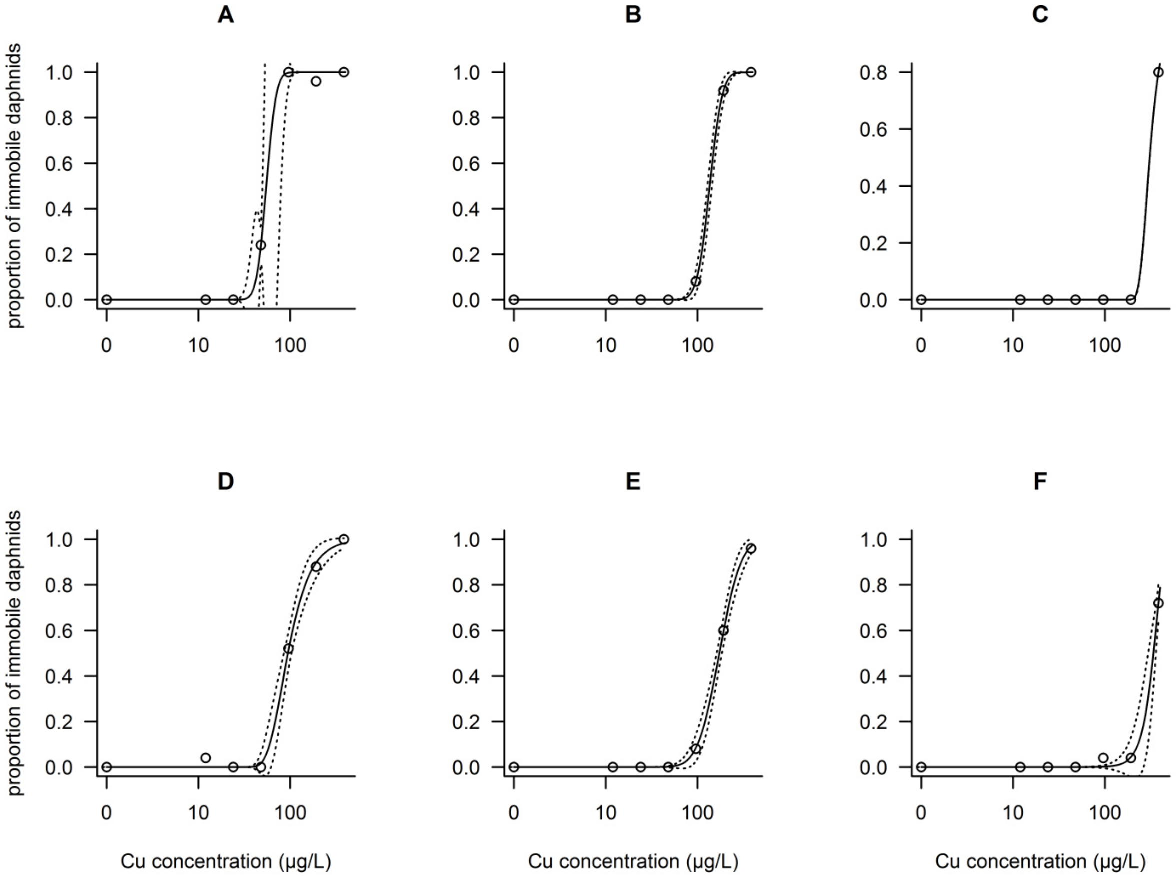

Figure S5. P25 × SR – Dose-response curves of Cu in absence (A-C) and presence (D-F) of $nTiO_2$ and the respective organic matter (OM) level (A+D: 0 mg TOC/L, B+E: 2 mg TOC/L, C+F: 8 mg/L) with 95% confidence intervals after 48 h.

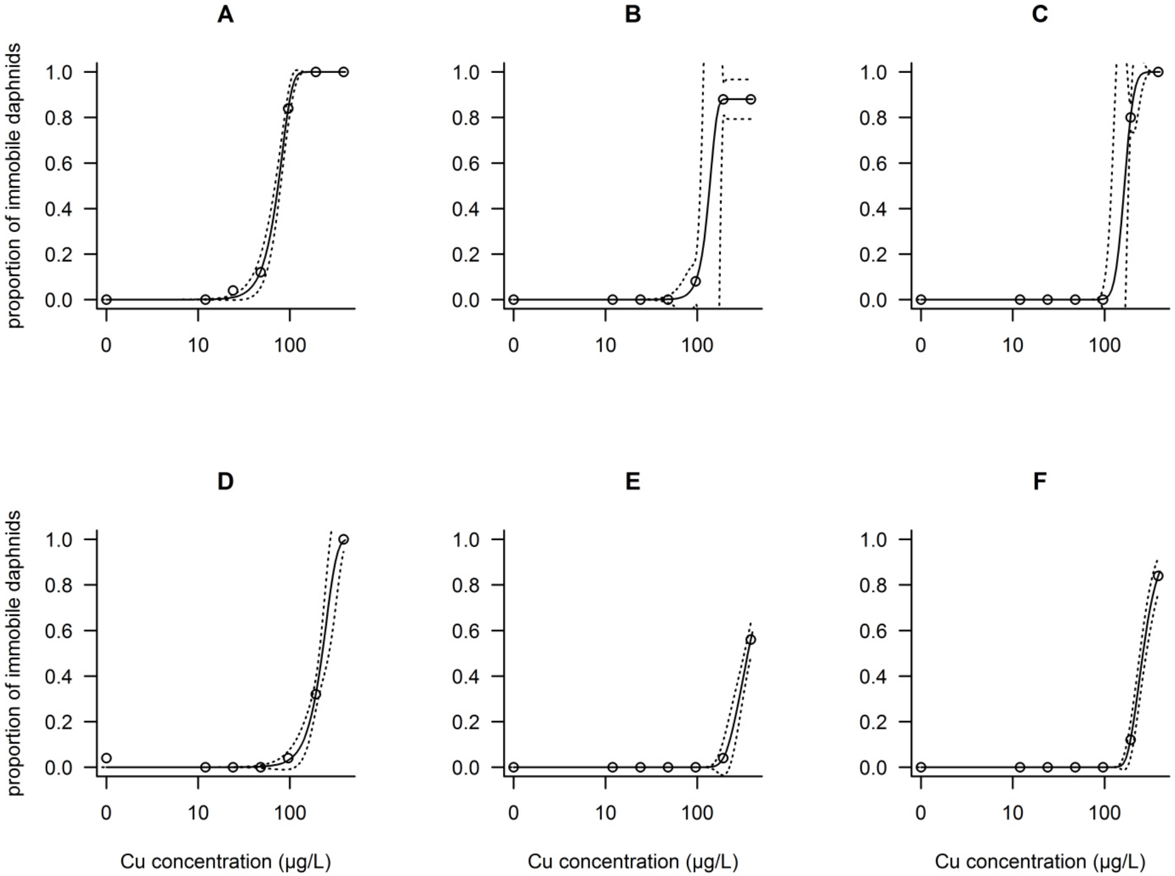

Figure S6. R050P × SR – Dose-response curves of Cu in absence (A-C) and presence (D-F) of $nTiO_2$ and the respective organic matter (OM) level (A+D: 0 mg TOC/L, B+E: 2 mg TOC/L, C+F: 8 mg/L) with 95% confidence intervals after 48 h.

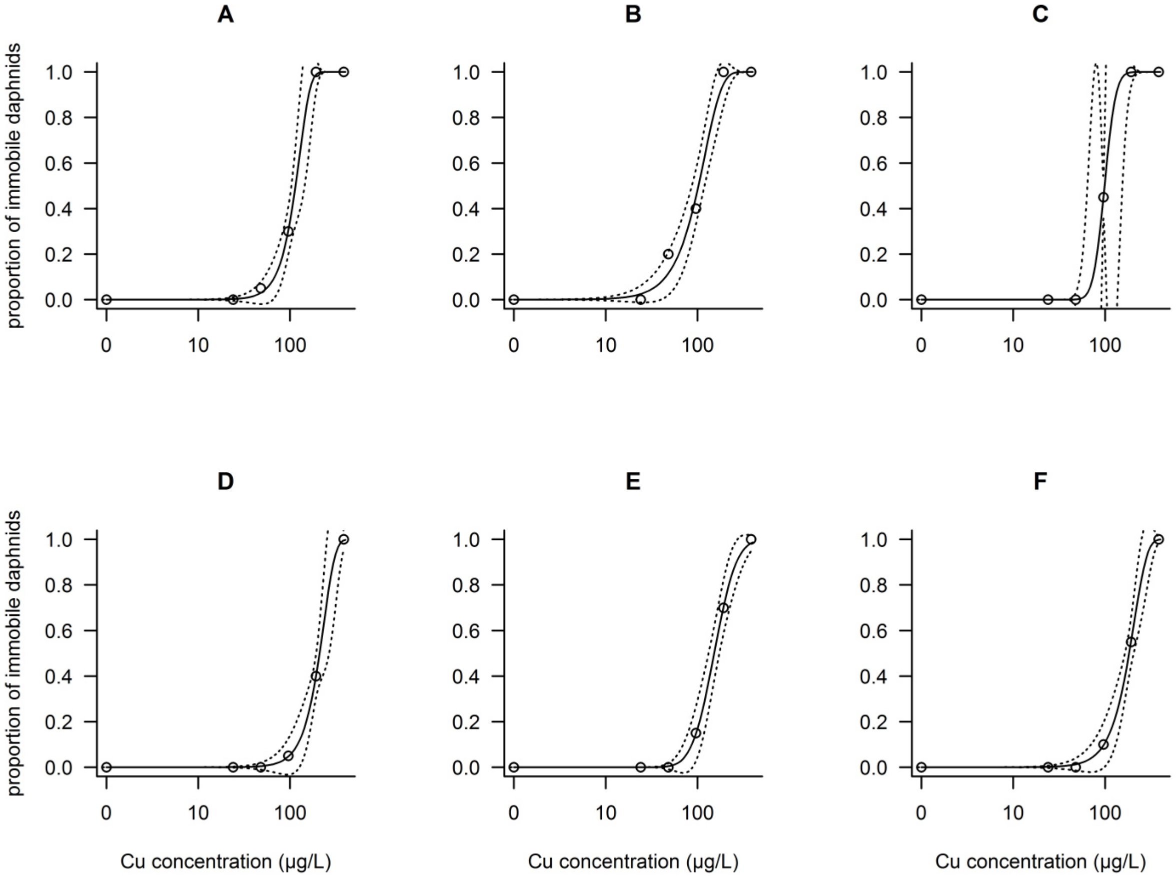

Figure S7. A-100 × SL – Dose-response curves of Cu in absence (A-C) and presence (D-F) of $nTiO_2$ and the respective organic matter (OM) level (A+D: 0 mg TOC/L, B+E: 2 mg TOC/L, C+F: 8 mg/L) with 95% confidence intervals after 48 h.

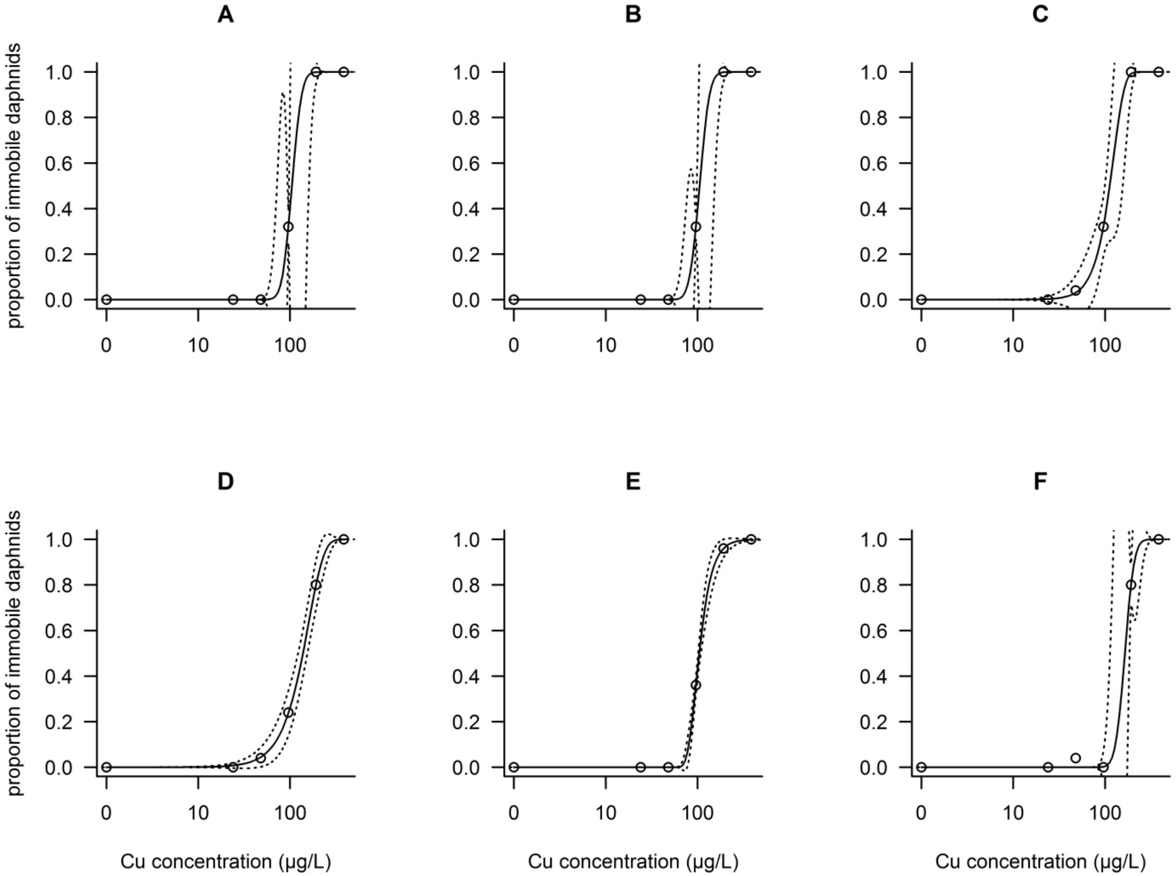

Figure S8. P25 × SL – Dose-response curves of Cu in absence (A-C) and presence (D-F) of $nTiO_2$ and the respective organic matter (OM) level (A+D: 0 mg TOC/L, B+E: 2 mg TOC/L, C+F: 8 mg/L) with 95% confidence intervals after 48 h.

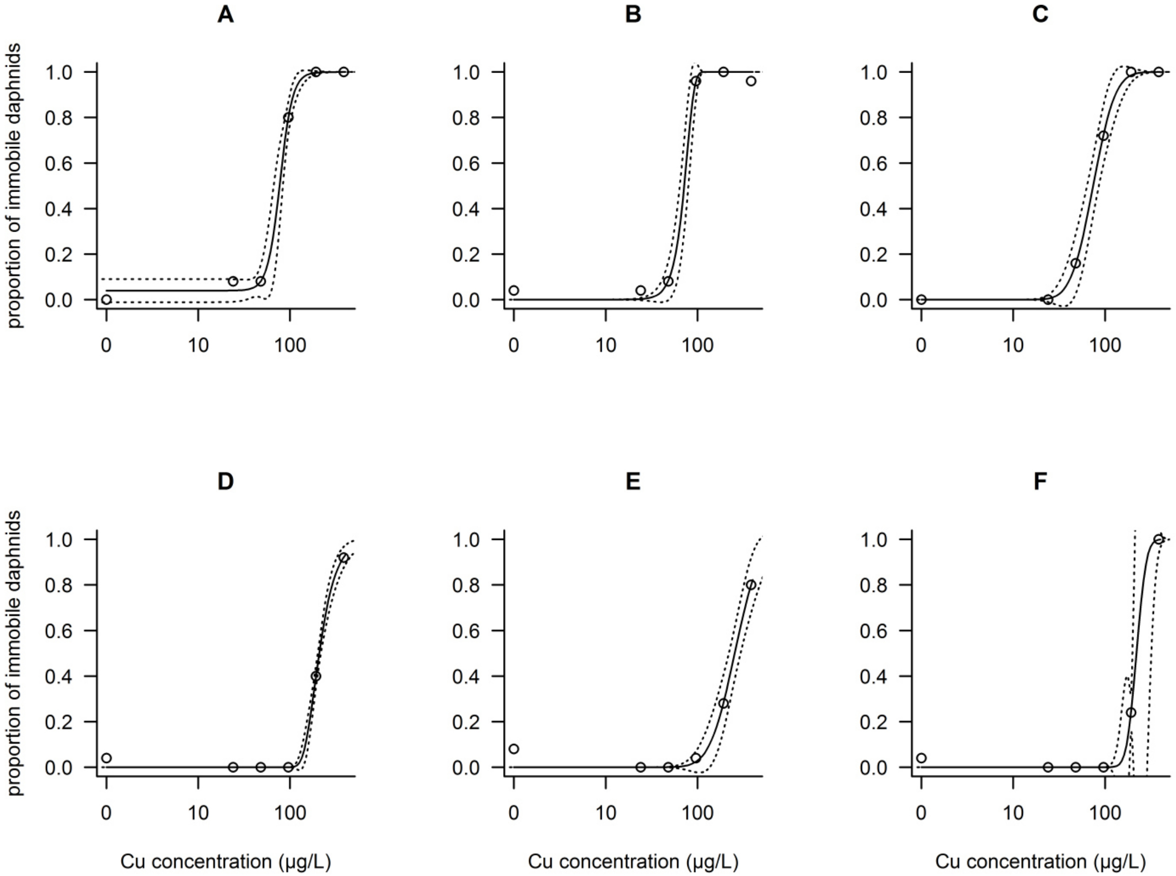

Figure S9. R050P × SL – Dose-response curves of Cu in absence (A-C) and presence (D-F) of $nTiO_2$ and the respective organic matter (OM) level (A+D: 0 mg TOC/L, B+E: 2 mg TOC/L, C+F: 8 mg/L) with 95% confidence intervals after 48 h.

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Publication II:
Nanosized titanium dioxide influences copper induced toxicity during aging as a function of environmental conditions

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Abstract

Titanium dioxide nanoparticles ($nTiO_2$) adsorb co-occurring heavy metals in surface waters modulating their toxicity for freshwater invertebrates. The processes triggering this interaction may, however, be influenced by several environmental parameters, while their relative importance remains unclear. Therefore, the present study assessed the implications of aging duration as well as ionic strength, pH and presence of different qualities of organic matter during aging on the joint acute toxicity of copper (Cu) and $nTiO_2$ for *Daphnia magna* over a study duration of up to 72 h. The results indicated that the presence of $nTiO_2$ reduced, under all of the aging scenarios assessed in the present study, the Cu-induced toxicity for daphnids – although with varying effect sizes, which was displayed by up to 3-fold higher EC_{50} values compared to the absence of $nTiO_2$. Moreover, the Cu speciation – as a result of the ionic composition and the pH as well as the presence of organic additives in the medium – strongly modulated the processes during aging with partly limited implications of the aging duration on the ecotoxicological response of *Daphnia*. Nonetheless, the present study underpins the remediation-like effect of $nTiO_2$ on the heavy metal toxicity in freshwater ecosystems under various environmental conditions, a pattern that, however, needs further verification using heavy metal ions with differing properties.

Keywords: Weathering – Adsorption – Transport processes – Factorial approach – Crustaceans

Introduction

Titanium dioxide nanoparticles ($nTiO_2$) show a broad range of beneficial properties and are therefore used in numerous consumer products including food, paints and sunscreens [1, 2]. This frequent involvement at high amounts poses the risk of their unintended release into the aquatic environment, with wastewater treatment plant effluents being their major entry-pathway [3]. Both during the wastewater treatment as well as after release into the receiving water body, $nTiO_2$ likely interact with other stressors, such as heavy metals, potentially modulating toxicity [4]. The heavy metal ions may be adsorbed onto the large surface area provided by the co-occurring $nTiO_2$ [5]. This process can reduce the ions' concentration in the water phase and thus their ecotoxicological potential [6-8], but leads at the same time to an accumulation of the element at the particles' surface, which may, if ingested, become bioavailable and thus toxic during gut passage [4]. In contrast, the settling of $nTiO_2$ agglomerates may potentially lower the risk of an ingestion by pelagic organisms [9] and hence the toxicity of the adsorbed metals, which may contribute to explain the discrepancy in published literature regarding the direction of $nTiO_2$'s effect on metal toxicity (e.g., [4, 9]).

As the time of interaction between $nTiO_2$ and metal ions prior to the exposure of the test organisms (=aging) triggers both the amount of ions adsorbed onto $nTiO_2$ and the agglomeration state of the particles themselves [8, 10], aging duration can be assumed to influence the joint toxicity of the two components. Moreover, the physicochemical parameters of the surrounding media complicate any prediction of the interaction between metal ions and $nTiO_2$. For instance, the ionic strength of the aging medium strongly influences the agglomeration processes of $nTiO_2$ [10]. Moreover, the mediums' ions potentially compete with heavy metals for adsorption to the particles' surfaces [11] and they probably form non-toxic complexes with the metals during aging [5]. Furthermore, the pH during aging determines the surface charge of $nTiO_2$ as well as the speciation of metal ions [12]. The surface charge of $nTiO_2$ in turn affects the nanoparticles' agglomeration dynamics as well as the adsorption of metal ions [13]. Moreover, organic matter adsorbed onto the nanoparticles (=coating) potentially stabilize $nTiO_2$ in the water phase [14] and alter the surface functionality of the particles as well as the adsorption efficiency [15], ultimately modulating the interaction between metal ions and $nTiO_2$. Taking these aspects together, $nTiO_2$ may adsorb higher loads of metal ions with aging duration in presence of natural organic matter. At the same time, organic matter complexes metal ions reducing their bioavailability [16].

To unravel the importance of these factors, the present study used copper (Cu) as a representative of heavy metals and the filter feeding pelagic crustacean *Daphnia magna* as a model organism. Daphnids were exposed towards mixtures of Cu and $nTiO_2$, while both stressors were assessed in a factorial test design involving seven levels of Cu and two levels of $nTiO_2$. Moreover, different aging scenarios were realized by varying the ionic strength, pH and organic matter quality and quantity of the media, while the aging duration ranged (depending on the scenario) from 0 to 168 h. By doing so it was hypothesized that (I) aging in presence of $nTiO_2$ reduces the toxicity of Cu compared to their absence, as observed for unaged combinations [9]. Since the interaction prior to the exposure decreases the amount of free Cu in a time-dependent manner [8], the expected reduction in toxicity should increase with increasing aging duration. It was also hypothesized that (II) an aging medium with a higher ionic strength lowers Cu toxicity in the presence of $nTiO_2$ more than medium with low ionic strength, which is driven by agglomeration and sedimentation. (III) Since H^+ competes for binding sites on $nTiO_2$ surfaces and these particles are more positively charged [17], less Cu can be expected to be adsorbed by $nTiO_2$ at a low pH suppressing the nanoparticles' remediation-like effects on Cu toxicity. It was finally assumed that (IV) the presence of organic matter stabilizes $nTiO_2$ [14] with adsorbed Cu in the water column, which increases Cu uptake and toxicity, while this process might vary with organic matter quality.

Material and methods

Test substances

The Cu solution was prepared prior to each application to the test medium by mixing the salt $Cu(NO_3)_2x3H_2O$ (Carl Roth GmbH + Co. KG, Germany) with deionized water in a volumetric flask (polypropylene) followed by a serial dilution to the nominal test concentrations. The Cu species occurring after dilution (see Table A.1) are henceforth subsumed as Cu.

The $nTiO_2$ suspension was prepared by stirred media milling (PML 2, Bühler AG, Switzerland) of the $nTiO_2$ powder P25 (AEROXIDE® TiO_2 P25; primary particle size = 21 nm; surface area = 50±15 m²/g; Evonik, Germany) in deionized water at the Institute for Particle Technology (TU Braunschweig, Germany) and was stabilized at a low pH displaying a monodisperse size distribution and an intensity weighted average particle diameter <100 nm (Table A.2; Figure A.1). The nanoparticles consisted of the crystalline phases anatase, rutile and amorphous in the ratio 78:14:8 [18]. Prior to each application for the experiments, the $nTiO_2$ stock suspension (2 g/L) was ultrasonicated for ten minutes to ensure a spatially uniform particle distribution.

Test organism

The test organism *D. magna* (clone V, Eurofins-GAB laboratories, Germany) was cultured in ASTM reconstituted hard water [19, 20] amended with selenium and vitamins (biotin, thiamine, cyanocobalamine, according to the OECD guideline 202 [21]) as well as 20 µL/L seaweed extract (Marinure, Glenside, Scotland). The medium was changed three times a week and the organisms were fed with the green algae *Desmodesmus* sp. equivalent to 200 µg carbon per organism on a daily basis. The culture was kept at 20±1°C and a light:dark rhythm of 16:8 h (800-1000 lux; OSRAM L 58W/21-840 ECO, Germany).

General test design

The influence of different factors during aging on the combined toxicity of Cu and $nTiO_2$ was assessed taking advantage of a 7×2 factorial test design. Therefore, the Cu concentrations of 0 (control), 1200, 2400, 4800, 9600, 19200 and 38400 µg/L were aged in 50-mL centrifuge tubes (polypropylene) in presence and absence of $nTiO_2$ (200 mg P25/L) under different conditions (see below) utilizing an overhead shaker. The concentrations during the aging corresponded to 100 times the concentration applied during the toxicity tests. The Cu concentrations were chosen to allow for a reliable determination of 48 and 72-h EC_{50} values (=median effective concentrations), while the $nTiO_2$ concentration equaled the 96-h EC_{50} for P25 [22] and thus is expected to affect the test species only marginally after 48 or 72 h [23].

After aging, each centrifuge tube was thoroughly vortexed for several seconds to resuspend potential agglomerates ensuring the transfer of the nominal $nTiO_2$ concentration (see Table 1). Afterwards 3 mL of the mixture were directly diluted in 300 mL of the test medium (reconstituted hard water; see section *Test organism*), which was shortly stirred and then distributed to the test beakers (polystyrene; n=5). Five juvenile daphnids (age <24 h) were placed in each beaker, initiating the respective acute toxicity test (according to the OECD guideline [21]; test duration: 72 h). The immobility of the test organisms was assessed after 48 and 72 h.

Factors: ionic strength and aging duration

Reconstituted hard water (see section *Test organism*; ionic strength: 9.25 mmol/L) without the addition of seaweed extract (=test medium) as well as deionized water was used as medium during the aging process. The aging duration was 0 (=approximately 5 min), 24, 72 and 168 h prior the initiation of the respective ecotoxicological assessment. In parallel to these treatments, unaged mixtures of Cu and $nTiO_2$ were assessed. With this test design it was feasible to characterize the potential effects of aging duration as well as ionic strength of the medium, in which the aging took place, on their combined toxicity. The ecotoxicological testing was performed as detailed above.

Factor: pH

To quantify the influence of the pH during the aging of Cu and $nTiO_2$ mixtures, the aging medium (here test medium) buffered with 1.046 g MOPS/L (3-(N-morpholino)propansulfonic acid; Carl Roth GmbH + Co. KG, Germany) – a non-toxic substance [24] – was adjusted to environmentally relevant pH values, i.e. 6.5 and 8.0 [25] using HCl or NaOH (2 N standard solution; Carl Roth GmbH + Co. KG, Germany). The aging was performed for 0, 24 and 72 hours, while the final ecotoxicological testing differed slightly from the procedure detailed above. Briefly, the aged mixtures were diluted and tested in (I) unbuffered test medium, (II) test medium buffered to pH 6.5 and (III) test medium buffered to pH 8.0. These experimental adjustments were realized to consider the implications of a pH shift on the desorption of Cu from the nanoparticles' surfaces after the transfer to the test beakers [26], which was assumed to be substantially higher at a pH of 6.5.

Factor: Organic matter

To characterize the influence of natural organic matter on the combined toxicity of $nTiO_2$ and Cu three different qualities of organic matter were assessed at 8 mg total organic carbon/L, namely sucralose (1,6-Dichloro-1,6-dideoxy-β-D-fructofuranosyl-4-chloro-4-deoxy-α-D-galactopyranoside, purity >98%; Sigma-Aldrich, USA), seaweed extract (*cp.* section *Test organism*; Marinure, Glenside, Scotland) and Suwannee River natural organic matter (International Humic Substances Society, USA). The aging was performed in deionized water amended with the organic matter for 72 h, while a supplemental experiment assessed the implication of the aging of one of the organic matter qualities (i.e., seaweed extract) in test medium to determine the transferability of the results to media with a higher ionic strength (see Appendix). Sucralose was applied during the present study as an artificial organic material, whose molecules likely coat $nTiO_2$ [27], but should, given their neutral charge, not complex Cu ions [5]. In contrast, seaweed extract is recommended for reproduction tests and continuous *D. magna* cultures by the

OECD guideline 211 [28], while Suwannee River natural organic matter was due to its detailed characterization used as a reference substance. This selection of organic materials hence represents a range of properties (e.g., regarding the quantity and quality of humic substances) reflecting their diversity in natural and anthropogenically influenced waters [29, 30]. Moreover, these materials show different capabilities of nanoparticle stabilization and impact on the interaction of $nTiO_2$ and Cu [30]. Again, the ecotoxicological testing was performed as detailed above.

Analytics

Water phase samples for the determination of total Cu and TiO_2 concentrations were taken exemplarily at the initiation of the acute toxicity tests assessing aging duration and ionic strength followed by an acidification with HNO_3. These samples were directly measured (mass: ^{65}Cu and ^{49}Ti) utilizing an inductively coupled plasma with quadrupole mass spectrometer (ICP-MS; XSeries 2, Thermo Fisher Scientific, Germany) as described elsewhere [9]. The concentrations of TiO_2 were calculated based on the measured Ti assuming the absence of other Ti-containing compounds.

Statistics

The statistical analyses were accomplished using the statistics program R version 3.0.1 [31] for Windows and several extension packages. The EC_{50} values (based on nominal concentrations) were determined by fitting adequate dose-response models, which were selected based on Akaike's information criterion and visual assessment. In the following only 48-h EC_{50} values are reported, whereas the EC_{50} values obtained after 72 h of exposure delivered a similar pattern, while EC_{50} values following 24 h of exposure were not determined as toxicity caused by Cu was too low. To evaluate the implication of $nTiO_2$ and the environmental parameters (e.g., ionic strength, pH, organic matter), as well as their combinations on Cu-induced toxicity, confidence interval (CI) testing [32] was applied to statistically compare the respective

EC_{50} values. If the 95% CIs of the difference between two EC_{50} values did not include zero, the difference was judged statistically significant.

To evaluate the contribution of the factors considered in the present study (i.e., $nTiO_2$ concentration, aging duration, ionic strength, pH during aging, exposure duration (including 48 and 72 h) and pH during the ecotoxicological tests), a multiple linear regression of box-cox transformed EC_{50} values [33] (n=197), weighted by the inverted relative standard error of the EC_{50} value, was performed (comparable methods are commonly used in quantitative structure-activity relationship analyses; cf. [34]). Please note that the presence of organic matter was not considered here, since this factor was not assessed in combination with enough other factors and their levels.

Results

Effect of ionic strength and aging duration

During aging in deionized water, $nTiO_2$ had no effect on the Cu concentration (irrespective of aging duration and the presence of $nTiO_2$. The Cu-level in the ecotoxicological test was always >89% of the nominal concentration), but after the aging in test medium the Cu concentration was higher in presence of $nTiO_2$ compared to their absence (Table 1): Following the aging in test medium, in presence and absence of $nTiO_2$, on average 87±4% and 44±29% of the nominal Cu concentration were detected, respectively (Table 1).

Table 1: The absolute (µg/L; mean±SD) and relative (% of nominal; mean±SD) concentration of Cu and TiO_2 measured in the water column after aging and subsequent dilution in test medium, but prior insertion of the test organism. The nominal concentrations were 48 µg Cu/L and 2000 µg TiO_2/L.

aging medium	aging duration (h)		Cu (µg/L)	Cu (%)	TiO_2 (µg/L)	TiO_2 (%)
deionized water	0	-nTiO_2	45.2±1.0	94.2±2.0	3.2±0.5	NA
		+nTiO_2	46.4±0.3	96.8±0.6	2155.2±32.6	107.8±1.6
	24	-nTiO_2	44.5±0.6	92.8±1.2	3.3±0.3	NA
		+nTiO_2	45.6±0.3	92.8±1.2	1862.4±33.8	93.1±1.7
	72	-nTiO_2	42.8±0.8	89.2±1.6	2.6±0.2	NA
		+nTiO_2	45.2±0.3	94.2±0.7	1630.2±40.5	81.5±2.0
	168	-nTiO_2	44.9±0.4	93.5±0.9	2.8±0.3	NA
		+nTiO_2	44.6±0.4	93.0±0.9	1504.0±50.1	75.2±2.5
test medium	0	-nTiO_2	44.8±0.2	93.3±0.5	3.2±0.2	NA
		+nTiO_2	44.8±0.2	93.3±0.3	2066.5±15.8	103.3±0.8
	24	-nTiO_2	15.5±0.2	32.3±0.4	3.1±0.1	NA
		+nTiO_2	40.1±0.5	83.5±1.1	1737.0±32.8	86.9±1.6
	72	-nTiO_2	12.1±0.4	25.2±0.9	3.5±1.4	NA
		+nTiO_2	42.4±0.6	88.3±1.4	1622.6±38.8	81.1±2.4
	168	-nTiO_2	12.0±0.1	24.9±0.3	3.0±0.2	NA
		+nTiO_2	39.8±0.6	82.9±1.3	1727.3±6.8	86.4±0.3

NA, not assessed

Without aging, the presence of nTiO_2 in the test medium reduced the toxicity of Cu, indicated by up to 60% higher 48-h EC_{50} values, compared to the absence of nTiO_2 (Figure 1). The same reduction of Cu toxicity in presence of nTiO_2 was observed after an aging for 72 h in deionized water (Figure 1 and A.2).

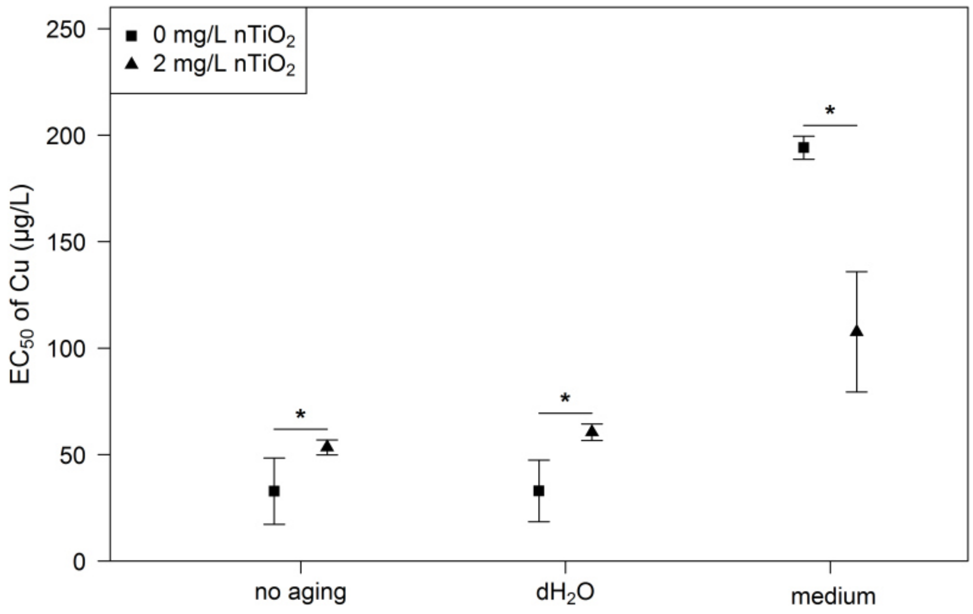

Figure 1: The effect of ionic strength. The 48-h EC_{50} values (±95% CIs) of Cu in absence (■) and presence (▲) of titanium dioxide nanoparticles ($nTiO_2$) without aging (=no aging) and after an aging for 72 h in deionized water (dH_2O) and test medium. Asterisks denote statistically significant differences between the absence and presence of $nTiO_2$.

In contrast, the aging of Cu and $nTiO_2$ in test medium for the same duration led to an increased toxicity of Cu, in comparison to the absence of $nTiO_2$, which is indicated by a 45% lower 48-h EC_{50} (Figure 1). This difference was only statistically significant after an aging duration of 72 h and could – as a result of the not computable EC_{50} in absence of $nTiO_2$, since the toxicity was to low – not be formally tested after 168 h of aging (Figure 2). Furthermore, the Cu toxicity dropped in absence as well as in presence of $nTiO_2$ with increasing aging duration in the test medium, but in presence of $nTiO_2$ the effect sizes were lower relative to their absence (Figure 2). Additionally, the difference between the effect sizes was statistically significantly increased after an aging duration of 72 h compared to 0 h (Figure 2). These phenomena were not observable for the aging of Cu and $nTiO_2$ in deionized water (Figure A.2).

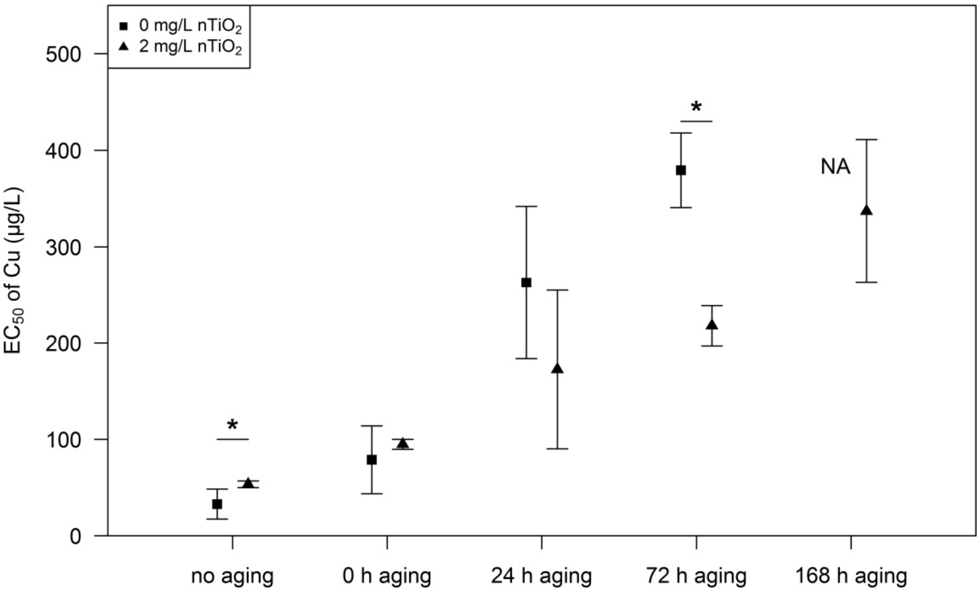

Figure 2: The effect of aging duration. The 48-h EC_{50} values (±95% CIs) of Cu in absence (■) and presence (▲) of titanium dioxide nanoparticles ($nTiO_2$) without aging (=no aging) and after an aging for 0 (≈ 5 min), 24, 72 and 168 h in test medium, respectively. Asterisks denote statistically significant differences between the absence and presence of $nTiO_2$. NA=The EC_{50} was above the chosen Cu concentration range.

Effect of pH

Irrespective of the pH of the final test medium, the toxicity of Cu was significantly reduced after 72 h of aging at a pH of 6.5 in presence of $nTiO_2$ compared to their absence (Figure 3A). This pattern was similar after the same aging duration at pH 8.0 (Figure 3B). Moreover, the toxicity of the aged Cu solution was – irrespective whether tested in absence or presence of $nTiO_2$ – higher if ecotoxicologically assessed in test medium with a pH of 6.5 compared to unbuffered medium and medium buffered to pH 8. In addition, the treatments aged in presence of $nTiO_2$ at pH 6.5 displayed an in average 39% (range: 10-65%) higher EC_{50} compared to their counterparts aged at pH 8.0 (Figures 3 and 4). However, for the treatments aged in absence of $nTiO_2$ these differences were on average only 8%.

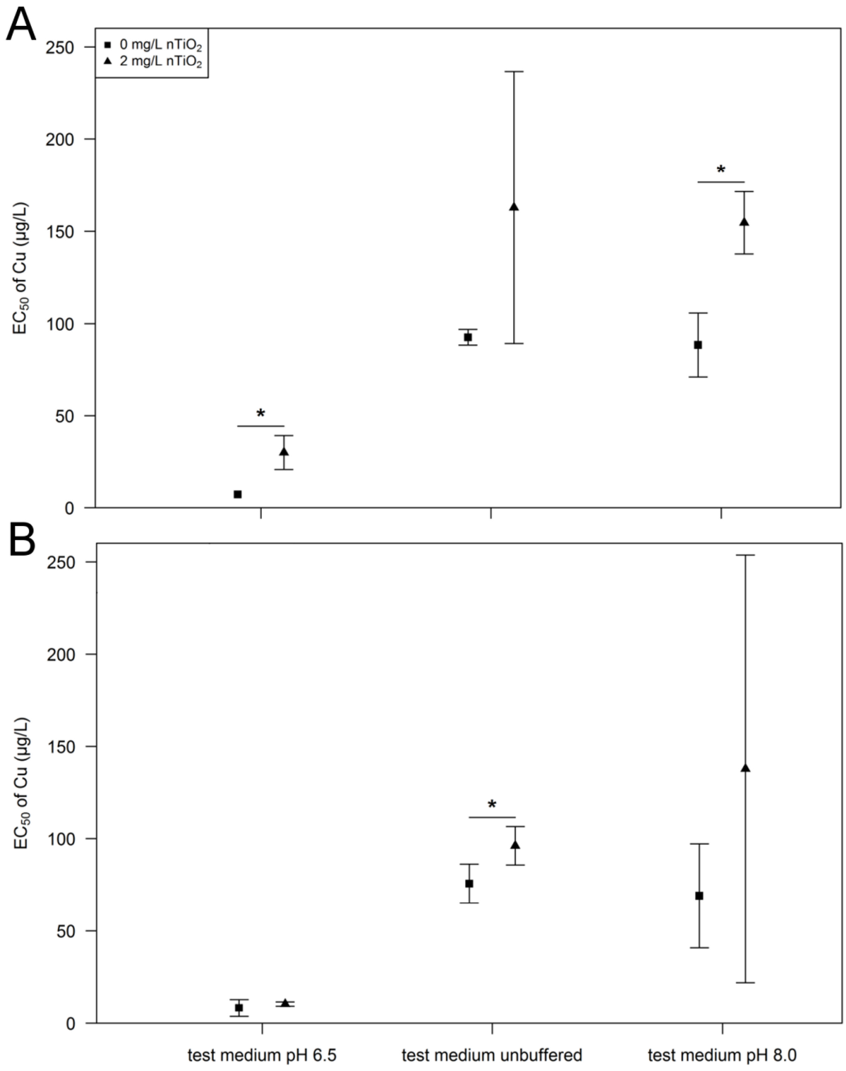

Figure 3: The effect of pH. The 48-h EC_{50} values (±95% CIs) of Cu in absence (■) and presence (▲) of titanium dioxide nanoparticles ($nTiO_2$) after an aging for 72 h in test medium buffered to either pH (A) 6.5 or (B) 8.0, assessed in test medium buffered to pH 6.5, unbuffered test medium and test medium buffered to pH 8.0, respectively. Asterisks denote statistically significant differences between the absence and presence of $nTiO_2$.

Effect of organic matter

Independent of the organic matter quality, Cu toxicity was significantly lower after 72 h of aging in presence of $nTiO_2$ in deionized water compared to their absence. This pattern was consistently observed with approximately 20% increased 48-h EC_{50} values (Figure 4).

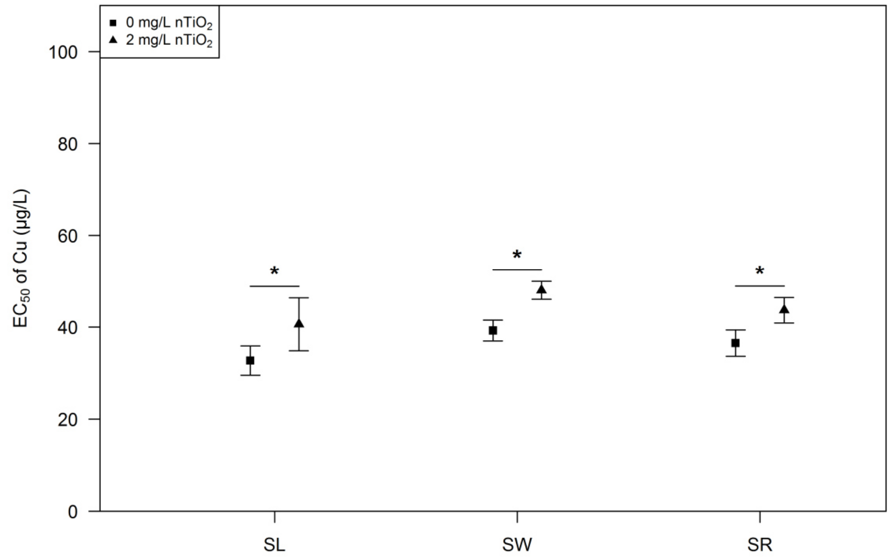

Figure 4: The effect of organic matter. The 48-h EC_{50} values (±95% CIs) of Cu in absence (■) and presence (▲) of $nTiO_2$ after an aging for 72 h in deionized water amended with organic matter (i.e., sucralose (SL), seaweed extract (SW), Suwannee River natural organic matter (SR); at 8 mg total organic carbon/L)), assessed in test medium. Asterisks denote statistically significant differences between the absence and presence of $nTiO_2$.

The contribution of each factor

The final model fit (multiple linear regression) of the box-cox transformed EC_{50} values had an adjusted R-squared of 0.8629, involving 197 EC_{50} values. The concentration of $nTiO_2$ was the only conditional factor for the increase of the Cu-based EC_{50} values (Table 2). Moreover, the significant interaction of $nTiO_2$ with pH during aging confirms the observed decrease of the Cu-based EC_{50} values with increasing pH and $nTiO_2$ concentration (Figure 3A and B). Similarly, the significant interaction of the pH and the ionic strength during aging showed a decreased EC_{50} value with the increase of both factors (Table 2). Finally, the most powerful increase in EC_{50} values could be attributed to the interaction of the pH during aging and the pH during the actual ecotoxicological testing.

Table 2: Model fitting results for the box-cox transformed nominal EC_{50} values. The multiple linear regression of the EC_{50} values of Cu against $nTiO_2$ concentration, ionic strength, pH during aging and during exposure as well as exposure duration after an aging.

Factors	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	0.7299	0.0370	19.7550	<0.0001
$nTiO_2$ concentration	0.1827	0.0483	3.7830	0.0002
$nTiO_2$ concentration : pH during aging	-0.0234	0.0066	-3.5700	0.0005
pH during aging : ionic strength	-0.0106	0.0006	-17.384	<0.0001
exposure duration : pH during exposure	-0.0002	0.0001	-5.0640	<0.0001
pH during aging : pH during exposure	0.0146	0.0007	21.3300	<0.0001
ionic strength : pH during exposure	0.0094	0.0006	15.2520	<0.0001

Estimate = strength of effect on EC_{50}-values

Discussion

Presence of $nTiO_2$ may increase Cu-toxicity with aging duration

In line with former studies [9, 30, 35], Cu-induced toxicity was reduced in the presence of $nTiO_2$ without aging (Figure 1). When, however, aged in medium with an ionic strength of 9.25 mmol/L (=test medium) prior to the ecotoxicological testing, Cu toxicity was increased by the presence, relative to the absence, of $nTiO_2$ (Figure 2). Moreover, the differences in toxicity (effect sizes) between these two aging scenarios increased with increasing aging duration (Figure 2) suggesting that the Cu toxicity was considerably less reduced in presence of $nTiO_2$ than in its absence as a function of aging duration.

This relative increase in Cu toxicity in presence of $nTiO_2$ during aging in test medium compared to their absence, which is in contrast to hypothesis I, may be explained by an enhanced transfer of Cu from the aging vessels to the ecotoxicological test system in presence relative to the absence of the nanoparticles (Table 1). This is an assumption, which is strongly supported when calculating the EC_{50} values based on the amount of Cu actually transferred into the ecotoxicological test systems (Table 1) and hence on the measured Cu concentrations (Table A.3). Thereby, a comparable reduction in Cu-induced toxicity (20-

100% higher EC_{50} values; Table A.3) was uncovered as, for instance, observed after aging in deionized water. This process could be related to the predominant adsorption of Cu onto the aging vessels in absence of $nTiO_2$, while in their presence Cu was primarily bound onto $nTiO_2$'s surfaces ensuring that both Cu and $nTiO_2$ were ultimately transferred to the ecotoxicological test system at levels very close to the nominal concentrations (Table 1) – despite the fact that $nTiO_2$ agglomerated (Table 3) and likely sedimented during the aging process. In accordance with these interpretations, supplemental experiments underpinned the relevance of the adsorption of Cu to the aging vessel for the results of the present study by showing that following the aging of Cu and $nTiO_2$ directly in the test beakers (see Appendix for details), Cu-induced toxicity was reduced in the presence of $nTiO_2$ relative to their absence (Figure A.3). Although large amounts of Cu were likely transferred to the ecotoxicological testing systems associated with $nTiO_2$ agglomerates that sedimented during the toxicity testing [23, 36], Cu still induced acutely toxic effects. This observation may be explained by the potential ingestion of the nanoparticles together with Cu by daphnids grazing at the bottom of the test beaker [37] or the Cu might have been desorbed from $nTiO_2$, when introduced into the ecotoxicological test [38].

Table 3: The particle size distribution, i.e. average diameter (nm, mean±SD), polydispersity index (PI), 10th 50th and 90th percentile, of $nTiO_2$ measured ($n \geq 3$) in the aging medium (i.e., test medium and deionized water) after 0, 24, 72 and 168 h.

aging medium	aging duration (h)	average diameter (nm)	PI (min)	PI (max)	10th percentile	50th percentile	90th percentile
deionized water	0	86.6±2.9	0.173	0.227	51.7	90.8	166.1
	24	695.3±28.3	0.314	0.329	160.5	600.4	2604.6
	72	1173.4±7.9	0.386	0.388	388.1	1417.0	4867.3
	168	1631.5±2.4	0.407	0.441	833.7	1555.0	10166.5
test medium	0	4240.9±278.3	0.591	0.657	1682.0	5278.8	18569.1
	24	4951.1±345.4	0.295	0.462	2558.3	4439.5	18664.2
	72	3192.9±111.1	0.579	0.761	1176.7	2678.6	42353.9
	168	4381.7±180.0	0.386	0.478	2296.8	3398.5	63921.1

The impact of environmental parameters during aging on Cu toxicity

Factor: Ionic strength

Partly in contrast to the results for aging in test medium and hypothesis II, the aging of Cu and $nTiO_2$ in deionized water (ionic strength: ≈0.0 mmol/L) resulted in a reduced Cu toxicity in presence of $nTiO_2$ compared to their absence – similar to the experiments performed without aging (Figures 1 and 2, *sensu* Seitz et al. [39]). The compliance of the experiments performed without aging and those with aging in deionized water indicated that no or only limited interactions between Cu and $nTiO_2$ as well as the aging vessel occurred over the aging duration of up to 168 h (Figure A.2). This pattern may be explained by a different composition of the Cu species in deionized water relative to the test medium used during aging (Table A.1) as a function of the ionic composition and the pH. The lower pH of deionized water (7.0±0.1) compared to test medium (8.2±0.1) has – due to a competition with H^+ for binding sites [26] – potentially reduced the adsorption of Cu to the aging vessels, but also to $nTiO_2$ (*cf.* [38, 40]). Similarly, at low ionic strength the formation of insoluble Cu complexes is reduced [5] likely stabilizing the amount of Cu in the water column and hence delaying the start of the interaction between Cu and $nTiO_2$ in deionized water until the initiation of the ecotoxicological test (i.e., the presence of anions and cations).

Factor: pH

To disentangle the role of pH during aging, further experiments assessed the combined toxicity of Cu and $nTiO_2$ after aging in test medium buffered to either pH 6.5 or pH 8.0 and hence avoiding any confounding effect of the ionic composition of the medium. In general, the aging at pH 8.0 and in presence of $nTiO_2$ led to a higher toxicity compared to aging at a pH of 6.5, while in absence of $nTiO_2$ this difference was negligible (<10%). This is in line with hypothesis III (less Cu can be expected to be adsorbed by $nTiO_2$ at a low pH suppressing the nanoparticles' remediation-like effects on Cu toxicity), and might be explained by a higher adsorption of copper under basic conditions, compared to acidic [6, 11].

Moreover, the toxicity of Cu was – irrespective of the pH value during aging and the actual toxicity test – in presence of $nTiO_2$ reduced compared to the absence of $nTiO_2$ (Figure 3A and B). These observations suggest that the difference in pH between deionized water and test medium may not explain the differences regarding the direction of $nTiO_2$-induced effect on the Cu toxicity. However, the involvement of a buffer (here MOPS) may have confounded the interaction of Cu and $nTiO_2$. This could be assumed since MOPS can coat $nTiO_2$ as well as the aging vessel and thereby alter their surface properties (e.g., via changes in surface charge or mechanical blocking) ultimately inhibiting the adsorption of Cu [27, 30], while similar effects have been observed for sucralose molecules [30]. These processes may have masked most of the real pH effects and query the role of the pH as a driving force during these experiments. Although, this phenomenon hindered an adequate assessment of pH's influence on the interaction between metal ions and $nTiO_2$, it may be concluded that the pH plays only a minor role in presence of organic molecules (i.e., MOPS).

Factor: Organic matter content

Besides MOPS, other organic matters can coat the surface of nanoparticles [41] and stabilize these particles in the medium [14], ultimately modulating the combined effect of Cu and $nTiO_2$. In this context, it was hypothesized that a coating of $nTiO_2$ by organic matter increases the transfer of Cu (from the aging vessels to the ecotoxicological test) and its effects on daphnids [39] (hypothesis IV). In contrast to this hypothesis, the presence of any of the qualities of organic matter (i.e., sucralose, seaweed extract and Suwannee River natural organic matter) during the 72-h aging reduced the Cu-induced toxicity to a similar extent as observed for unaged $nTiO_2$ and Cu mixtures (Figures 1 and 4) suggesting a limited impact of organic matter quality. This observation is in line with Seitz et al. [39] showing that $nTiO_2$ aged in deionized water in presence of organic matter causes the same effects as unaged $nTiO_2$. Similarly, if the aging in presence of organic matter (i.e., seaweed extract) was conducted by using test medium, the same pattern, although at higher EC_{50} values, was observed (Figure A.4). This pattern can most likely be explained by the adsorption and complexation processes discussed above. Furthermore, the adsorption of organic matter changes the surface properties of metal oxides such as TiO_2 [15] as well as the aging vessel potentially decreasing the adsorption of Cu and thus the loss of Cu prior to the initiation of the ecotoxicological testing.

Conclusion

The present study revealed, in line with the multiple linear regression on 197 EC_{50} values, that the presence of $nTiO_2$ had the strongest impact on the Cu-induced toxicity, while $nTiO_2$ decreased Cu toxicity in all of the environmental conditions assessed. This suggests that these nanoparticles may indeed be a suitable remediation tool for heavy metal contamination in freshwater ecosystems, which, however, needs further verification using heavy metal ions with differing properties. It remains, however, an open question whether similar patterns can be observed at lower, more field relevant $nTiO_2$ concentrations. Furthermore, it is obvious from the present study that many factors need to be considered when assessing the combined toxicity of stressors and nanoparticles. These factors include besides the loss of the chemical stressors' availability during aging (e.g., adsorption to aging vessels) also the implications of chemical buffers or, more general, medium additives (here MOPS) on the interactions between stressor(s) and nanoparticles.

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Supplementary information

Table A.1: Abundance of copper species (%) during aging in test medium and deionized water for the highest and lowest concentration (determined via visual MINTEQ 3.0 (Gustafsson, 2011)).

medium	Cu concentration (µg/L)	Cu^{2+}	$CuOH^+$	$Cu_2(OH)_2^{2+}$	$Cu_3(OH)_4^{2+}$	$CuCO_3$(aq)	$Cu(CO_3)_2^{2-}$	sum
deionized	1200	79.0	17.1	3.5	<1.0	<1.0	<1.0	99.6
water	38400	40.4	7.9	25.7	25.7	<1.0	<1.0	99.7
test	1200	1.6	4.9	<1.0	<1.0	87.2	4.5	98.2
medium	38400	1.1	3.3	5.1	36.2	51.1	2.3	99.1

Nanoparticle characterization in the stock suspension

The transmission electron microscope images (200 keV Zeiss 922 Omega) displayed a primary particle size corresponding to the suppliers' information (Figure A.1). Moreover, the sizes of the agglomerates are in the range of the measured particle size distribution (Table A.1). Moreover, the particle size distribution of the stock suspension displayed a polydispersity index <0.2, indicating its monodisperse particle distribution (Table A.1.).

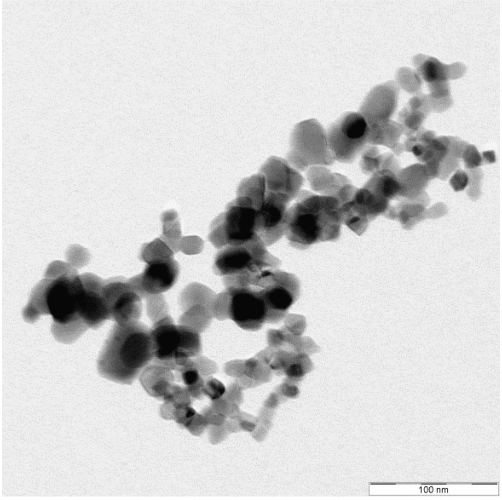

Figure A.1: Transmission electron microscopy image of the applied P25 titanium dioxide nanoparticle stock suspension (200 keV Zeiss 922 Omega; scale bar=100 nm).

Table A.2: The particle size distribution, i.e. average diameter (nm, mean±SD), polydispersity index (PI), 10th, 50th and 90th percentile (D10%, D50%, D90%) of P25 titanium dioxide nanoparticles measured ($n \geq 3$) in the stock suspension.

average diameter (nm)	PI (min)	PI (max)	10th percentile	50th percentile	90th percentile
93.9±1.2	0.185	0.193	53.1	98.5	188.1

Table A.3: The 48-h EC_{50} values (µg/L), based on the nominal and the measured Cu concentrations in absence and presence of titanium dioxide nanoparticles ($nTiO_2$) after an aging for 0 (≈ 5 min), 24, 72 and 168 h in test medium, respectively. NA=The EC_{50} was above the highest Cu concentration tested.

aging duration		EC_{50} (nominal)	EC_{50} (measured)
0h	$-nTiO_2$	78.8	73.6
	$+nTiO_2$	94.9	88.6
24h	$-nTiO_2$	262.9	84.9
	$+nTiO_2$	172.6	144.1
72h	$-nTiO_2$	379.3	95.6
	$+nTiO_2$	217.9	192.6
168h	$-nTiO_2$	NA	NA
	$+nTiO_2$	337.3	279.6

Aging in deionized water

In parallel to the aging in test medium, Cu and $nTiO_2$ were jointly aged in deionized water for 0, 24, 72 and 168 h. This process only marginally influenced the toxicity compared to the unaged treatment (Figure A.2).

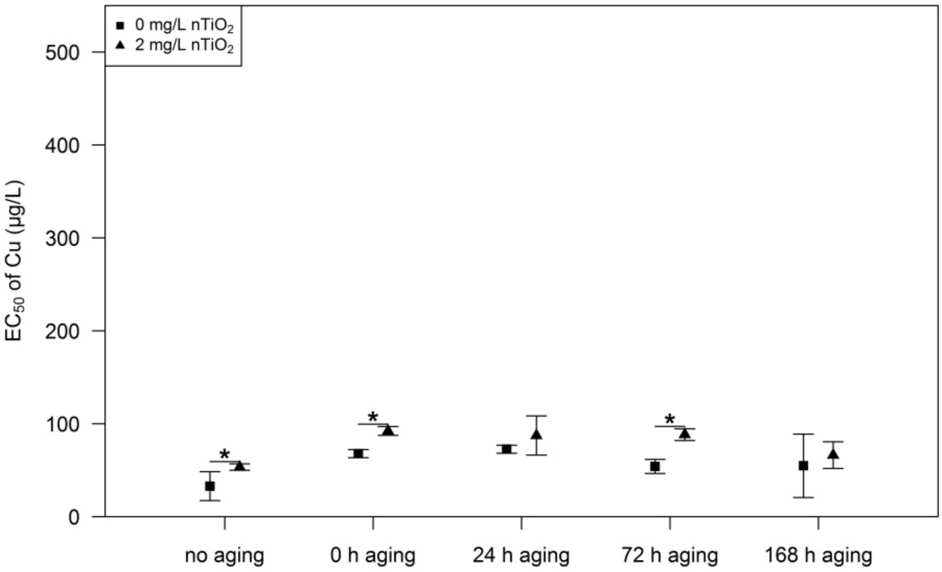

Figure A.2: The effect of aging duration. The 48-h EC_{50} values (±95% CIs) of Cu in absence (■) and presence (▲) of $nTiO_2$ without previous aging and after an aging for 0, 24, 72 and 168 h in dionized water, respectively. Asterisks denote statistically significant differences between the absence and presence of $nTiO_2$.

Enhanced transport of Cu as main effect

An experiment was conducted during which the mixtures of Cu and $nTiO_2$ were directly aged in the test beakers for 72 hours prior to the test initiation with the daphnids, which excluded the use of additional aging vessels and hence the loss of Cu during aging. The results displayed a reduced Cu toxicity after an aging in presence of $nTiO_2$ compared to their absence.

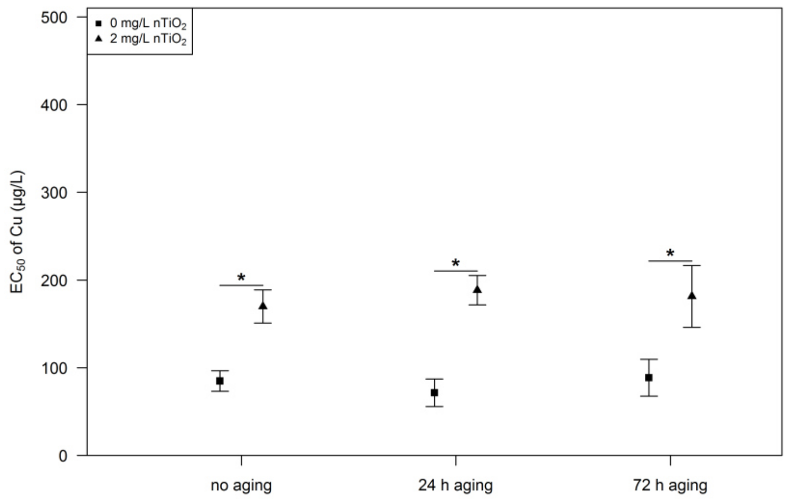

Figure A.3: The effect of the transfer. The 48-h EC_{50} values (±95% CIs) of Cu in absence (■) and presence (▲) of titanium dioxide nanoparticles ($nTiO_2$) without aging and after an aging for 24 and 72 h in test medium directly in the test beakers without subsequent dilution. Asterisks denote statistically significant differences between the absence and presence of $nTiO_2$.

Aging in test medium with different levels of seaweed extract

To complement the tests aiming to characterize the influence of natural organic matter on the joint toxicity of $nTiO_2$ and Cu an aging with 0, 2 and 8 mg total organic carbon/L of seaweed extract (*cp.* section *Test organism*; Marinure, Glenside, Scotland) in test medium was performed. The results uncovered a comparable pattern as observed following the aging of $nTiO_2$ and Cu in deionized water (compare Figure A.4 with Figures 1 and 2). Furthermore, the quantity of organic matter seemed to play a minor role.

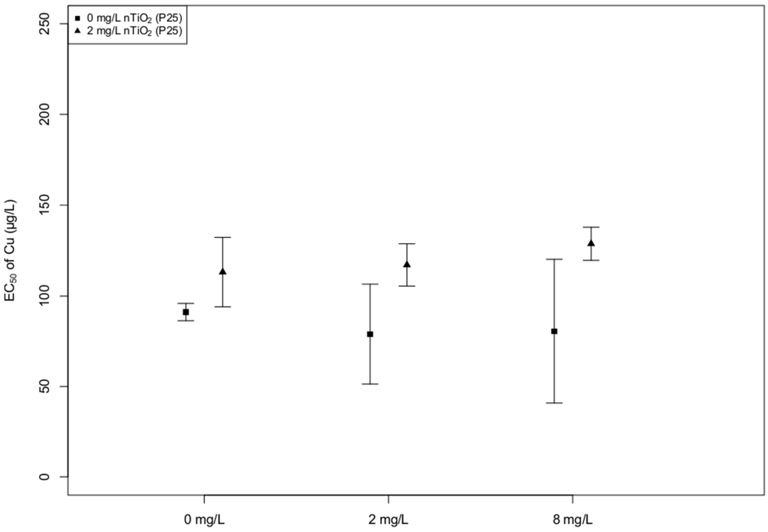

Figure A.4: The effect of aging in test medium with different levels of seaweed extract. The 48-h EC_{50} values (±95% CIs) of Cu in absence (■) and presence (▲) of titanium dioxide nanoparticles ($nTiO_2$) with 0, 2 and 8 mg TOC/L seaweed extract aged for 72 h in test medium. Asterisks denote statistically significant differences between the absence and presence of $nTiO_2$.

Publication III:
Does the presence of titanium dioxide nanoparticles reduce copper toxicity? A factorial approach with the benthic amphipod Gammarus fossarum

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Abstract

In aquatic ecosystems, titanium dioxide nanoparticles (nano-TiO_2) may adsorb co-occurring chemical stressors, such as copper (Cu). This interaction has the potential to reduce the concentration of dissolved Cu due to surface binding to the nanoparticles. The subsequent sedimentation of nano-TiO_2 agglomerates may increase the exposure of benthic species towards the associated Cu. This scenario was assessed by employing the amphipod *Gammarus fossarum* as model species and taking advantage of a 2×2-factorial design investigating absence and presence of 2 mg nano-TiO_2/L and 40 µg Cu/L (n=45; t=24 d) in darkness, respectively. Nano-TiO_2 alone did not affect mortality and leaf consumption, whereas Cu alone caused high mortality (>70%), reduced leaf consumption (25%) and feces production (30%) relative to the control. In presence of nano-TiO_2, Cu-induced toxicity was largely eliminated. However, independent of Cu, nano-TiO_2 decreased the gammarids' assimilation and weight. Hence, nano-TiO_2 may be applicable as Cu-remediation agent, while its potential long-term effects need further attention.

Keywords: Heavy metal – Benthic invertebrates – Energy processing – Remediation – Combined toxicity

Introduction

The production of titanium dioxide nanoparticles (nano-TiO_2) has already reached an annual production of approximately 38,000 tons in the United States (Hendren et al., 2011) and more than 10,000 tons in the European Union (Sun et al., 2014), due to their broad application in many household and industrial products (e.g., textiles, sunscreens and facade paints; Windler et al., 2012). This may lead to an unintentional release of nano-TiO_2 into aquatic ecosystems *via* point sources such as wastewater treatment plant effluents (WWTPs; Gottschalk et al., 2013; Sun et al., 2014). However, following their release, nano-TiO_2 may interact with other chemical stressors by adsorbing for instance metal ions onto their surface (Bekkouche et al., 2012; Hua et al., 2012; Rashidi et al., 2010). In this context, copper (Cu) an ubiquitous aquatic contaminant with concentrations partly above 200 µg/L in surface waters (Süß et al., 2006) may be of particular relevance. This is further strengthened by Cu's high potential to adversely affect lenthic and lothic freshwater ecosystems as indicated by acute toxicity data for *Daphnia magna* (48-h EC_{50} regarding immobility: 78 µg Cu/L; Rosenfeldt et al., 2015) and *Gammarus fossarum* (7-d LC_{50}: 78 µg Cu/L; Zubrod et al., 2014), respectively.

By adsorbing Cu onto nano-TiO_2, the concentration of Cu^{2+}, the most toxic Cu species (Flemming and Trevors, 1989), is reduced in the water column (Rosenfeldt et al., 2015). As a consequence, the toxicity of Cu for aquatic organisms may be altered. Current literature is inconclusive on the direction of the effect (Fan et al., 2011; Rosenfeldt et al., 2014). For the pelagic standard test organisms *D. magna*, it was suggested that a reduction of Cu toxicity is attributed to Cu^{2+} removal from the water column as a result of the agglomeration and sedimentation of nano-TiO_2 together with the absorbed metal ions (Rosenfeldt et al., 2014). This observation ultimately raises concerns whether a comparable pattern, namely a reduction of Cu toxicity by nano-TiO_2, or a different pattern, in fact an increase in Cu toxicity in presence of nano-TiO_2, can be observed for benthic invertebrates.

To address this question, an ecologically important benthic species, namely the leaf shredding amphipod *G. fossarum* (Dangles et al., 2004), was selected as a test organisms. The hypothesis, that benthic organisms show, opposed to pelagic organisms, an increased Cu accumulation and toxicity in presence of nano-TiO_2, was assessed by utilizing a 2×2 factorial test design. Therefore, a 24-day semi-static experiment was conducted involving two levels of nano-TiO_2 and two environmentally relevant Cu concentrations, which are expected to cause no effect and sublethal effects in gammarids, respectively. During the course of this study mortality as well as endpoints related to the organic matter processing of *G. fossarum* were assessed. Moreover, after the termination of the experiment, the lipid content (a proxy for energy reserves) and the dry weight of the test organisms were determined as indicators of physiological fitness. These data were supplemented by the quantification of TiO_2 and Cu, in the water column and the test organisms, allowing the estimation of their fate during the experiment.

Material and methods

Test substances

A Cu solution was prepared, prior to the applications to the test medium (SAM-5S; Borgmann, 1996), by mixing $CuSO_4 \times 5H_2O$ salt (purity ≥99%; Fluka, Germany) with 1 L deionized water in a volumetric flask. Subsequently, a dilution in test medium was carried out to achieve the nominal test concentration of 40 µg Cu/L, subsuming different Cu species (84.1% $CuCO_3$(aq), 9.2% $CuOH^+$, 3.6% Cu^{+2}, 1.6% $Cu(CO_3)_2^{-2}$, 1.2% $Cu(OH)_2$ (aq); determined *via* visual MINTEQ, version 3.0; Gustafsson, 2011).

The additive free nano-TiO_2 suspension had an intensity weighted average particle size of 59.5±0.7 nm (Table 1), measured using a DelsaNano C Particle Analyzer (Beckman Coulter, Germany). It was prepared at the Institute for Particle Technology (TU Braunschweig, Germany) by stirred media milling (PML 2, Bühler AG, Switzerland) of the purchased P25 powder (AEROXIDE® TiO_2 P 25; Evonik, Germany) using deionized water as medium (Dabrunz et al., 2011). The primary particle size of P25 was 21 nm, while the advertised surface area of the powder was 50±15 m²/g (Brunauer-Emmett-Teller). The suspension was pH stabilized and has shown a monodisperse size distribution (Table 1; Figure S.1). Prior to the applications the stock suspension (2 g/L) was ultrasonicated for ten minutes to ensure a spatially uniform particle distribution.

Table 1. Particle size distribution (i.e., average diameter, polydispersity index (PI), 10th and 90th percentile) of nano-TiO_2 measured ($n \geq 3$) in the stock suspension and the bioassay after 0, 24 and 48 h.

time	average diameter (nm)	PI	D10%	D90%
stock	59.5±0.7	0.101-0.173	37.3±1.0	101.9±4
0 h	289.5±0.8	0.229-0.259	143.6±3.8	597.4±98.7
24 h	1315.1±68.5	0.450-0.516	487.1±10.0	16759.5±2314.5
48 h	1043.3±58.7	0.402-0.449	284.6±19.3	12275.3±1406.8

The nano-TiO_2 in test medium (see section *Test organisms*), showed an intensity weighted average particle size of 289.5±0.8 nm (*n*=3; Table 1), which was measured using a DelsaNano C Particle Analyzer (Beckman Coulter, Germany) some minutes after test initiation. After 24 the average particle size was above 1000 nm (*n*=3; Table 1). Additionally, the particle size distribution was measured in test medium without gammarids in absence and presence of leaf discs (Table A.1). This procedure verified the stabilization of the particles, most likely as a results of the organic materials released by the leaf discs (Patil et al., 2012; the average particle diameter was less than 50% of the diameter in pure medium after 24 and 48 h). Nevertheless, nano-TiO_2 reached sizes above one micrometer and sedimented in all treatments (visual observation).

Test organisms

The test organism *G. fossarum* was kick-sampled from the stream Hainbach (near Frankweiler, Germany; N49° 14' 50" E8° 2' 27") upstream of any agricultural activity, settlement, and WWTP (Feckler et al., 2012). Afterwards, gammarids were gradually acclimatized to test medium over a time period of seven days. During this period, organisms were maintained under aeration in a climate-controlled chamber at 16±1°C in total darkness and fed *ad libitum* with pre-conditioned black alder leaves. Only male adult gammarids (identified by their position in precopula pairs) with a cephalothorax diameter between 1.6 and

2.0 mm (corresponding to an average dry mass of 5.7±0.7 mg, mean±SD, *n*=50) were used in the present study in order to reduce variability in the assessed endpoints (*cf.* Naylor et al., 1989; Pascoe et al., 1995). All organisms were visually free of acanthocephalan parasites.

Preparation of leaf discs

The leaf discs were prepared as described in Zubrod et al. (2010). Briefly, black alder (*Alnus glutinosa* (L.) Gaertn.) leaves were collected in the surroundings of Landau, Germany (N49°11 E8°05). Leaf discs of 2 cm diameter were cut from the leaves and subjected to microbial colonization (i.e., conditioning) for ten days in aerated nutrient medium (Dang et al., 2005) using leaf material previously exposed in another near-natural stream (Rodenbach, Germany; N49° 33' 59" E8° 02' 33") as inoculum. After conditioning, leaf discs were dried to a constant weight (~24 h at 60°C) and weighed to the nearest 0.01 mg.

Test design

To assess the influence of nano-TiO_2 on the toxicity of Cu, a 24-d bioassay was conducted applying a 2×2 factorial test design. The first factor was the absence or presence of nano-TiO_2 (P25) utilizing a concentration of 2 mg/L, which has shown to trigger no sublethal effects in previous experiments (Seitz et al., 2014). The second factor, namely Cu, was also applied at two levels, one without and one with a 40 µg/L exposure concentration. The Cu concentration is environmentally relevant (Bereswill et al., 2012; Süß et al., 2006) and is expected to cause sublethal effects in gammarids according to Zubrod et al. (2014). The combination of all factor levels resulted in four treatments, while each of them was replicated 45 times at test initiation. One replicate consisted of a 225-mL plastic beaker (polystyrene) filled with SAM-5S medium reflecting the respective treatment, an individual male gammarid as well as two leaf discs. To account for microbial and physical leaf mass loss, five additional beakers were set up for each treatment without gammarids. All beakers were aerated and kept at 16±1°C in complete darkness. In order to ensure a constant exposure, the medium of each treatment was renewed every third day. Following the careful transfer of the test organisms as well as the leaf material to fresh medium, the old medium was filtered individually for each beaker over a pre-weighed glass fiber filter (pore size: 1-3 µm; GF 6, Whatman GmbH, Germany). The filter was dried and weighed to quantify feces production. Furthermore, during every second water exchange leaf discs were replaced, while the removed leaf material was dried and weighed as detailed above. During each test medium renewal mortality was recorded. At the time of test termination, all remaining gammarids were carefully blotted dry, frozen in liquid nitrogen and then stored at -75°C until further processing.

Parameters related to physiological fitness

Following freeze-drying, individuals were weighed to the nearest 0.01 mg. The dry weight of gammarids was determined at the termination of the test. The total lipid content of the gammarids was quantified by the sulfo-phospho-vanillin reaction, which was described by Knight et al. (1972) using soy bean oil (Sojola, Vandemoortele, Belgium) as general reference standard for lipids (Zöllner and Kirsch, 1962). All samples (individual gammarids; *n*=5 per treatment) were treated as described by Van Handel (1985), while this procedure was validated for the assessment of energy resources in organisms exposed to nano-TiO_2 (see SI). Briefly, 0.5 mL of chloroform/methanol mixture (1:1) was added to each of the dried samples, which were kept in 2-mL glass vials with polytetrafluoroethylene (PTFE) caps. After three days samples were transferred (residues were rinsed with the solvent mixture) into micro mortars (Roth, Germany) and homogenized. These samples were then centrifuged for 60 s (short spin; up to 2000 rpm) and the supernatants were transferred into glass culture tubes with PTFE caps. The chloroform/methanol mixture was evaporated from the sample tubes using a water bath (95°C). Afterwards 0.2 mL sulfuric acid was added to each sample. After sealing the tubes, they were heated for 10 minutes in the water bath (95°C) again. Subsequently, the samples were cooled to room temperature, followed by the addition of 5 mL vanillin-phosphoric acid reagent (600 mg vanillin were solved in 100 mL hot deionized water and afterwards mixed with 400 mL of 85-% phosphoric acid) to each sample and thorough vortexing. For quantification, 200 µL of each sample and the calibration standards were transferred into wells of a 96-well plate (BRANDplate®, BRAND GmbH & Co. KG, Germany) and the absorption was measured with an Infinite® 200 M microplate reader (Tecan Group Ltd., Switzerland) using the i-control software (Tecan Group Ltd., Switzerland) at a wavelength of 490 nm.

Calculations and statistics

The endpoints related to the (organic) matter processing of *G. fossarum*, namely leaf consumption, feces production and assimilation, were quantified every sixth day and averaged, individually for each replicate, over the whole test duration for further statistical analysis. Leaf consumption (*C*) was expressed as mg consumed leaf dry mass per gammarid and day and was calculated as follows (Naylor et al., 1989):

$$C = \frac{L_a \times (1 - \frac{L_s - L_e}{L_s}) - L_r}{t}$$

where L_a is the initial dry weight of leaf discs available for gammarids, L_s is the initial mean dry weight of the leaf discs of the five replicates without gammarid, L_e is the mean dry weight of the leaf discs of the five replicates without gammarid after six days, thereby L_s and L_e served as correction factors for the microbial and leaching mediated leaf mass loss, L_r is the dry weight of leaf discs' residues after feeding in the same replicate, and *t* is the observation time in days. Feces production (*F*) was expressed as mg feces dry weight per gammarid and day and was calculated as follows (after Naylor et al., 1989, modified by Zubrod et al., 2010):

$$F = \frac{F_f - F_i - F_c}{t}$$

where F_f is the final dry weight of the filter, F_i is the initial dry weight of the filter, and F_c is the mean change in dry weight of five additional filters, serving as correction factor to account for general filter weight changes due to method dependent factors (e.g., microbial leaf decomposition, potential adsorption of nano-TiO_2 agglomerates and potential mass loss of the filter due to washing out). Both correction factors involved in these calculations were independent of the nano-TiO_2 exposure. Assimilation was expressed as mg assimilated amount of food per gammarid and day and is defined as the difference between C and F (Naylor et al., 1989).

Statistical analyses were accomplished using the statistics program R version 3.0.1 for Windows (R Core Team, 2013). The sublethal endpoints (i.e., leaf consumption, feces production, assimilation) and endpoints related to the physiological fitness (total lipid content and dry weight) were evaluated by two-factor ANOVAs, which allow, in contrast to one-way ANOVA, to assess for the individual effects of the two factors Cu and nano-TiO_2 as well as for their interaction, instead of assessing solely for the differences between treatment groups. Normal distribution and homogeneity of variances were assessed using the Shapiro-Wilk Normality Test and the Bartlett Test of Homogeneity of Variances, respectively. If the data did not meet the requirements for parametric testing the data set was rank-transformed prior to further assessment. The estimation of effects on mortality was addressed by pairwise comparisons for proportions using chi-square tests with Bonferroni-Holm correction for multiple comparisons. Furthermore, the measured differences between the total body burdens of Cu were assessed with a Kruskal-Wallis Rank Sum Test followed by a Wilcoxon Rank Sum Test for multiple comparisons (Bonferroni adjustment).

Chemical analysis

Ten milliliter samples for the determination of TiO_2 and Cu concentrations in the water phase were taken prior and after the water exchange, followed by an acidification with HNO_3 and storage at 5°C. Samples were measured for ^{65}Cu and ^{49}Ti present in the water column using inductively coupled plasma with a quadrupole mass spectrometer (ICP-MS; XSeries 2, Thermo Fisher Scientific, Germany) as described elsewhere (Rosenfeldt et al., 2014). In all cases, the concentrations of TiO_2 were calculated based on the measured Ti, while assuming the absence of other Ti-containing compounds.

Additionally, the total body burden of TiO_2 (including parts potentially adsorbed to the exterior of the organisms) in freeze-dried, weighed and subsequently incinerated (at 400°C) gammarids (after 24 d exposure) was analyzed *via* electro thermal vaporization (ETV 4000 System, Spectral Systems, Germany) coupled to the ICP-MS (Duester et al., 2010). The ICP-MS was run in the collision cell mode with -3 V and 6 mL He/H_2 cell gas in order to avoid polyatomic interferences and 25 mL Ar/O_2 were applied as additional gas to oxidize the introduced carbon (Rosenfeldt et al., 2014). The total body burden of Cu was determined with ICP-MS in freeze-dried and weighed gammarids sampled at the termination of the experiment following an acid digestion using aqua regia for four days with subsequent increases in temperature (30-80°C). The reference materials (i.e., GBW07602; bush twigs and leafs; Institute of Geophysical and Geochemical Exploration, China and TMDA-53.3; surface water; Environment Canada, Canada) showed a recovery of 102.5±12.2% (mean±SD). The detection limit of this method was 0.16 µg/L and the quantification limit 0.42 µg/L.

Results and discussion

Effects of nano-TiO_2

Titanium dioxide nanoparticles caused a near-significant increase in feces production by *G. fossarum* compared to the control (two-factor ANOVA: $p=0.052$; Table 2; Figure 1). The median difference, the conventional non-parametric measure to assess the difference between two samples (e.g., in the course of the Wilcoxon rank sum test; Altman et al., 2000), was in this case 0.02 mg/gammarid/day, which is not easily observable from the difference between the samples' medians as displayed in Figure 1. Together with the leaf consumption, this strong tendency to an increase in feces production may explain the significantly reduced assimilation (by approximately 40%) in the presence of nano-TiO_2 compared to the control (two-factor ANOVA: $p<0.001$; Table 2; Figure 1).

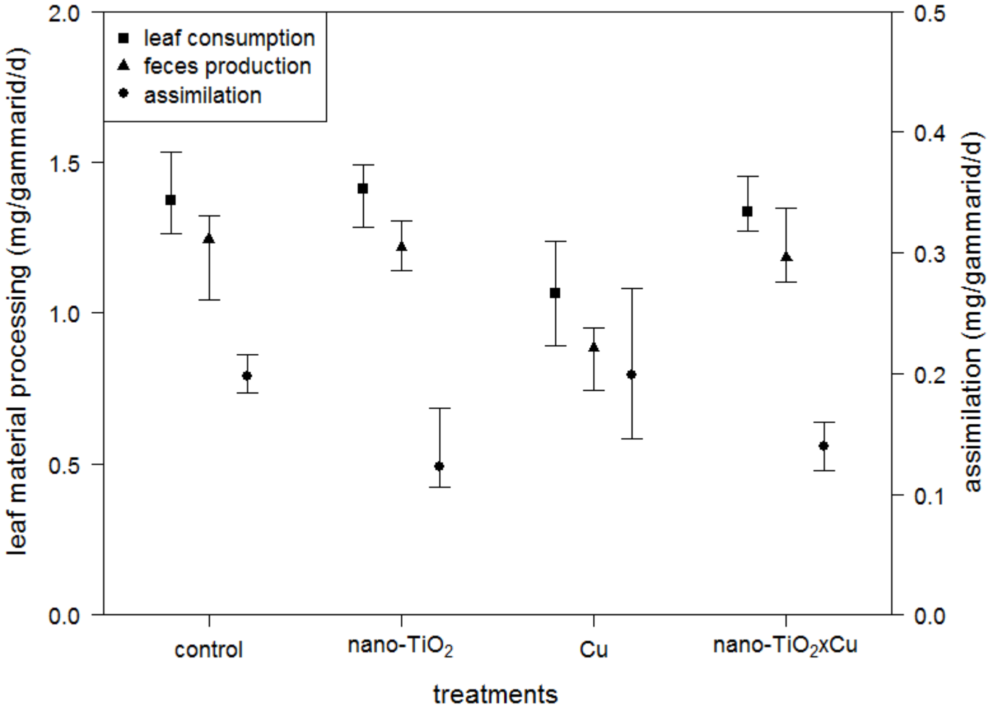

Figure 1. Medians (with 95-% confidence intervals) of the endpoints related to the processing of (organic) matter (i.e., leaf consumption □, feces production ▲ and assimilation ●; all in mg/gammarid/d) of *G. fossarum* exposed to the control, 2 mg nano-TiO_2/L, 40 µg Cu/L as well as the combination of nano-TiO_2 and Cu over the entire study duration of 24-d. The statistical significance of the variables and their interaction can be extracted from Table 2.

Table 2. Output of two-factor ANOVAs for leaf consumption, feces production, assimilation, weight and lipids.

	Df	Sum Sq	Mean Sq	F value	*p*-value
leaf consumption					
nano-TiO_2	1	0.033	0.034	0.370	0.544
Cu	1	0.401	0.401	4.425	0.037
nano-TiO_2×Cu	1	0.441	0.441	4.867	0.029
residuals	130	11.781	0.091		
feces production					
nano-TiO_2	1	0.343	0.343	3.862	0.052
Cu	1	0.285	0.285	3.206	0.076
nano-TiO_2×Cu	1	0.583	0.583	6.563	0.012
residuals	130	11.541	0.089		
assimilation					
nano-TiO_2	1	16865	16865	11.949	<0.001
Cu	1	150	150	0.106	0.745
nano-TiO_2×Cu	1	2	2	0.002	0.967
residuals	130	183480	1411		
weight					
nano-TiO_2	1	1.950	1.951	3.214	0.075
Cu	1	6.210	6.215	10.238	0.002
nano-TiO_2×Cu	1	1.010	1.010	1.664	0.199
residuals	131	79.520	0.607		
lipids					
nano-TiO_2	1	3883	3883	6.786	0.019
Cu	1	8	8	0.015	0.906
nano-TiO_2×Cu	1	140	140	0.244	0.628
residuals	16	9154	572		

Df, degrees of freedom
Sum Sq, sum of squares
Mean Sq, mean squares

These data suggest an inefficient digestion of the leaf material due to prioritizing the excretion of the nanoparticles over the assimilation for energy (Schaller et al., 2011). Another explanation for this phenomenon might be the clogging of the digestive tract by nanoparticles as shown for daphnids exposed to P25 (Campos et al., 2013). Irrespective of the underlying mechanism, the present study indicates that the uptake of nano-TiO_2 (Table 3) negatively affects gammarids over the long term as a result of the less efficient utilization of the ingested food.

Table 3. Total body burden (ng and ng/mg; mean±SD) of TiO_2 and Cu in *G. fossarum* after test termination.

	TiO_2 (ng)	TiO_2 (ng/mg)	Cu (ng)	Cu (ng/mg)
control	0.0±0.3	0.0±0.1	324.0±12.8	61.9±7.7
nano-TiO_2	481.2±179.6	129.4±34.0	NA	NA
Cu	1.2±1.5	0.1±0.4	674.8±117.5	138.9±23.6
nano-TiO_2×Cu	497.6±437.5	84.3±75.3	424.6±74.9	115.7±18.7

NA, not assessed

p-values for the differences in total body burden of Cu are available in the SI (Table S.2)

This assumption coincides – although not significant – with the reduced weight of the gammarids at the termination of the experiment by 10% relative to the control (two-factor ANOVA: p=0.075; Figure 2A). In contrast to the reduced weight, the lipid content of gammarids exposed to nano-TiO_2 was significantly increased by more than 60% in presence of nano-TiO_2 (two-factor ANOVA: p=0.019; Figure 2B; see also Dabrunz et al., 2011).

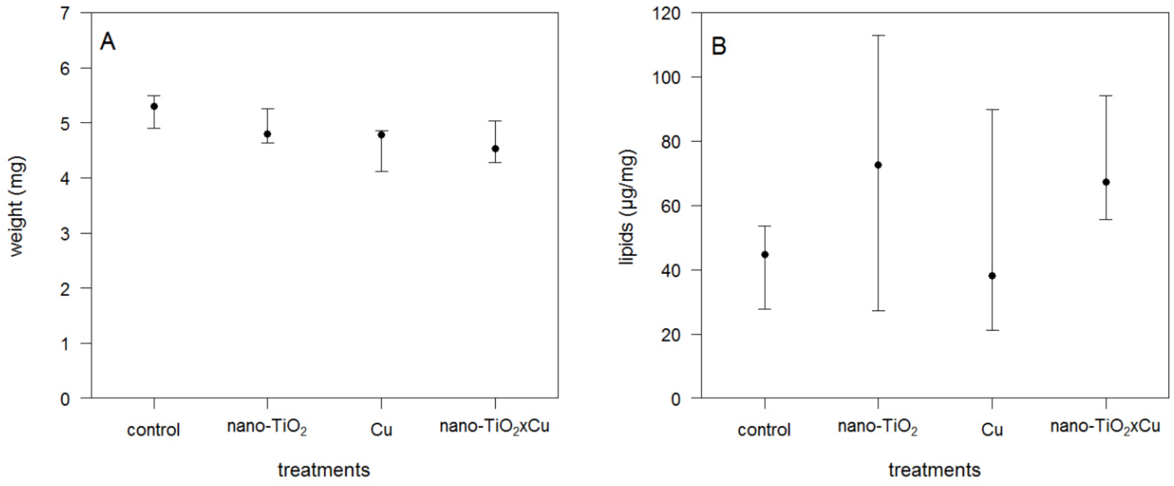

Figure 2. Medians (with 95-% confidence intervals) of the dry weight (A) and the lipid content (B) of *G. fossarum* exposed to the control, 2 mg nano-TiO_2/L, 40 µg Cu/L as well as the combination of nano-TiO_2 and Cu at the termination of the experiment (for both endpoints *n*≥5). The statistical significance of each of the independent variables and their interaction can be extracted from Table 2.

This increase in lipid content may be explained by an adsorption of lipids by nanoparticles within the body of the exposed organisms (Fortunelli and Monti, 2008). Lipids attached to the surface of nano-TiO_2 are probably not useable for the organism, ultimately accumulating in their body. Since nanoparticles can be translocated from the gut into lipid storage droplets in daphnids (Rosenkranz et al., 2009) and such droplets are also present in amphipods like *G. fossarum* (Schill and Köhler, 2004), this pathway seems possible. Even though nano-TiO_2 caused rather limited effects during this 24-d experiment; however, the observations, suggest detrimental effects over a longer time period due to the potential of nanoparticle exposure to hamper the assimilation and usability of energy from food and its storage, respectively.

Effects of Cu

Gammarids exposed to an environmentally relevant Cu concentration of 40 µg/L (Bereswill et al., 2012; Süß et al., 2006) showed a high mortality (71%) as well as sublethal effects on leaf consumption, which was significantly reduced (23%; two-factor ANOVA: p=0.037; Figure 1). At the same time, feces production of *G. fossarum* was reduced by approximately 30% (two-factor ANOVA: p=0.076; Figure 1), which resulted in an assimilation comparable to the control. Even though the assimilation was not affected, Cu exposed organisms exhibited a significantly reduced weight compared to animals from the control (approximately 10%; two-factor ANOVA: p=0.002; Figure 2A) at the termination of the experiment, while no influence on the lipid content was detected (Figure 2B; *cf.* Zubrod et al., submitted). These results suggest the expenditure of energy from the consumed leaf mass for detoxification processes instead of an increased utilization of the stored energy resources (i.e., lipids; Schill and Köhler, 2004). Thus, the excretion of Cu *via* feces was potentially stalled to enable sufficient energy assimilation from the consumed leaf material. The alternative to direct excretion was probably the detoxification of excess Cu *via* complexation with metallothioneins and sequestration as solid metallic sulfur granules (Ahearn et al., 2004; Barka et al., 2010; Khan et al., 2012). At the same time, gammarids seemed unable to supply energy for other physiological mechanisms, likely explaining the weight loss.

Combined effects

The results of the present study demonstrated for benthic invertebrates, using *G. fossarum* as model species, a reduced Cu toxicity in presence of nano-TiO_2. This observation contradicts the common misgivings, which assume an increased Cu toxicity in presence of nano-TiO_2 as a result of Cu adsorption to the nanoparticles surface, followed by nanoparticle agglomeration and sedimentation (Table 1 and 4).

Table 4. Titanium dioxide and Cu measured in the water column compared to the nominal concentrations (%; mean±SD), shortly after application and prior to the medium exchange.

treatments	freshly prepared		after 3 days	
	TiO_2	Cu	TiO_2	Cu
nano-TiO_2	95.4±4.4	NA	0.9±0.1	NA
Cu	NA	98.4±1.0	NA	36.9±0.4
nano-TiO_2×Cu	98.4±2.5	101.1±0.8	0.5±0.1	15.6±0.1

NA, not assessed

Rosenfeldt et al. (2015) observed an adsorption of more than 40% of the Cu by nano-TiO_2. This process was hypothesized to increase the Cu uptake (as nano-TiO_2 covered with Cu ions) by benthic species *via* a co-ingestion with their food (Bundschuh et al., 2011) and finally increasing Cu induced toxicity. However, the results of the present study indicate that the mortality of *Gammarus* at the termination of the experiment was reduced more than 4-fold in the combined exposure scenario relative to the absence of nano-TiO_2 (chi-square tests with correction for multiple comparisons: $p<0.001$; Table 5).

Table 5. Observed mortality in absolute values and as percentages.

	control	nano-TiO_2	Cu	nano-TiO_2×Cu
absolute mortality	1	3	32	7
mortality (%)	2.2	6.7	71.1	15.6

This reduction of Cu toxicity induced by nano-TiO_2 is further supported by a leaf consumption at the control level in the presence of nano-TiO_2, which is underpinned by the significant interaction term of the two-factor ANOVA ($p=0.0291$; Table 2). These observations may be related mainly to the, with approximately 30%, significantly lower total body burden of Cu measured in presence of nano-TiO_2 compared to its absence (Wilcoxon rank sum test for multiple comparisons: $p=0.026$; Table 3). Potentially, this lower total body burden was facilitated by the excretion of nanoparticles with the adsorbed Cu before an uptake of Cu ions through the intestinal wall occurred (Table 3; Schaller et al., 2011). However, these observations suggest that the bioavailability of Cu was reduced after the adsorption to nano-TiO_2, irrespective of their sedimentation and the probably increased exposure of the benthic gammarids.

Furthermore, adverse effects, which were also observed in the treatment containing nano-TiO_2 alone, namely the 30% reduction in assimilation and the 15% reduction in body weight at the termination of the experiment, indicate the substantial impact of nanoparticles on the sublethal and physiological responses of the test organisms. Moreover, gammarids exposed to the mixture of nano-TiO_2 and Cu exhibited lipid contents as high as in organisms only being exposed to nanoparticles, indirectly supporting the argumentation regarding lipid adsorption to nano-TiO_2, which was detailed above. This indicates that, nano-TiO_2 could reduce the toxicity of Cu, while on the other hand, their long term effects on the physiological fitness of *Gammarus* might lead to decreased population sizes. For instance this might happen as a result of the reduced assimilation of food and the unavailability of lipids due to nano-TiO_2, which might hamper reproduction (Charron et al., 2014; Clarke et al., 1985; De Coen et al., 1997). In addition, male gammarids might have not enough energy for mate guarding (Plaistow et al., 2003), which could also result in a decrease in population size. Thus, the local leaf litter decomposition potentially decreases (Dangles et al., 2004) and downstream communities (e.g., collectors) might additionally be affected since they depend on the fine particulate organic matter provided by shredders (Cummins and Klug, 1979).

Conclusions

Nano-TiO_2 reduced Cu toxicity, which seems to be triggered by adsorption of free Cu. Moreover, this phenomenon is transferable among pelagic and benthic invertebrate species. Despite this mitigating effect, nano-TiO_2 affected gammarids' physiological fitness ultimately impacting their population development. Since *Gammarus* is a key species in the ecosystem function of leaf litter decomposition, such impairments may alter the local food web and related energy pathways. Therefore, it seems sensible to thoroughly investigate for such potential side-effects before nano-TiO_2 products are assessed as safe or are even suggested for Cu-remediation (Hua et al., 2012).

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Zubrod, J.P., Baudy, P., Schulz, R., Bundschuh, M., 2014. Effects of current-use fungicides and their mixtures on the feeding and survival of the key shredder *Gammarus fossarum*. Aquatic Toxicology 150, 133-143.

Supplementary information

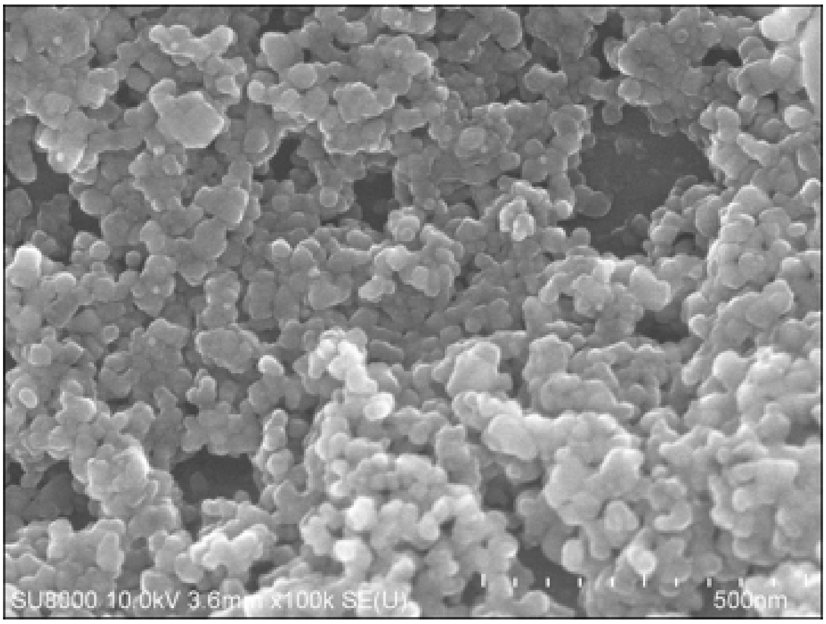

Figure S.1. Scanning electron microscopy image of the applied P25 nano-TiO_2 stock suspension (Hitachi SU8030; scale bar=500 nm).

Table S.1. Particle size distribution (i.e., average diameter, polydispersity index (PI), 10th, 50th and 90th percentile) of nano-TiO_2 measured ($n \geq 3$) after 0, 24 and 48 h during the bioassay in presence of leaf disks only (L) and in pure test medium (M).

time	treatment	average diameter (nm)	PI	D10%	D50%	D90%
0 h	L	363.6±23.6	0.226-0.268	181.7±3.0	367.3±21.1	770.2±88.0
	M	364.8±19.9	0.257-0.274	172.5±19.4	390.1±17.1	907.9±43.1
24 h	L	1137.6±30.3	0.404-0.439	417.9±13.8	886.4±14.0	12644.9±949.4
	M	3037.0±215.5	0.737-0.825	784.8±43.5	3317.9±239.9	18194.4±2291.2
48 h	L	983.6±9.3	0.372-0.394	373.1±8.7	755.3±11.8	10754.9±530.4
	M	2584.7±95.2	0.759-0.877	615.9±37.0	3338.8±1082.4	42579.9±5436.0

Indirect validation of the sulfo-phospho-vanillin reaction for determining lipid content in presence of nano-TiO_2

Freeze dried daphnids were weighed using an ultrafine balance SE 2-OCE (Sartorius) in DSC-pans (aluminum) with a perforated cap. The samples were subsequently measured in the differential scanning calorimeter Q1000 (TA Instruments) with a RCS 90 (TA Instruments) cooler. Thereby, the thermal decomposition of the daphnids was investigated. This was conducted by a constant heating rate of 10

K/min, from -50°C up to 550°C. The purge gas used in the measurement cell consisted of synthetic air with a flux of 50 mL/min. As reference pan an imperforated and hermetically closed aluminum pan was used. The enthalpy was calculated on the weighing mass (J/g) and summed up for the temperature from 250°C up to 450°C.

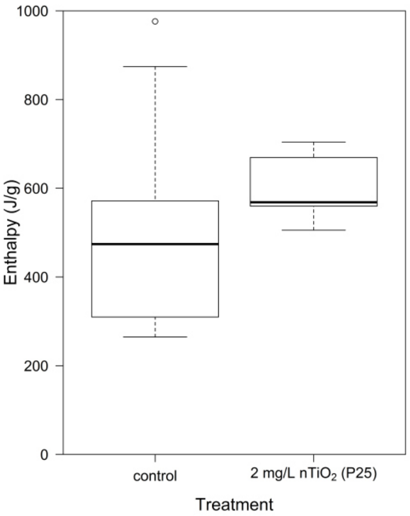

Figure S.2. Enthalpy (J/g) measured in pooled samples (*n*=9 with approximately 10 juvenile daphnids each) via differential scanning calorimetry after an exposure of *Daphnia magna* towards 2 mg/L nano-TiO_2 for 48 h.

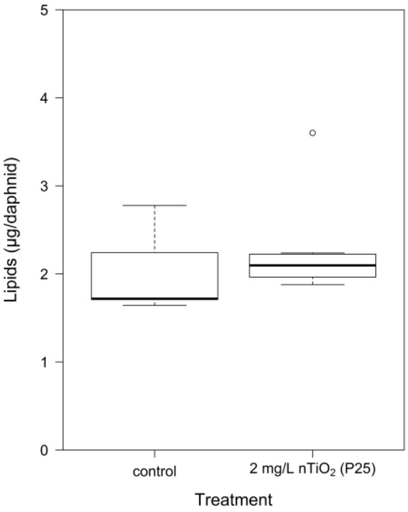

Figure S.3. Lipids (µg/daphnid) measured in pooled samples (*n*=7 with approximately 10 juvenile daphnids each) via the sulfo-phospho-vanillin reaction after an exposure of *Daphnia magna* towards 2 mg/L nano-TiO$_2$ for 48 h.

The difference in the enthalpy between the medians was 153.8 J/g dry weigh (37% of the control value), while the median of the control was 414.6±93.0 J/g and the median of the nano-TiO$_2$ treatment was 568.3±153.1 J/g (Figure S.2; Welch Two Sample t-test *p*=0.066). A comparable result was observed for the lipid analysis as performed in the present study, which is the sulfo-phospho-vanillin reaction (Figure S.3). In this case the difference between the medians was 0.38 µg lipids/daphnid (22% of the control value; Welch Two Sample t-test *p*=0.356). These results indicate that the lipid determination method used during the present study is not biased by the potential of nano-TiO$_2$ to interfere with the spectrophotometric measurements.

Table S.2. Output (*p*-values) of the Wilcoxon Rank Sum Test for multiple comparisons of the differences in total Cu body burden.

	control	Cu
Cu	<0.001	-
x	<0.001	0.026

Curriculum vitae

Personal data

Name: M.Sc. (Ecotoxicology) Ricki Richard Rosenfeldt

Birth date/-place: 08.09.1985 Kandel, Germany

Father: Rosenfeldt, Michael – master carpenter and managing director

Mother: Rosenfeldt, Sigrid nee Herrmann – administrative officer

Address: An der Neumühle, 76855 Annweiler

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School

1996-14.02.2000: Realschule (Staufer-Schulzentrum) Annweiler

15.02.2000-2005: Trifels-Gymnasium Annweiler

2005: Abitur

2005: Award of the Deutsche Physikalische Gesellschaft for excellent performance in physics

Studies

2005: Aerospace technology (Dipl.) at the University of Stuttgart

2006-2009: Environmental Sciences (Dipl.) at the University of Koblenz-Landau

2008: Research trip: Tuapse/Russia (University of Tyumen) – TEMPUS Joint European Project (JEP 26172-2005) “Securing Water Resources through Educational Change” (S.W.R.E.C.)

2009: Intermediate diploma (graduation grade: 1,8)

2009: Transition in the master program Ecotoxicology (M.Sc.) at the University of Koblenz-Landau

2010: Bachelor thesis – “Effect of titanium dioxide nanoparticles ($nTiO_2$) on lipid content, as energy reserve, in *Daphnia magna*.”

B.Sc. Environmental Sciences (graduation grade: 1,6)

2011: Master thesis – “Do TiO_2 nanoparticles alter heavy metal toxicity? – A factorial approach using *Daphnia magna*.”

M.Sc. Ecotoxicology (graduation grade: 1,4)

2011- recent: PhD thesis – “Combined toxicity of nanoparticulate titanium dioxide products and heavy metals considering various environmental parameters.”

Internships

2005: Metal processing – Kardex, Bellheim

2005: Foundry – Ronal, Landau

2010: Laboratory internal validation of the OECD guideline 231 – BASF SE, Ludwigshafen: Development and conduction of a reference study based on OECD guideline 231 (Amphibian Metamorphosis Assay) for the assessment of potential endocrine activity of chemicals

(Research) assistant

2009: Project “Dynamik Fischfauna“

- Sampling of sediments in several cut-off meanders of the river Rhine
- Particle size analyses of sediments
- Microwave digestion of the sediments, for the assessement of heavy metal residues
- Liquid-liquid extraction of potential polycyclic aromatic hydrocarbons from the sediments

2010: Project “Dynamik Fischfauna“

- Co-supervision of the course “Aquatische Systeme“

2011: Project “Internano“

- Conduction of several studies with nanoparticles

2015-recent: Project “AufLand”

Career

2015-recent: Co-founder of nEcoTox Consult

Publications

Note: The following articles were 106 times cited (without self-citations 65 times), resulting in an h-index of 6 (Web of science, 03.08.2015).

Peer-reviewed articles (17)

- **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*. *Aquatic Toxicology*, 165, 154-159.

- Zubrod, J. P., Englert, D., **Rosenfeldt, R.R.**, Wolfram, J., Lüderwald, S., Wallace, D., Schnetzer, N., Schulz, R., Bundschuh, M., 2015. The relative importance of diet-related and waterborne effects of copper for a leaf-shredding invertebrate. *Environmental Pollution*, 205, 16-22.

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

- Zubrod, J.P., Feckler, A., Englert, D., Koksharova, N., **Rosenfeldt, R.R.**, Seitz, F., Schulz, R., Bundschuh M. 2015: Inorganic fungicides as routinely applied in organic and conventional agriculture can increase palatability but reduce microbial decomposition of leaf litter. *Journal of Applied Ecology*, 52, 310-322.

- Bundschuh, M., Newman, M.C., Zubrod, J.P., Seitz, F., **Rosenfeldt, R.R.**, Schulz, R. 2015: Addendum to the article: Misuse of null hypothesis significance testing: Would estimation of positive and negative predictive values improve certainty of chemical risk assessment? *Environmental Science and Pollution Research* 22, 3955-3957.

- **Rosenfeldt, R.R.**, Seitz, F., Senn, L., Schilde, C., Schulz, R., Bundschuh, M., 2015: Nanosized titanium dioxide reduces copper toxicity – the role of organic material and the crystalline phase. *Environmental Science and Technology* 49 (3), 1815-1822.

- Seitz, F., **Rosenfeldt, R.R.**, Strom, K., Metrevelli, 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.

- Feckler, A., **Rosenfeldt, R.R.**, Seitz, F., Schulz, R., Bundschuh, M. 2015: Photocatalytic properties of titanium dioxide nanoparticles affect habitat selection of and food quality for a key species in the leaf litter decomposition process. *Environmental Pollution*, 196, 276-283.

- Seitz, F., **Rosenfeldt, R.R.**, Schneider, S., Schulz, R., Bundschuh, M., 2014. Size-, surface- and crystalline structure composition-related effects of titanium dioxide nanoparticles during their aquatic life cycle. *Sciences of the Total Environment*, 493, 891-897.

- **Rosenfeldt, R.R.**, Seitz, F., Schulz, R., Bundschuh, M. 2014: Heavy metal uptake and toxicity in the presence of titanium dioxide nanoparticles: A factorial approach using *Daphnia magna*. *Environmental Science & Technology*, 48 (12), 6965-6972.

- Kalčíková, G., Englert, D., **Rosenfeldt, R.R.**, Seitz, F., Schulz, R., Bundschuh, M. 2014: Combined effect of UV-irradiation and TiO_2-nanoparticles on the predator-prey interaction of gammarids and mayfly nymphs. *Environmental Pollution*, 186, 136-140.

- Noss C., Dabrunz A., **Rosenfeldt R.R.**, Lorke A., Schulz R., 2013 Three-dimensional analysis of the swimming behavior of *Daphnia magna* exposed to nanosized titanium dioxide. *PLoS ONE*, 8, e80960.

- Bundschuh, M., Newman, M.C., Zubrod, J.P., Seitz, F., **Rosenfeldt, R.R.**, Schulz, R., 2013. Misuse of null hypothesis significance testing: Would estimation of positive and negative predictive values improve certainty of chemical risk assessment? *Environmental Science and Pollution Research.*

- Seitz, F., Bundschuh, M., **Rosenfeldt, R.R.**, Schulz, R., 2012. Nanoparticle toxicity in *Daphnia magna* reproduction studies: The importance of test design. *Aquatic Toxicology*, 126, 163-168.

- Bundschuh M., Seitz F., **Rosenfeldt R. R.**, Schulz R., 2012. Titanium dioxide nanoparticles increase sensitivity in the next generation of the water flea *Daphnia magna*. *PLoS ONE*, 7, e48956.

- 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(10): 1563-1567.

- Dabrunz, A., Duester, L., Prasse, C., Seitz, F., **Rosenfeldt, R.R.**, Schilde, C., Schaumann, G.E., Schulz, R., 2011. Biological surface coating and molting inhibition as mechanisms of TiO_2 nanoparticle toxicity in *Daphnia magna*. *PLoS ONE*, 6, e20112.

Oral presentations (15)

- Seitz F., Lüderwald S., **Rosenfeldt R. R.**, Müller M., Schulz R., Bundschuh M., 2015. Quantity and quality of natural organic matter trigger the ecotoxicity of titanium dioxide nanoparticles to *Daphnia magna*, 25th Annual Meeting SETAC Europe, Barcelona, Spain.

- **Rosenfeldt R. R.**, Seitz F., Zubrod J. P., Feckler F., Lüderwald S., Merkel T., Bundschuh R., Schulz R., Bundschuh M., 2015. Nanosized titanium dioxide mitigates copper toxicity, 25th Annual Meeting SETAC Europe, Barcelona, Spain.

- Zubrod J. P., Wolfram, J., Englert, D., Bundschuh, R., **Rosenfeldt, R. R.**, Seitz F., Schulz, R., Bundschuh, M., 2015. Long-term effects of inorganic and synthetic fungicides on leaf-associated microorganisms and leaf-shredding macroinvertebrates – an artificial stream study, 25th Annual Meeting SETAC Europe, Barcelona, Spain.

- Schulz R., Bundschuh M., **Rosenfeldt R. R.**, Seitz F., 2015. Aquatic ecotoxicology of nanomaterials, NanoVision, Stuttgart, Germany.

- Seitz F., **Rosenfeldt R. R.**, Schneider S., Schulz R., Bundschuh M., 2014. Particle characteristic dependent effects of titanium dioxide nanoparticles on *Daphnia magna* and *Gammarus fossarum.* 24th Annual Meeting SETAC Europe, Basel, Switzerland.

- Feckler A., **Rosenfeldt R. R.**, Seitz F., Schulz R., Bundschuh M., 2014. Habitat selection of a leaf shredding amphipod affected by photocatalytic properties of $nTiO_2$. 24th Annual Meeting SETAC Europe, Basel, Switzerland.

- Seitz F., **Rosenfeldt R. R.**, Schneider S., Schulz R., Bundschuh M., 2014. Particle characteristic related effects of titanium dioxide nanoparticles on *Daphnia magna* and *Gammarus fossarum,* International Workshop - Nanoparticles in Soils and Waters: Fate, Transport and Effects, Landau, Germany.

- **Rosenfeldt R. R.**, Seitz F., Höger J., Haigis A.-C., Schulz R., and Bundschuh M., 2014. Factors triggering the combined toxicity of nanosized titanium dioxide and heavy metals, International Workshop - Nanoparticles in Soils and Waters: Fate, Transport and Effects, Landau, Germany.

- Bundschuh M., Seitz F., **Rosenfeldt R. R. [presenting author]**, Schulz R., 2013. Titanium dioxide nanoparticles increase sensitivity in the next generation of the water flea *Daphnia magna*. SETAC Europe 23rd Annual Meeting, Glasgow, Scotland, UK.

- Seitz F., **Rosenfeldt R.R.,** Storm, K., Schulz R., Bundschuh M. 2013. Environmental conditions alter the ecotoxicity of silver nanoparticles to *Daphnia magna*. SETAC Europe 23rd Annual Meeting, Glasgow, Scotland, UK.

- Seitz F., **Rosenfeldt R. R.**, Schneider S., Schulz. R., Bundschuh M., 2013. Product and size specific ecotoxicity of titanium dioxide nanoparticles to *Daphnia magna*. 3rd SETAC Young environmental scientists meeting, Krakow, Poland.

- **Rosenfeldt R.R.**, Bundschuh M., Seitz F., Schulz R., 2012. Environmental parameters influence the combined toxicity of TiO_2 nanoparticles and heavy metals: A factorial approach using *Daphnia magna.* 7th International Conference on the Environmental Effects of Nanoparticles and Nanomaterials, Banff, Canada.

- **Rosenfeldt R.R.**, Bundschuh M., Seitz F., Schulz R., 2011. Do TiO_2 nanoparticles alter heavy metal toxicity? – A factorial approach using *Daphnia magna*. SETAC GLB 16th Annual Meeting, Landau, Germany.

- Seitz F., Bundschuh M., **Rosenfeldt R.R.**, Schulz R., 2011. Chronische Effekte von TiO_2-Nanopartikeln auf Daphnia magna: Durchfluss- und semistatisches Testsystem im Vergleich. SETAC GLB 16th Annual Meeting, Landau, Germany.

- Schulz R., Bundschuh M., Seitz F., **Rosenfeldt R.R.**, 2011. Ökotoxizität anorganischer Nanopartikel auf aquatische Organismen – Studien unter Verwendung von $nTiO_2$. Nanotechnologie und Wasserwirtschaft, Düsseldorf, Germany.

Poster presentations (19)

- Lüderwald S., Seitz F., **Rosenfeldt R. R.**, Schulz R., Bundschuh M, 2015. influence of condition specific aging on nanoparticle toxicity – the role of time, SETAC Europe, Barcelona, Spain.
- Metreveli G., Frombold B., Seitz F., Grün A., Phillipe A., **Rosenfeldt R. R.**, Bundschuh M., Schulz R., Manz W., Schaumann G. E., 2015. Aggregation dynamics of silver nanoparticles in ecotoxicological test media, SETAC Europe, Barcelona, Spain.
- Schell T., **Rosenfeldt R. R.**, Seitz F., Schulz R., Bundschuh M., 2015. Influence of differing nTiO2 concentrations on copper toxicity for Daphnia magna, SETAC Europe, Barcelona, Spain.
- Steinmetz Z., Metreveli G., Vogel H.-J., Bundschuh M., Manz W., Baumann T., Schulz R., Lang F., Klitzke S., Seitz F., **Rosenfeldt R. R.**, Schaumann G. E., 2015. A floodplain mesocosm system linking fate and effects of engineered nanoparticles at the aquatic-terrestrial interface, SETAC Europe, Barcelona, Spain.
- Seitz F., Lüderwald S., **Rosenfeldt R. R.**, Schulz R., Bundschuh M, 2015. Aging of nanoparticles modifies the toxicity to aquatic organisms, NanoVision, Stuttgart, Germany.
- **Rosenfeldt R. R.**, Seitz F., Schulz R., Bundschuh M, 2015. Do titanium dioxide nanoparticles reduce heavy metal toxicity, NanoVision, Stuttgart, Germany.
- **Rosenfeldt R. R.**, Seitz F., Bundschuh M, Schulz R, 2015. Ecotoxicological assessment of nanoparticles and more, NanoVision, Stuttgart, Germany.
- Seitz F., **Rosenfeldt R. R.**, Schneider S., Schulz R., Bundschuh M., 2014. Particle characteristic dependent effects of titanium dioxide nanoparticles on *Daphnia magna* and *Gammarus fossarum.* 24th Annual Meeting SETAC Europe, Basel, Switzerland.

- **Rosenfeldt R. R.**, Seitz F., Höger J, Haigis A-C, Schulz R., Bundschuh M., 2014. Factors triggering the combined toxicity of nanosized titanium dioxide and heavy metals. [poster spotlight] 24th Annual Meeting SETAC Europe, Basel, Switzerland.

- Lüderwald S., Seitz F., **Rosenfeldt R. R.**, Schulz R., Bundschuh M., 2014. Acute and chronic toxicity of aged titanium dioxide nanoparticles on *Daphnia magna*, International Workshop - Nanoparticles in Soils and Waters: Fate, Transport and Effects, Landau, Germany.

- Schell T., **Rosenfeldt R. R.**, Seitz F., Schulz R., Bundschuh M., 2014. Implications of low titanium dioxide nanoparticle concentrations on the ecotoxicological potential of copper for *Daphnia magna*, International Workshop - Nanoparticles in Soils and Waters: Fate, Transport and Effects, Landau, Germany.

- Seitz F., **Rosenfeldt R. R.**, Storm K., Schulz. R., Bundschuh M., 2013. Varying environmental conditions alter the ecotoxicity of silver nanoparticles (nAg) to *Daphnia magna*. 8th International Conference on the Environmental Effects of Nanoparticles and Nanomaterials, Aix-en-Provence, France.

- **Rosenfeldt R. R.**, Seitz F., Schulz R. and Bundschuh M., 2013. Factors triggering the mixture toxicity of nanosized titanium dioxide and heavy metals. 8th International Conference on the Environmental Effects of Nanoparticles and Nanomaterials, Aix-en-Provence, France.

- **Rosenfeldt R.R.**, Seitz F., Schmuecking K., Schulz R., Bundschuh M., 2013. Time of aging as an important factor triggering the combined toxicity of titanium dioxide nanoparticles and heavy metals. SETAC Europe 23rd Annual Meeting, Glasgow, Scotland, UK.

- **Rosenfeldt R. R.**, Seitz F., Ehlert A., Schulz R. and Bundschuh M., 2013. The influence of natural organic matter on the combined toxicity of nanosized titanium dioxide and heavy metals. 3rd SETAC Young environmental scientists meeting, Krakow, Poland.

- Seitz F., **Rosenfeldt R.R.**, Schneider S., Schulz R., Bundschuh M., 2012. Product- and size-specific toxicity of titanium dioxide nanoparticles to the water flea *Daphnia magna*. 7th International Conference on the Environmental Effects of Nanoparticles and Nanomaterials, Banff, Canada.

- Seitz F., Bundschuh M., **Rosenfeldt R.R.**, Schulz R., 2012. Do titanium dioxide nanoparticles induce adverse effects in the reproduction of *Daphnia magna*? SETAC World, Berlin, Germany.

- **Rosenfeldt R.R.**, Bundschuh M., Seitz F., Schulz, R., 2012. Do TiO_2 nanoparticles alter heavy metal toxicity? – A factorial approach using *Daphnia magna*. [poster corner] 6th SETAC World Congress/ SETAC Europe 22nd Annual Meeting, Berlin, Germany.

- **Rosenfeldt R.R.**, Seitz F., Dabrunz A., Schulz, R., 2011. Establishment of an improved lipid determination method for *Daphnia magna* and its value in acute nanoparticle toxicity tests. 2nd SETAC Young environmental scientists meeting, Aachen, Germany.

Memberships

since 2006: World Wide Fund For Nature Deutschland (WWF)
since 2008: Naturschutzbund Deutschland e.V. (NABU)
since 2010: Society of Environmental Toxicology and Chemistry (SETAC)