

Comparative multi-generation study on long-term effects of pristine and wastewater-borne silver and titanium dioxide nanoparticles on key lifecycle parameters in *Daphnia magna*

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ABSTRACT

The rising production volume of engineered nanoparticles (NPs) leads to an increasing risk of environmental pollution. After passing sewage treatment plants (STPs), a significant concentration of NPs may end up in the aquatic environment where NPs can accumulate in the aquatic food chain and may cause harmful effects on aquatic organisms. However, when passing STPs some NPs such as silver nanoparticles (AgNPs) are transformed and enter the aquatic environment mostly as sulphide species with lower bioavailability and reduced toxicity compared to pristine NPs. For the environmental risk assessment of NPs, it is thus crucial to consider the transformation processes of nanomaterials during STP processes. For other NPs, such as titanium dioxide nanoparticles (TiO₂NPs), knowledge about the acute and chronic toxicity of NPs from STP effluents on aquatic organisms is still missing. Chronic studies, such as the *Daphnia* reproduction test following OECD TG 211, cover a period of only 21 days and hence allow only to evaluate the reproduction performance of a single generation. Multi-generation studies provide a more realistic exposure scenario and offer the opportunity to identify transgenerational effects which may possess a significant impact on the population dynamic. Hence, the aim of this study was to assess the impact of wastewater-borne AgNPs and TiO₂NPs on the aquatic invertebrate *Daphnia magna* in a multi-generation approach covering six generations. The effects of long-term exposure to pristine AgNPs (NM-300K; 14.9 ± 2.4 nm) and TiO₂ NPs (NM-105; 21 ± 9 nm) on the reproductive success (number of offspring), mortality, time to first brood and body size of adult *Daphnia* were measured and compared to those caused by wastewater-borne AgNP and TiO₂NPs. In all six generations, the exposure to environmentally relevant concentrations (determined by inductively coupled plasma mass spectrometry, ICP-MS) of pristine AgNPs caused a significant reduction in the mean number of offspring compared to the control. However, wastewater-borne AgNPs had no effects on reproduction in any generation. STEM analysis shows, that the AgNPs particles were transformed to Ag₂S while passing the STP. No effects could be detected following exposure to environmentally relevant concentrations (determined by inductively coupled plasma optical emission spectrometry, ICP-OES) of pristine TiO₂NPs and wastewater-borne TiO₂NPs. The present study is the first multi-generation study on long-term effects of pristine and wastewater-borne nanoparticles on *Daphnia*. No transgenerational effects of wastewater-borne AgNPs nor TiO₂NPs were observed. The results confirm that realistic exposure conditions are required in order to allow for a reliable environmental risk assessment of NPs.

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1. Introduction

Sewage treatment plants (STPs) are the main source for the release of nanoparticles into the aquatic environment (Gottschalk and Nowack, 2011). Although a considerable fraction of NPs in wastewater ends up in sewage sludge, which is often used as fertilizer in agriculture (Fytli and Zabaniotou, 2008; Gottschalk and Nowack, 2011), a significant amount of NPs may still reach the freshwater ecosystems through STP effluents. Silver nanoparticles (AgNPs) and titanium dioxide nanoparticles (TiO₂NPs) have been identified as compounds for which high concentrations in STP influent and effluent are to be expected (Nowack et al., 2012). The predicted environmental concentrations (PECs) in surface water for AgNPs and TiO₂NPs range from 0.088 to 10.000 ng/L and 0.021–10 µg/L, respectively (Gottschalk et al., 2009; Maurer-Jones et al., 2013; Nowack et al., 2012). Even higher PECs with 0.0164–17 µg/L for AgNPs and up to 100 µg/L for TiO₂NPs are expected for STP effluents (Maurer-Jones et al., 2013).

From studies analysing single generations, it is well known that AgNPs affect the reproduction in *Daphnia* species (Blinova et al., 2013; Mackevica et al., 2015; Ribeiro et al., 2014; Seitz et al., 2013; Zhao and Wang, 2011). For instance, Mackevica et al. (2015) showed that the exposure to 40 µg/L AgNP leads to a significantly lower mean number of offspring while the study of Ribeiro et al. (Ribeiro et al., 2016) estimated a 21 day EC₅₀ value of 1.0 µg/L for AgNPs. By contrast, the exposure to AgNO₃ leads to a higher toxicity with a 21 day EC₅₀ value of 0.385 µg/L for reproduction (Ribeiro et al., 2016). This difference of the effects and toxicity of AgNP and ionic silver (in form of AgNO₃) were further found by a study with *Daphnia magna* (Zhao and Wang, 2011) and zebrafish larvae *Danio rerio* (Asharani et al., 2008). However, the mechanism of the toxicity of AgNPs is not fully understood (Völker et al., 2013a) but can be mostly explained by the release of ionic silver (Yang et al., 2012). Ionic silver is one of the most toxic metals for freshwater organisms, especially for amphipods and cladocerans (Bianchini et al., 2002; Ratte, 1999). Silver ions can inhibit the Na⁺/K⁺/ATPase transport system leading to a fatal failure of ion-regulation (Bianchini and Wood, 2002). The release of Ag⁺ ions from the surface of nanoparticles may thus explain the toxic effects of AgNPs observed in acute and chronic studies with *Daphnia* (Bundschuh et al., 2016; Völker et al., 2013b; Zhao and Wang, 2011).

Especially for TiO₂NPs, test concentrations applied in ecotoxicological studies for testing toxic effects of NPs on aquatic organisms usually exceeded the related PEC values. For example, for the green algae *Desmodesmus subspicatus* and *Pseudokirchneriella subcapitata* EC₅₀ (median effective concentration) values of 44 mg/L (Hund-Rinke and Simon, 2006) and 5.83 mg/L (Aruoja et al., 2009) were determined, respectively. Chronic exposure of nanosized TiO₂ to the aquatic invertebrate *Daphnia magna* according to OECD TG 211 resulted in an estimated EC₅₀ value of 0.46 mg/L for reproduction and an estimated LC₅₀ (median lethal concentration) value of 2.62 mg/L (Zhu et al., 2010). The mechanism for toxicity of TiO₂NPs is based on physiological and mechanical damage (Bundschuh et al., 2016). Physiological damage is caused by the production of reactive oxygen species (ROS) (Bundschuh et al., 2016) and oxidative stress mediated toxicity, a major source of ROS. A mechanical damage is caused due to sorption of TiO₂NPs onto aquatic organisms, resulting in reduced filtering efficiency, decreased swimming speed and an increase in mortality due to an inhibition of moulting (Bundschuh et al., 2016).

Ecotoxicological studies for testing toxic effects of NPs have been mainly carried out with test media supplemented with pristine NPs. However, transformation processes during the STP process may lead to differences in the toxicity of pristine and wastewater-borne NPs. For instance, it is known from several studies that sulfidation is one of the major transformation processes of AgNPs into Ag₂S, while passing through a STP, which can be detected in both sludge and effluents (Kaegi et al., 2011; Kim et al., 2010; Levard et al., 2012). Ag₂S has a low water solubility which results in a reduced bioavailability and a

reduced formation of Ag⁺ ions leading to a decreased toxicity of silver to aquatic organisms (Levard et al., 2013; Kaegi et al., 2011; Bianchini et al., 2002; Ratte, 1999). Adam et al. (2018) calculated the release of AgNPs to the aquatic environment and estimated that 53% of the particles in the effluent of a STP are present in a transformed form, mostly Ag₂S, 22% are dissolved and only 18% of the NPs are released as nanoparticles (Adam et al., 2018). Furthermore, organic compounds like humic acids in the medium reduce the toxicity and the behaviour of silver (Fabrega et al., 2010; Ratte, 1999) due to adsorption to the surface of AgNPs (Cedervall et al., 2012; Kühn et al., 2018). Therefore, realistic exposure conditions are required for ecotoxicological studies in order to allow for a reliable environmental risk assessment of NPs. Muth-Köhne et al. (2013) reported that the toxicity of AgNPs to zebrafish embryos increased after passing through a model STP. By contrast, acute and chronic exposure studies reported that a STP effluent containing AgNPs led to a reduced toxicity in the freshwater crustaceans *D. magna* and *Hyalella azteca* compared to pristine AgNPs (Georgantzopoulou et al., 2018; Kühn et al., 2018). No information is currently available on the chronic toxicity of TiO₂NPs in STP effluents to aquatic organisms, such as the invertebrate *D. magna*. Chronic exposure studies following OECD TG 211 cover only a period of 21 days and thus evaluate the reproductive performance of only a single generation. An approach covering multi-generations of *D. magna* would provide an environmental relevant and more realistic exposure scenario, since the fitness of neonates plays an important role for population dynamics (Baun et al., 2008; Hammers-Wirtz and Ratte, 2000; Muyssen and Janssen, 2001). Völker et al. (2013b) discovered in a multi-generation study with *D. magna* an increased toxicity of AgNPs in the treatment with the highest concentration (10 µg/L) on population level after five consecutive generations. A multigenerational study with the nematode *Caenorhabditis elegans* indicated that the continuous exposure to PVP-coated AgNPs and AgNO₃ caused a pronounced approx. 10-fold sensitization in the F2 generation, which was present until F10 (Schultz et al., 2016). Bundschuh et al. (2012) showed that the acute exposure of juveniles to TiO₂NPs of pre-exposed adult *Daphnia* led to a significantly lower 96 h-EC₅₀ (median effective concentration after 96 h of exposure) value compared to juveniles of unexposed adults, thus indicating a transgenerational effect (Bundschuh et al., 2012). Looking at long-term studies over multiple generations in *Daphnia magna*, the exposure to TiO₂NPs at concentrations above approximately 1.8 mg/L induced a population collapse after five successive generations (Jacobasch et al., 2014). Multi-generation studies in the presence of environmental relevant concentrations of wastewater-borne NPs may help to further elucidate chronic effects under more realistic environmental conditions.

The aim of this study was, therefore, to investigate the impact of pristine and wastewater-borne AgNPs and TiO₂NPs on *D. magna* over six successive generations based on key life cycle parameters such as reproductive success, mortality, time to first brood and adult's body length. All studies were carried out with environmentally relevant concentrations of total Ag and Ti supplemented as AgNPs (NM-300 K) and TiO₂NPs (NM-105) to the corresponding test media. Due to the different exposure scenarios and potential changes in media concentrations in the course of the studies, total Ag and Ti concentrations were determined (with inductively coupled plasma mass spectrometry, ICP-MS, and ICP optical emission spectrometry, ICP-OES, respectively) in the STP effluents used to prepare the test media as well as the fresh and aged test media collected in representative samples (generation F2 and F4).

2. Material and methods

2.1. Study species

Populations of the freshwater cladoceran *Daphnia magna* (clone V; Federal Environment Agency, Berlin, Germany) were cultured in an air-

conditioned room ($20 \pm 2^\circ\text{C}$) with a 16:8 h (light:dark) photoperiod. The animals were kept in 2 L glass beakers with 1.8 L culture medium and 30 adult *Daphnia* per unit. As culture medium, ASTM reconstituted hard freshwater (192 mg/L NaHCO_3 , 120 mg/L $\text{CaSO}_4 \cdot 2\text{H}_2\text{O}$, 120 mg/L MgSO_4 , 8 mg/L KCl) (ASTM, 2007), enriched with vitamins (biotin, thiamine hydrochloride, cyanocobalamin) and selenium (65.7 mg/L Na_2SeO_3) (Seitz et al., 2013) was used. Culture medium was renewed once a week and in addition, juveniles were removed three times a week to avoid a high density. *Daphnia* were fed daily with the green algae *Desmodesmus subspicatus*. Algae were cultured in an air-conditioned room ($24 \pm 1^\circ\text{C}$) with a 16:8 h (light:dark) photoperiod. Culture medium was prepared as described elsewhere (Bringmann and Kühn, 1980). Before use, the algae stock culture was centrifuged and resuspended in ultra-pure water to provide an appropriate food source.

2.2. Preparation of test media and particle characterisation

2.2.1. Silver-nanoparticles (NM-300K)

All experiments with AgNPs were performed with NM-300K, which is one of the reference nanomaterials within the OECD Working Party on Manufactured Nanomaterials (WPMN) Sponsorship (Klein et al., 2011). The aqueous dispersion of NM-300K contained nominally 10% (w/w) silver and two stabilizing agents (4% each of polyoxyethylene glycerol trioleate and polyoxyethylene (20) sorbitan monolaurate (Tween 20)). The average particle size was reported to be 15 nm (provided by the manufacturer) with a narrow size distribution of 99% of the particle number concentration having a diameter of < 20 nm (Klein et al., 2011). Before use, the NM-300K stock vial was sonicated in an ultrasonic bath for 10 min (Bransonic 221 ultrasonic cleaner, Branson Ultrasonic, USA) to disperse AgNP agglomerates and air bubbles (Muth-Köhne et al., 2013). Afterwards, a working stock dispersion (nominal concentration: 50 mg Ag/L ASTM-medium) was prepared. In addition to the test media containing pristine AgNPs, a matrix control containing the AgNP-free dispersing agent, NM-300K-DIS, in ASTM-medium (dispersant stock; nominal concentration: 50 mg NM-300K DIS/L) was prepared as a matrix control. All dilutions were done by volume in PP (polypropylene) vials (VWR, International, Langenfeld, Germany).

2.2.2. Titanium dioxide-nanoparticles (NM-105)

The experiments with TiO_2 NPs were conducted with the OECD reference nanomaterial (WPMN programme) NM-105 ($> 99\%$ purity, $\sim 14\%$ rutile and $\sim 86\%$ anatase, uncoated, dry powder) with a nominal primary particle size of 21 nm for anatase and 15 nm for rutile (Rasmussen et al., 2014). Before use, the TiO_2 NP powder was dispersed in PP vials (VWR International, Langenfeld, Germany) in ASTM-medium to reach a working stock dispersion with a nominal concentration of 500 mg/L. The dispersion was sonicated for 16 min using an ultrasonic homogenizer (Bandelin SONOPLUS HD2200, Berlin, Germany) equipped with a 13 mm horn (MS 72) at 40% amplitude (Verleyen et al., 2014). Dilutions were done in PP vials (VWR International, Langenfeld, Germany). The suspension was used immediately.

2.2.3. Particle characterisation

A FEI Talos F200X electron microscope (Thermo Fisher Scientific, Waltham, USA) operating at 200 kV was used for (scanning) transmission electron microscopy (S/TEM) analysis. Imaging was carried out with a high-angle annular dark-field detector to enhance the contrast of nanoparticles consisting of heavy elements and a Super-X EDX detector was used for elemental mapping analysis. 5 μL of a pristine Ag or TiO_2 NP stock solution were deposited onto an amorphous carbon-coated copper grid (200 mesh, Plano) and dried in a desiccator overnight (0.1 mbar, Ar atmosphere). Particles from wastewater-borne samples were extracted from their saline media via the cloud point extraction as described by Hartmann et al. (2013) prior to analysis. Diluted extracted media were then centrifuged onto copper grids (2 h,

40°C , 12300 g) and organic residues were carefully rinsed off with absolute ethanol ($\geq 99.8\%$, VWR Germany).

2.3. Model sewage treatment plant (STP)

Several model sewage treatment plants (STPs) were used to produce effluents in two independent runs with AgNPs or TiO_2 NPs according to OECD TG 303a (OECD, 2001) as previously described (Kampe et al., 2018; Kühn et al., 2018). The STPs allowed to simulate transformation processes of NPs within a full-scale STP. The model STPs consisted of three reactors each (denitrification, nitrification and sedimentation) and were fed with active sludge (2.5 g dry mass/L) from a municipal STP ($51^\circ 09' \text{N}$ $8^\circ 16' \text{E}$, Schmallingberg, Germany). Under temperature-controlled conditions up to six STPs were continuously fed with artificial wastewater (flow: 750 mL/h; retention time: 6 h) with a defined composition according to OECD TG 303a (OECD, 2001). Physico-chemical properties (pH, O_2 saturation, ammonium, nitrate, and nitrite) of the wastewater were monitored periodically. Oxygen saturation and pH were measured manually using a multimeter (MultiLine® Multi 3410 IDS, WTW, Germany). Nitrite, nitrate and ammonium were measured photometrically with a digital photometer (Nanocolor® 500D, Macherey-Nagel, Germany) and the respective Nanocolor® test kits for nitrite, nitrate and ammonium (Macherey-Nagel, Germany) were used. After adding the sieved sludge (≤ 2 mm) to the STPs, an adaption phase of 5–6 days was necessary until the model STPs reached stable conditions [elimination rates of dissolved organic carbon (DOC) $> 80\%$ and constant concentrations of ammonium, nitrite and nitrate]. Based on the validity criteria of OECD TG 303a, the mean concentration in the effluents should be < 1 mg/L ammonia-N and < 2 mg/L nitrite-N (OECD, 2001). These values served as an orientation for the stable performance of the STPs. Subsequently, test substances were added to the STPs. AgNPs were prepared every 3–4 days as stock dispersions (10-fold concentrated as nominal inlet concentrations, Table 1) and pumped via a tube system (PLP 33; SP04/3.5 K, behr Labor-Technik) into the denitrification reactor of the STP units together with tap water and artificial wastewater (10-fold concentrated). To avoid sedimentation, TiO_2 NP dispersions were prepared daily and pipetted (nominal concentration equal to inlet concentration) manually into the model STPs. In total, six and four STPs ran for studies with AgNPs and TiO_2 NPs, respectively, with one STP each serving as control (without NPs). The effluents of all STPs were collected after 6–10 days and stored at 4°C until usage. Nominal inlet concentrations of the respective NPs are shown in Table 1. For the analysis of total silver and total titanium concentrations, samples of all effluents were collected and prepared for ICP-MS and ICP-OES analyses (see Section 2.6).

2.4. General test design of the multi-generation study

The multi-generation study was performed as a set of chronic exposure tests carried out according to OECD TG 211 (OECD, 2012). Biological effects of nanoparticles on *Daphnia* were investigated with AgNPs and TiO_2 NPs under two different exposure scenarios: exposure to (i) pristine and exposure to (ii) wastewater-borne NPs. All tests were started with juvenile *Daphnia* younger than 24 h. The procedure of our multi-generation study was comparable to the method used by Völker et al. (2013b) and Jacobasch et al. (2014). We exposed the parental generation of *D. magna* over a period of 21 days and used the third brood to start the next generation. In total, we received six consecutive generations (F0 - F5). For every generation, 10 replicates per treatment were exposed. In all experiments, a single juvenile was placed in a glass beaker (100 mL, Rotilabo, Carl Roth GmbH + Co. KG, Karlsruhe, Germany), filled with 50 mL of test medium. Test media were replaced manually three times a week (semi-static test approach). The *Daphnia* were fed daily with suspensions of the green algae *Desmodesmus subspicatus* (0.2 mg C/daphnid/day). The number of offspring was counted and removed six times a week. Once a week we measured temperature,

Table 1
Exposure scenarios and preparation of test media for the different treatments with respective nominal Ag and Ti concentrations. Concentrations [$\mu\text{g/L}$] of total Ag and total Ti in the STP effluents ($\pm$ U) measured by ICP-MS (Ag) and ICP-OES (Ti).

Exposure scenario	Treatment	Medium	STP: nominal sewage inlet conc. [mg/L]	STP: Effluent total conc. [$\mu\text{g/L}$] $\pm$ U	Effluent dilution factor	Nominal test media conc. [$\mu\text{g/L}$]
(i _a) pristine AgNPs	p-C 1	ASTM-medium	n/r	n/r	n/r	–
	p-M 1	ASTM-medium spiked with NM-300K DIS	n/r	n/r	n/r	–
	p-Ag 1	ASTM-medium spiked with NM-300K	n/r	n/r	n/r	1.25
	p-Ag 2	ASTM-medium spiked with NM-300K	n/r	n/r	n/r	2.5
	p-Ag 3	ASTM-medium spiked with NM-300K	n/r	n/r	n/r	5.00
(i _b) pristine TiO ₂ NPs	p-Ag 4	ASTM-medium spiked with NM-300K	n/r	n/r	n/r	10.00
	p-C 2	ASTM-medium	n/r	n/r	n/r	–
	p-TiO ₂ 1	ASTM-medium spiked with NM-105	n/r	n/r	n/r	25
	p-TiO ₂ 2	ASTM-medium spiked with NM-105	n/r	n/r	n/r	50
	p-TiO ₂ 3	ASTM-medium spiked with NM-105	n/r	n/r	n/r	100
(ii _a) wastewater-borne AgNPs	STP-C 1	Control effluent from STP	–	n/m	1:23	–
	STP-Ag 1	AgNP-spiked effluent	1	53.98 $\pm$ 5.30	1:43	1.25
	STP-Ag 2	AgNP-spiked effluent	2.5	64.45 $\pm$ 4.83	1:25	2.5
	STP-Ag 3	AgNP-spiked effluent	3.5	140.70 $\pm$ 5.56	1:28	5.00
	STP-Ag 4	AgNP-spiked effluent	6.5	239.03 $\pm$ 7.24	1:23	10.00
(ii _b) wastewater-borne TiO ₂ NPs	STP-C 2	Control effluent from STP	–	–	1:3	–
	STP-TiO ₂ 1	TiO ₂ NP-spiked effluent	1	104.34 $\pm$ 2.55	1:7	25
	STP-TiO ₂ 2	TiO ₂ NP-spiked effluent	2.5	113.67 $\pm$ 1.43	1:4	50
	STP-TiO ₂ 3	TiO ₂ NP-spiked effluent	5	464.27 $\pm$ 6.66	1:8	100

Note: For exposure scenario ii_b the total concentration of the effluent is present as $\mu\text{g/L} \pm$ sd; n/r – not required; n/m – not measured; U – expanded uncertainty for measured concentrations.

dissolved oxygen and pH in fresh medium in one replicate of the control and in one replicate of each treatment with a WTW Multi 3430 (WTW GmbH, Weilheim, Germany) which fulfilled the valid criteria of OECD TG 211 (OECD, 2012) (Table A.1). For each generation, the endpoints ‘cumulative mean number of offspring’, ‘time to first brood’, ‘mortality’, and ‘body length’ (measured as distance from naupliar eye to the base of the dorsal spine) of adult *Daphnia* were measured. The ‘cumulative mean number of offspring’ was determined after 21 days at the end of each exposure period. To measure body length, we took pictures of the *Daphnia* after 21 days with a digital camera (Nikon Coolpix L830, Chiyoda, Tokyo, Japan) and analysed body length using the software AxioVision (Carl Zeiss, Jena, Germany).

2.4.1. Exposure scenario (i): pristine NPs

Exposure to pristine AgNPs (i_a) was carried out with ASTM-medium. Treatments included an ASTM-control (p-C 1), a matrix control (p-M 1) and four Ag-treatments (p-Ag 1–4) with different concentrations of AgNPs. The matrix control (p-M 1) was prepared to contain the same amount of dispersing agent equivalent to the highest AgNP concentration to identify possible harmful effects of the dispersing agent. The exposure scenario with pristine TiO₂NPs (i_b) was performed with a control (p-C 2) containing ASTM-medium only and three TiO₂-treatments (p-TiO₂ 1–3) with different concentrations of TiO₂NPs. An overview of the preparation of the exposure scenario with pristine NPs are shown in Table 1.

2.4.2. Exposure scenario (ii): wastewater-borne NPs

For the scenario with wastewater-borne AgNPs (ii_a) and with wastewater-borne TiO₂NPs (ii_b), the collected effluents from the model STPs were shaken for 2 min before use to get a homogeneous suspension. Effluents from model STP runs without NPs were diluted with ASTM-medium at the lowest dilution factor applied for the treatment preparation and used as control medium (Table 1). Effluents with AgNPs were diluted in ASTM-medium to achieve similar concentrations in comparison to the pristine exposure scenario. Treatments with AgNPs included a control (STP-C 1) and four wastewater-borne Ag-treatments (STP-Ag 1–4) with different concentrations of AgNPs. The dilution factors applied to reach the final test concentration are presented in Table 1. No matrix control was included. Wastewater-borne TiO₂ (scenario ii_b) was tested by using the control (STP-C 2) and three wastewater-borne TiO₂-treatments (STP-TiO₂ 1–3) with different concentrations of TiO₂NPs. Dilution steps for the wastewater-borne TiO₂NP (ii_b) treatments and information on media preparation are presented in Table 1.

2.5. Collection of media samples for determination of total Ag and Ti concentrations

For the STP effluent with AgNPs and TiO₂NPs, aqueous test samples were taken of the fresh collected effluents, directly after the run of each STP. During the semi-static tests, test media were renewed twice a week after two days and once a week after three days of exposure. In generation F2, samples of freshly prepared media were taken to verify concentrations of total Ag in the different treatments. During the same generation, further samples were taken after three days of exposure representing the longest exposure period without media change. In total, three sets of fresh and aged media were available to specify potential changes in media concentrations in the course of the multi-generation studies with Ag. Similarly, a single set of fresh and aged test media was collected within generation F4 to determine total Ti concentrations. All aqueous samples were stored at 4 °C for four weeks before the analysis.

2.6. Determination of total Ag and Ti using ICP-MS and ICP-OES

Determination of total Ag concentrations in media samples and in

STP effluent as well as determination of total Ti concentrations in media samples were carried out at the University of Siegen as described below for aqueous test samples. Analysis of total Ti concentrations in STP effluent was carried out by Fraunhofer IME, Schmallenberg.

Total silver content of the aqueous samples collected during the multi-generation study was determined by ICP-MS (iCAP Qc, Thermo Fisher Scientific, Bremen, Germany). Before analyses, samples were taken out of the fridge and shaken for 30 min with a shaking machine (Edmund Bühler, Bodelshausen, Germany). The samples for the total silver analysis were digested with concentrated nitric acid (70%, Analytical Reagent Grade, Fisher Scientific, Loughborough, UK) for 90 min and afterwards diluted 100 times to obtain a concentration of 2.85% (w/v) HNO_3 . The calibration of the instrument was done on the same day with Ag^+ standard solution (Inorganic Ventures, Christiansburg, VA, USA). All aqueous test samples were measured 10 times and quantified using isotope $^{107}\text{Ag}^+$. Indium (Inorganic Ventures, Christiansburg, VA, USA) served as an internal standard. All concentrations were calculated from calibration graphs using the internal standard correction. Limit of detection (LOD) and limit of quantification (LOQ) for $^{107}\text{Ag}^+$ were ranging from 0.06 to 0.12 $\mu\text{g/L}$ and from 0.19 to 0.38 $\mu\text{g/L}$, respectively, depending on the experimental setup. Average concentrations of fresh and aged media were sampled during generation F2.

All aqueous titanium dioxide samples collected during the multi-generation study were measured at the University of Siegen by ICP-OES (ARCOS, SPECTRO Analytical Instruments GmbH, Kleve, Germany) according to the method of Khosravi et al. (2012) with minor changes. Aqueous test samples were taken within generation F4 as described above for the total silver measurements. For the sample preparation, 15.0 mL of each sample were evaporated in porcelain crucibles (Carl Roth GmbH + Co. KG, Karlsruhe, Germany), and 1.00 g of ammonium persulfate (> 98% p.a. ACS, Carl Roth GmbH + Co. KG, Karlsruhe, Germany) was added to the crucibles. A Bunsen burner was used to fume the crucibles for 5 min. After cooling down, the crucibles were

filled with bi-distilled water and placed on a hot plate to boil for 10 min. The obtained digest was transferred to 15 mL PP centrifuge tubes (VWR International, Langenfeld, Germany), nitric acid was added to the samples to achieve a concentration of 2% (w/v). The samples were shaken and analysed on the same day. All aqueous test samples were measured three times and quantified based on Ti 334.941 nm. All samples contained 200 $\mu\text{g/L}$ scandium (Inorganic Ventures, Christiansburg, VA, USA) as internal standard to perform internal standard correction. All concentrations were calculated from calibration graphs using the internal standard correction. LOD and LOQ for Ti 334.941 nm were ranging from 0.56 to 1.84 $\mu\text{g/L}$ and from 1.88 to 6.14 $\mu\text{g/L}$, respectively, depending on the experimental conditions.

The main ICP-MS and ICP-OES instrumental parameters are presented in Table A.2. Expanded uncertainty (U , $k = 2$) was calculated for all measured concentrations from standard deviations using the error propagation; taking into account the dilutions, uncertainties of the calibration, and the instrumental uncertainties. The coverage factor ($k = 2$) corresponds to the 95% confidence interval.

For the analysis of total Ti concentrations in STP effluent, samples (4 mL) were acidified with 0.8 mL nitric acid (69%, Suprapur®, Carl Roth, Germany) and 0.2 mL hydrofluoric acid (40%, Suprapur®, Merck, Germany) in Teflon vials and digested in a microwave UltraClave II (MLS GmbH, 25 min heating up to 220 °C, 30 min on 220 °C, max pressure 80 bar). For the complexation of hydrofluoric acid, 1 mL of boric acid 4% (Merck, Germany) was added after the digestion process and the samples were filled up to 15 mL with ultrapure water. The analysis for total Ti concentrations was performed by ICP-OES (Agilent 720, Agilent Technologies, Waldbronn, Germany). Commercially available Ti ICP standard solutions (Merck Certipur® 1000 mg/L Ti in 10% (v/v) nitric acid, Merck, Darmstadt, Germany) were used for the preparation of matrix adjusted calibration standards and stock solutions. A linear regression was used by the software (Agilent MassHunter workstation) to calculate calibration function and LOD. The LOQ was calculated as three times the LOD as described for ICP-MS

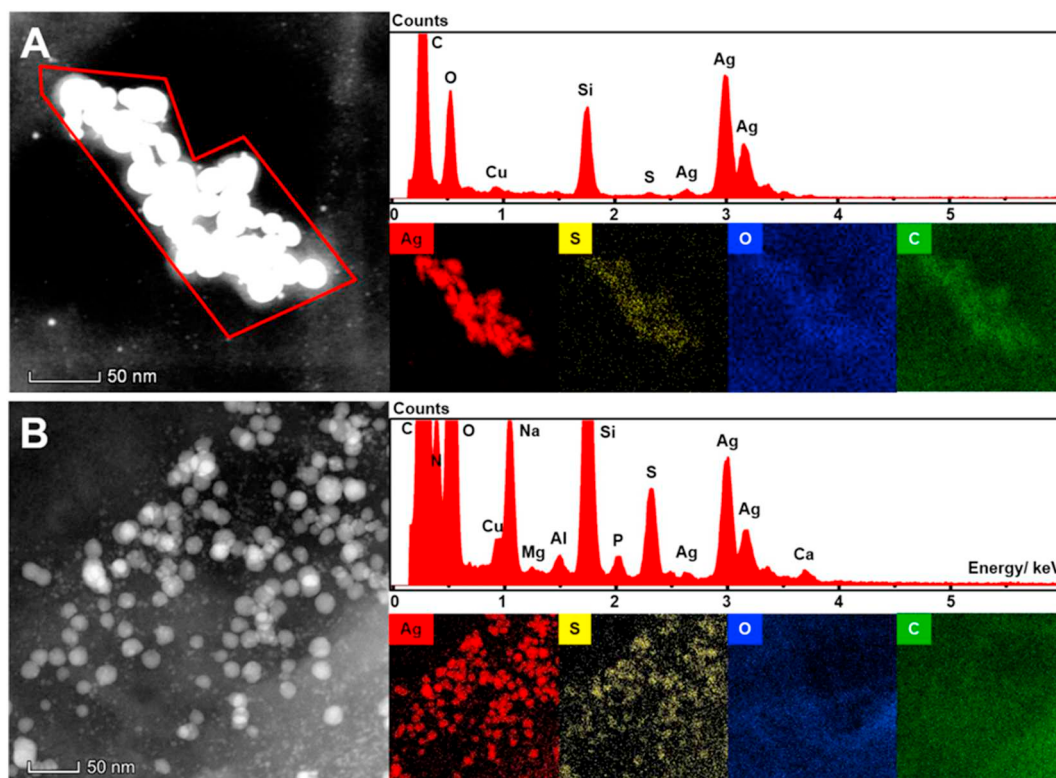


Fig. 1. STEM images with the corresponding EDX spectra and elemental maps of pristine (A) and wastewater-borne AgNPs (B). The spectrum in A corresponds to the highlighted area in the STEM image, while the spectrum in B corresponds to the entire STEM image.

measurement. All samples were measured in triplicate (internal triplicate measurement).

2.7. Statistical analysis

The statistical analysis was performed using the statistics program R version 3.2.4 for Windows (R Core Team, 2016). For each exposure scenario and each generation (F0 - F5), the cumulative mean number of offspring ($\pm$ sd), the mean body length (mm $\pm$ sd), mortality [%] and the time to first brood (days $\pm$ sd) were calculated. All data were checked for normal distribution (Shapiro-Wilk test) and for homogeneity of variances (Bartlett's test). Parametric tests to identify statistical differences were applied if data fulfilled both requirements. In this case a one-way analysis of variances (ANOVA) followed by a Dunnett's post hoc-test for multiple comparisons was performed. If one of the requirements was not fulfilled, the nonparametric alternative, the Kruskal-Wallis test followed by a Wilcoxon rank sum test for unpaired samples was used. The *p*-values were adjusted with Bonferroni correction. Significant *p*-values were marked with asterisks (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$). All *p*-values are two tailed.

3. Results

3.1. Particle characterisation

TEM analyses of pristine AgNPs showed that the spherical particles had a modal diameter of 14.9 ± 2.4 nm and were well dispersed, whereas TiO₂NPs formed aggregates of up to several 100 nm due to the absence of stabilizing agents. In both cases, the size of the nanomaterial did not change during the STP process indicating their physical stability. NPs were analysed via scanning transmission electron microscopy (STEM) combined with energy-dispersive x-ray analysis (EDX) mapping with a dark-field detector to determine the chemical transformation of Ag and TiO₂NPs in their pristine state (ASTM-medium) and after

passage through a model STP. The respective EDX spectra showed that wastewater-borne AgNPs were always associated with sulphur, indicating the chemical transformation during the STP process from pristine Ag towards Ag₂S (silver sulphide) which was supported by the atomic Ag/S ratio of ~ 2 (Fig. 1B). Other possible transformation products with different chemical moieties such as Cl can be ruled out due to the absence of the corresponding EDX signals. In contrast to that there is no evidence for the chemical transformation of TiO₂NPs. Titanium in its highest oxidation state of +IV is chemically stable and therefore not affected by sulphur, which is exclusively associated with the surrounding organic matrix (Fig. 2B). The small amounts of sulphur found in samples of pristine NPs most likely stem from the sulphate ions which were a component of the ASTM-medium. Since the relative amount of sulphur for pristine AgNPs was (i) substantially lower than for wastewater-borne AgNPs (Ag/S ratio of 30 instead of 2) and (ii) comparable to pristine, chemically inert TiO₂NPs, it can be assumed that no sulphidation process occurred. This transformation typically requires a reaction with sulphides (S²⁻) or thiols (R-SH) which is more likely to occur during wastewater treatment but not in ASTM media. Hence, AgNPs were still in their pristine state and, therefore, able to generate toxic Ag⁺ ions. Copper and silicon signals were either spurious x-rays from the TEM grid or originate from contaminations, whereas the remaining elements (Na, Ca, Mg, Al, P) were residues from the wastewater medium.

3.2. Total Ag and Ti concentration in STP effluents and test media

The analysis of the total Ag and Ti content of the STP effluent are shown in Table 1. The results are in accordance with previous studies, which found that up to 95% of Ag and 70–85% of Ti were removed from the effluent and end up into the wastewater biomass (Kaegi et al., 2011; Kiser et al., 2009). To confirm the nominal concentration used in the study, the concentrations of total Ag and Ti were measured by elemental analysis and are shown in Table 2. In generation F2, the total

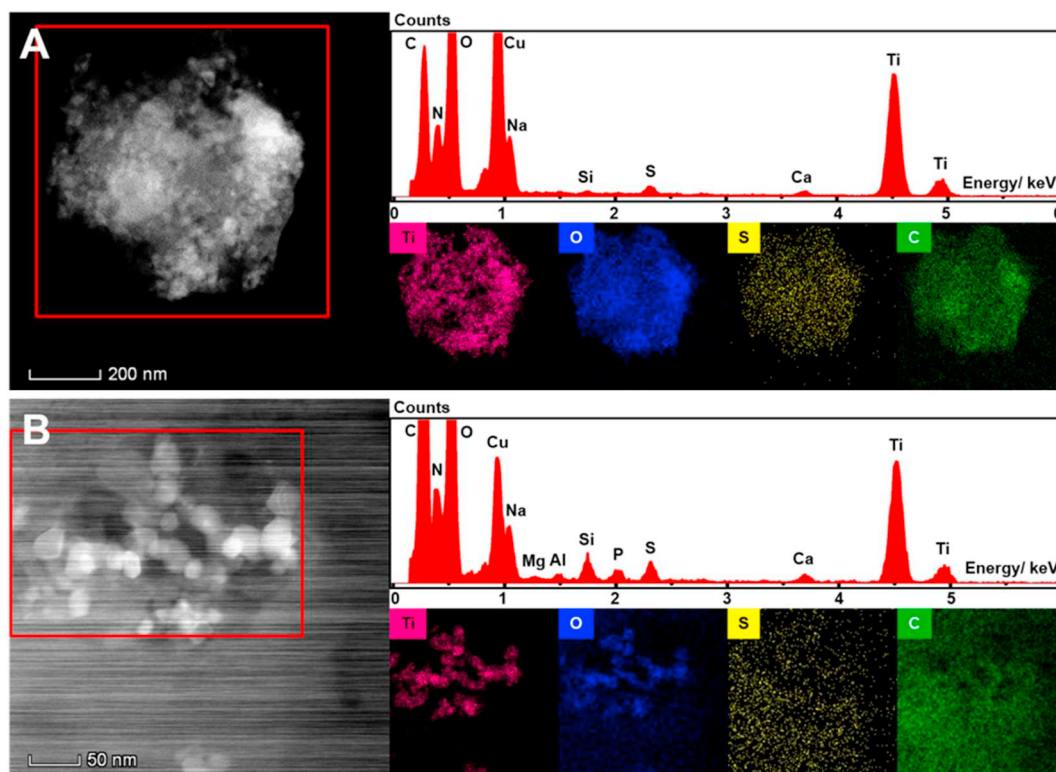


Fig. 2. STEM images with the respective corresponding EDX spectra and elemental maps of pristine (A) and wastewater-borne TiO₂NPs (B). The spectrum in A and B were captured in the highlighted areas in the STEM images in A and B.

Ag contents of the treatments with pristine Ag (i_a ; ASTM-medium spiked with NM-300K) were 1.98 $\mu\text{g/L}$ (p-Ag 1), 3.35 $\mu\text{g/L}$ (p-Ag 2), 6.78 $\mu\text{g/L}$ (p-Ag 3) or 10.34 $\mu\text{g/L}$ (p-Ag 4, Table 2). The effluent spiked with AgNPs (i_a ; STP-Ag 1–4) achieved similar total Ag concentrations after the dilution with ASTM-medium in different treatments (Table 2). The Ag concentrations ranged from 0.93 $\mu\text{g/L}$ (STP-Ag 1) to 10.07 $\mu\text{g/L}$ (STP-Ag 4). The total Ag contents for the ASTM-control treatment were 3.56 $\mu\text{g/L}$ (p-C 1), for the control effluent 0.61 $\mu\text{g/L}$ (STP-C 1) and for the matrix control 1.32 $\mu\text{g/L}$ (p-M 1). The analysis of the total Ti contents (in generation F4) for the pristine TiO_2 treatments (i_b ; ASTM-medium spiked with NM-105) determined concentrations of 7.59 $\mu\text{g/L}$ (p-TiO₂ 1), 13.47 $\mu\text{g/L}$ (p-TiO₂ 2) and 68.85 $\mu\text{g/L}$ (p-TiO₂ 3) and for the wastewater-borne TiO_2 treatments (i_b) < 6.14 $\mu\text{g/L}$ (STP-TiO₂ 1), 11.59 $\mu\text{g/L}$ (STP-TiO₂ 2) and 25.20 $\mu\text{g/L}$ (STP-TiO₂ 3), respectively (Table 2). The total Ti content of the ASTM-control treatment was estimated with < 0.56 $\mu\text{g/L}$ (p-C 2) and of the control effluent with 14.71 $\mu\text{g/L}$ (STP-C 2). The analysis of the aged medium showed for nearly all tested treatments a decrease in the total Ag and Ti content (Table 2). The concentration of p-Ag 1 and STP-TiO₂ 1 were higher in comparison to the fresh media contents. Additionally, the pooled expanded uncertainty (U) for all measured total Ag and Ti contents are presented in Table A.3.

3.3. Multi-generation study

3.3.1. Reproduction and body length

3.3.1.1. Exposure scenario (i): pristine NPs. In the experiment with pristine AgNPs (i_a), we found no significant differences in none of the key parameters in focus between the ASTM-control (p-C 1) and the matrix control (p-M 1) in all tested generations (F0 - F5) (data not shown). Hence, we combined the ASTM-control with the matrix control and refer to them in the following as control.

In all generations (F0 – F5) exposed to pristine AgNPs, a significant lower cumulative mean number of offspring per *Daphnia* in comparison to the control was observed with increasing AgNP concentrations (Fig. 3). The cumulative mean number of offspring in generation F0 was affected by all tested concentrations (Kruskal-Wallis test, $\chi^2 = 28.949$, $P \leq 0.001$, Fig. 3), except for the lowest concentration treatment (p-Ag 1). In the control, on average 61.67 neonates were released, which were significantly more than in treatment p-Ag 2 with 55.20 (p-Ag 2, Wilcoxon rank sum test for unpaired samples, $W = 151.9$; $P = 0.01$), treatment p-Ag 3 with an average of 51.56 neonates (Wilcoxon rank sum test for unpaired samples, $W = 161$; $P \leq 0.001$), and treatment p-Ag 4 with an average of 46.88 neonates (Wilcoxon rank sum test for unpaired samples, $W = 143.5$; $P \leq 0.001$). No significant differences could be detected for treatment p-Ag 1 with an average of 59.6 neonates per female *Daphnia* to the control (Wilcoxon rank sum test for unpaired samples, $W = 99$; $P = 1$). In the F1 generation, no concentration dependent effect on reproduction was observed. A significant lower mean number of offspring compared to the control was found in the lowest and the two highest concentrations (Wilcoxon rank sum test for unpaired samples, $P \leq 0.01$, Fig. 3). Generations F2 - F4 showed a similar pattern as in F0, the mean number of offspring was significantly lower at the three highest concentrations compared to the control (Wilcoxon rank sum test for unpaired samples; $P \leq 0.01$). Only in the F5 generation, a concentration dependent effect was observed showing a higher reproduction in the control than in the lowest test concentration (p-Ag 1, one-way ANOVA and Dunnett's test, $P \leq 0.05$). In the control on average 69.16 neonates were released whereas in treatment p-Ag 1 only 61.6 neonates were counted. Further bidirectional comparisons are listed in Table A.4.

The body length of adult female *Daphnia* did not differ between the control and all tested pristine AgNP concentrations at the end of generations F0 – F4 (one-way ANOVA and Dunnett's test, $P \geq 0.05$, Table A.5), except for generation F5 where the highest concentration (p-Ag 4) resulted in a significantly larger body length compared to the control

(Wilcoxon rank sum test for unpaired samples, $W = 0$, $P \leq 0.001$).

The exposure of *Daphnia* to pristine TiO_2 NPs (i_b) had no effect on the reproductive success nor on the adult's body length in any of the treatments (Fig. 4, Table A.4 and A.5) and over all tested generations (F0 – F5).

3.3.1.2. Exposure scenario (ii): wastewater-borne NPs. In comparison to pristine AgNPs, the exposure of female *Daphnia* to wastewater-borne AgNPs (i_a) had no effects on the reproduction success nor on adult's body length in treated *Daphnia* compared to *Daphnia* of the control over six generations (F0 – F5) (Fig. 5, Table A.4 and A.5). Since no matrix control was used for the wastewater-borne AgNP exposure scenario, the control effluent treatment served as the control.

In the wastewater-borne exposure scenario with TiO_2 NPs (i_b), *Daphnia* of generations F0, F3 - F5 released a similar cumulative mean number of offspring as *Daphnia* of the control and, thus, no significant differences were observed. Only in generation F1 and F2, female *Daphnia* treated with the lowest test concentration (STP-TiO₂ 1) released a significantly lower cumulative mean number of offspring compared to the control (Wilcoxon rank sum test for unpaired samples, $W = 95.5$, $P = 0.001$, Fig. 6, Table A.4). No concentration-dependent effect could be observed in all generations. Moreover, in generations F0 – F5, adult's body length of *Daphnia* in the control and in treatments showed no significant differences, except for *Daphnia* in treatment STP-TiO₂ 3 in generation F5 (Table A.5). Here, the mean body length was significantly larger than the mean body length of *Daphnia* in the control (Wilcoxon rank sum test for unpaired samples, $W = 9.5$, $P = 0.007$, Table A.5).

3.3.2. Mortality and time to first brood

Mortality did not differ over all tested generations and between exposure scenarios. However, the time to first brood was significantly affected in some of the treatments (F2: p-Ag 3, F3: p-Ag 2, p-TiO₂ 1, p-TiO₂ 2; STP-Ag 3, STP-Ag 4, F4: p-TiO₂ 1, p-TiO₂ 3 STP-TiO₂ 1, F5: STP-TiO₂ 1, STP-TiO₂ 2, STP-TiO₂ 3), however, no clear relation between

Table 2

Total Ag and Ti concentrations ($\mu\text{g/L}$) of freshly prepared media and aged media samples collected during generation F2 and F4 after 72 h of exposure. Given are measured concentrations as mean ($\pm$ sd) of three individual samples.

Exposure scenario	Treatment	Nominal concentration ($\mu\text{g/L}$)	Mean measured concentration $\pm$ sd ($\mu\text{g/L}$)	
			Fresh media	Aged media
(i_a) pristine AgNPs	p-C 1	–	3.56 $\pm$ 2.61*	1.96 $\pm$ 2.11*
	p-M 1	–	1.32 $\pm$ 1.13	0.47 $\pm$ 3.68
	p-Ag 1	1.25	1.98 $\pm$ 0.61	4.86 $\pm$ 3.65
	p-Ag 2	2.5	3.35 $\pm$ 0.33	3.31 $\pm$ 1.67
	p-Ag 3	5.00	6.78 $\pm$ 2.58	3.36 $\pm$ 3.48
	p-Ag 4	10.00	10.34 $\pm$ 1.38	5.83 $\pm$ 1.49
(i_b) pristine TiO_2 NPs	p-C 2	–	< 0.56	n/m
	p-TiO ₂ 1	25	7.59	n/m
	p-TiO ₂ 2	50	13.47	< 1.84
	p-TiO ₂ 3	100	68.85	< 6.14
(ii_a) wastewater-borne AgNPs	STP-C 1	–	0.61 $\pm$ 0.75	0.59 $\pm$ 0.49
	STP-Ag 1	1.25	0.93 $\pm$ 0.28	0.48 $\pm$ 0.09
	STP-Ag 2	2.5	2.93 $\pm$ 0.11	1.27 $\pm$ 0.36
	STP-Ag 3	5.00	6.39 $\pm$ 0.47	2.80 $\pm$ 1.36
(ii_b) wastewater-borne TiO_2 NPs	STP-Ag 4	10.00	10.07 $\pm$ 2.96	4.21 $\pm$ 0.53
	STP-C 2	–	14.71	< 6.14
	STP-TiO ₂ 1	25	< 6.14	11.34
	STP-TiO ₂ 2	50	11.59	8.61
	STP-TiO ₂ 3	100	25.20	18.07

Note that mean total silver concentrations ($n = 3$), except for the value “*” marked, where $n = 2$. Only one replicate was measured for total Ti content; experiments were performed parallel, according to the NP; n/m – not measured.

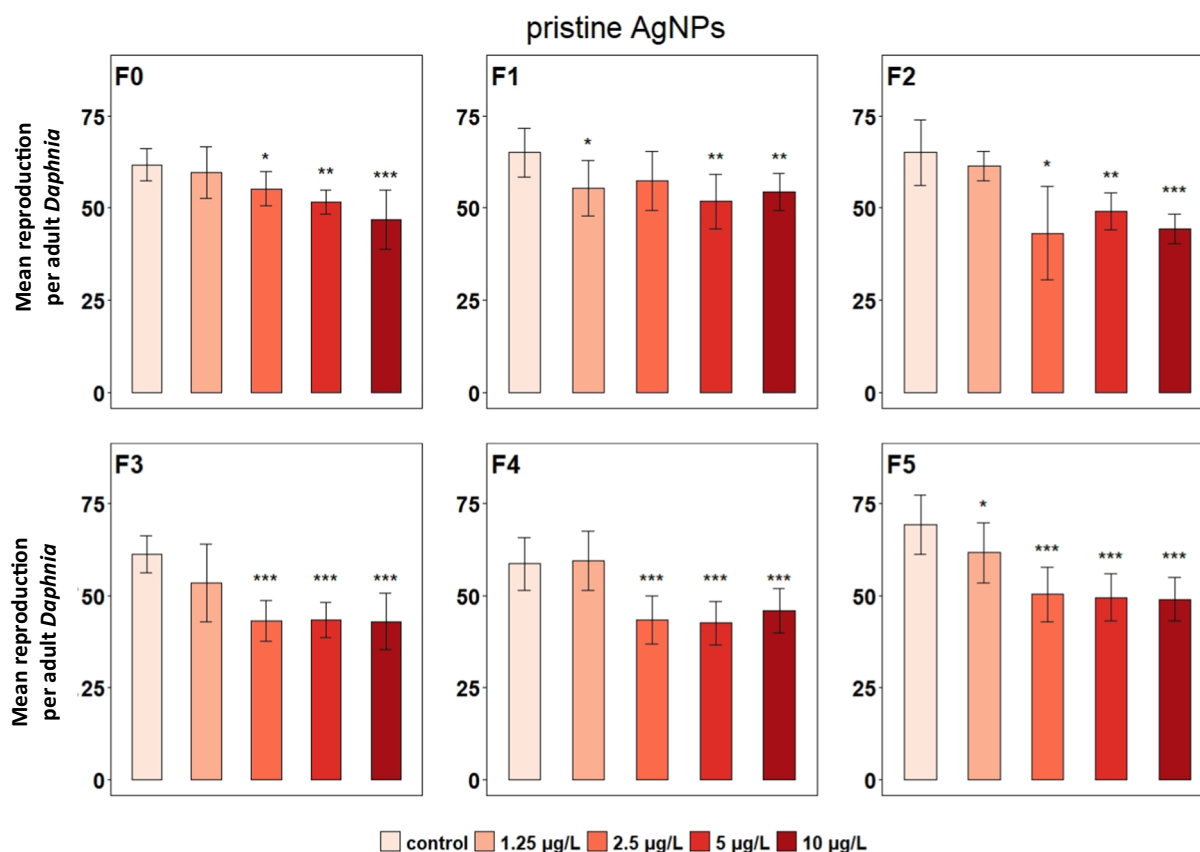


Fig. 3. Cumulative mean reproduction (as mean number of offspring) per adult *Daphnia* ($n = 10$; mean $\pm$ sd) treated with pristine AgNPs in a multi-generation approach (F0 - F5). Concentrations in treatments are given as nominal concentrations of total Ag in $\mu\text{g/L}$. Asterisks indicate significant differences compared to the control group: * = $P < 0.05$, ** = $P < 0.01$, *** = $P < 0.001$.

NP-exposure and this endpoint could be detected. The results are listed in Table A.6.

4. Discussion

In this study we investigated long-term effects of pristine and wastewater-borne Ag- and TiO_2 NPs on key lifecycle parameters in *D. magna* in a multi-generation test approach. When *Daphnia* was treated with pristine AgNPs, the number of offspring was significantly negatively affected in all tested generations (F0 - F5). In contrast, no effects on reproductive success were observed when animals were exposed to wastewater-borne AgNPs. This is in accordance with the results of former ecotoxicological studies with freshwater invertebrates using the effluent of lab-scale STPs containing wastewater-borne AgNPs (Georgantzopoulou et al., 2018; Kühr et al., 2018). The release of ionic Ag from the AgNP surface into the test media is probably the main mechanism behind the toxic effects observed for pristine AgNPs. As described by Kaegi et al. (2011), the differences between the toxicity of AgNPs detected in the pristine and in the wastewater-borne scenario is most likely explainable by transformation processes during the STP procedure and the presence of organic compounds in the effluent, both leading to a reduced release of ionic silver.

Multi-generation studies allow to identify potential long-term effects of AgNPs in a more realistic scenario than the usually applied chronic studies (e.g. OECD TG 211) where potential transgenerational effects cannot be recorded and evaluated. The current study has shown that wastewater-borne AgNPs do not affect the reproductive success in *Daphnia* over an extended period of six generations. No amplification of potential initially concealed toxicological effects could be observed. In the same way, exposure to pristine AgNPs showed a clear effect on the studied key lifecycle parameters already during the first generation,

however, effects occurring over the successive generations rather mirrored the previously observed effects. No indications for transgenerational effects leading to a reduction or further increase of the toxicity of pristine AgNPs were found. The current study is the first study on reproductive success and transgenerational effects in *D. magna* following long-term exposure to wastewater-borne AgNPs. The measured key lifecycle parameters are crucial factors for the long-term development of *Daphnia* population structure, which has significant implications on fish populations. The results of the multi-generation study provide clear indications that AgNPs in STP effluents represent a lower risk for the aquatic environment than pristine AgNPs. The characterisation of AgNPs within the effluent of the STP showed that AgNPs are to a large extent sulfurized to Ag_2S , which is in accordance to previous studies showing that AgNPs co-localized with sulphur in effluent samples and also in the sludge of STPs (Adam et al., 2018; Georgantzopoulou et al., 2018; Ma et al., 2014; Kaegi et al., 2011). Hence, based on the chemical characteristics of Ag_2S , i.e. low water solubility and reduced release of Ag^+ , the reduced toxicity of wastewater-borne AgNPs to the reproduction of *D. magna* is attributed to transformation processes during passage through a STP. This study hence shows that it is essential to consider transformation processes of nanomaterials during STP processes to allow for a realistic environmental risk assessment of AgNPs.

The results of this multi-generation study with environmentally relevant concentrations of TiO_2 NPs demonstrated that neither pristine nor wastewater-borne TiO_2 NPs caused any significant effects regarding the reproductive success of *D. magna*. Furthermore, both exposure scenarios with TiO_2 NPs did not lead to a population collapse as previously described by Jacobasch et al. (2014). However, the majority of studies published on ecotoxicological effects of TiO_2 NPs focused on the investigation of the mechanisms and the toxic effects of pristine TiO_2 NPs. Concentrations up to 100 times higher compared to this study

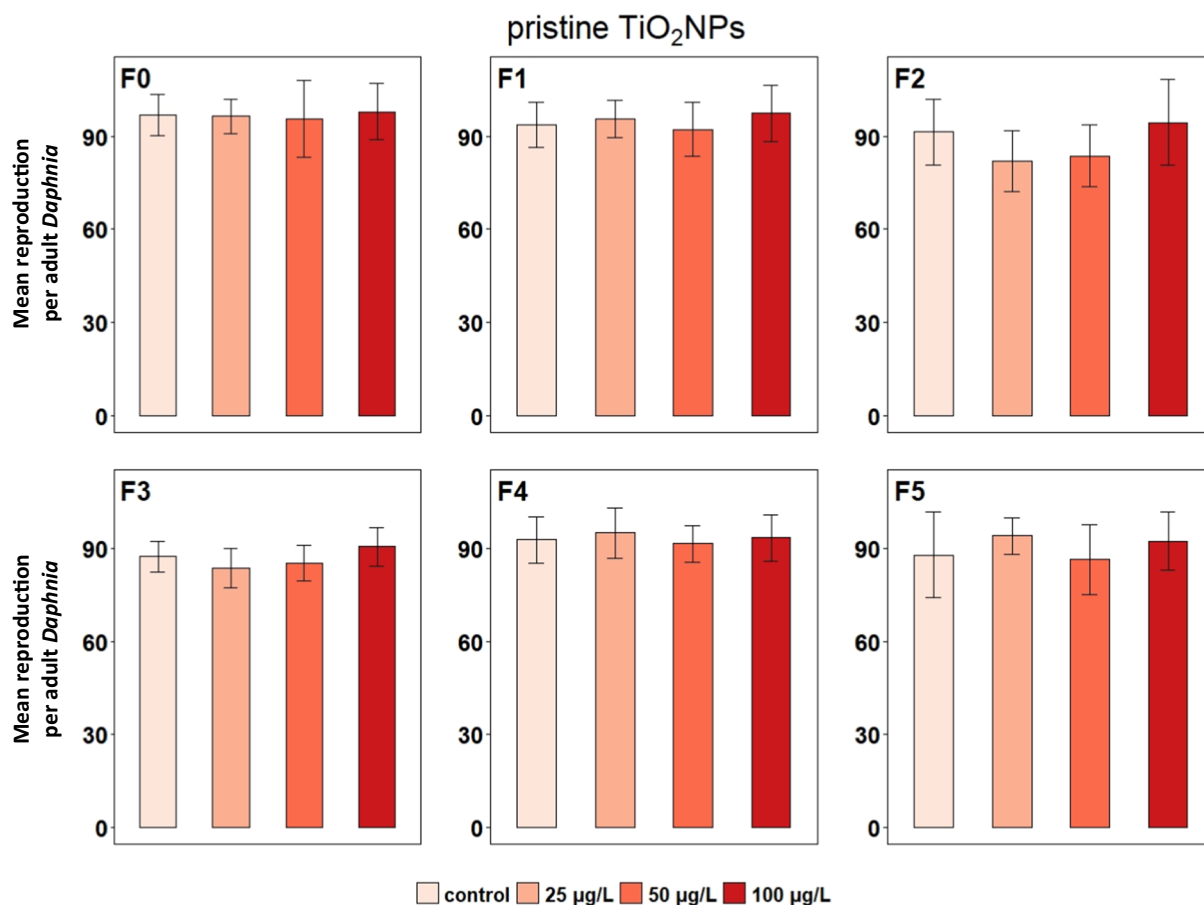


Fig. 4. Cumulative mean reproduction (as mean number of offspring) per adult *Daphnia* ($n = 10$; mean $\pm$ sd) treated with pristine TiO₂NPs in a multi-generation approach (F0 - F5). Concentrations in treatments are given as nominal concentrations of total Ti in µg/L. No statistically significant differences could be detected.

were applied. To our knowledge, this is the first study on the toxicity of TiO₂NPs in the range of environmentally relevant concentrations. In general, the removal efficiency of Ti in a full-scale STP is estimated to be 70–85% whereby the removed Ti accumulates in biosolids (Kiser et al., 2009). A material flow analysis showed that 95% of the TiO₂NPs in the effluent of a STP are present as pristine particles and only 5% are matrix-embedded particles (Adam et al., 2018). However, the analysis did not distinguish between pristine and free, agglomerated and aggregated particles. The EDX results of the current study showed that the TiO₂ particles are not associated with sulphur and form large agglomerates while no chemical transformation processes occurred during the STP passage. These results are in accordance with the findings of Adam et al. (2018), who reported that it is most likely that TiO₂NPs in STP effluents are released in pristine form. Since both tested scenarios, exposure to pristine or wastewater-borne TiO₂NPs, respectively, led to similar results, it seems that the toxicity of TiO₂NPs does not depend on the exposure pathway but rather on the used test concentrations and the formation of large agglomerates. The results clearly indicate that environmentally relevant concentrations of TiO₂NPs do not lead to physiological nor mechanical damage in *Daphnia*. This is in contradiction to the calculated risk quotient (RQ) of TiO₂NPs for water systems between > 0.73 and 16 which has to be considered critical for the aquatic environment (Mueller and Nowack, 2008). Further studies are required in this respect to elucidate in more detail the fate and the ecotoxicological impact of wastewater-borne TiO₂NPs on aquatic organisms.

In this study the toxicity of pristine and wastewater-borne NPs was evaluated based on the nominal concentrations of Ag and Ti in the applied media. The concentrations of total Ag and Ti were determined with state-of-the-art analytical instruments in aqueous samples of fresh

and aged media collected during one of the multiple test generations. The results show that it is important to determine the actual exposure concentrations during the study instead of nominal concentration only, which would clearly overrate the applied dosage. However, based on the large scale of the study and the long duration (13 weeks per experiment), no sample analysis could be performed at each water change over six generations due to the high workload. Furthermore, test concentrations decreased during the semi-static tests between the media replacements after three days of exposure. Therefore, we recommend to exchange water media every day during the complete period of testing to derive an even more detailed picture of the exposure conditions in such long-term chronic or/and multi-generation studies and to improve the ecotoxicological risk assessment for NPs in aquatic environment.

The use of wastewater-borne NPs for ecotoxicological testing represents a more realistic exposure scenario compared to test media supplemented with pristine NPs. However, care should be generally taken with respect to the use of laboratory test systems which may influence the effects induced by NPs. For instance, AgNPs show a strong adsorption to borosilicate glass surfaces (Struempfer, 1973). The adsorption may be only partially reversible during cleaning, therefore, glass vessels, which were previously exposed to Ag ions or AgNPs may exhibit partial desorption of silver from the glass walls. The use of polycarbonate containers for testing AgNPs as recommended for ionic Ag (110^mAgNO₃) should be considered (Sekine et al., 2015). In this multi-generation approach, however, it was not possible to use new glass vessels for every media replacement. Thus, beakers were carefully cleaned before re-use by inserting into a 70% nitric acid bed and afterwards, washing two times in a dishwasher and finally dried in a warming chamber at 120 °C. Nevertheless, background contaminations as measured in the control treatments of the chronic study with pristine

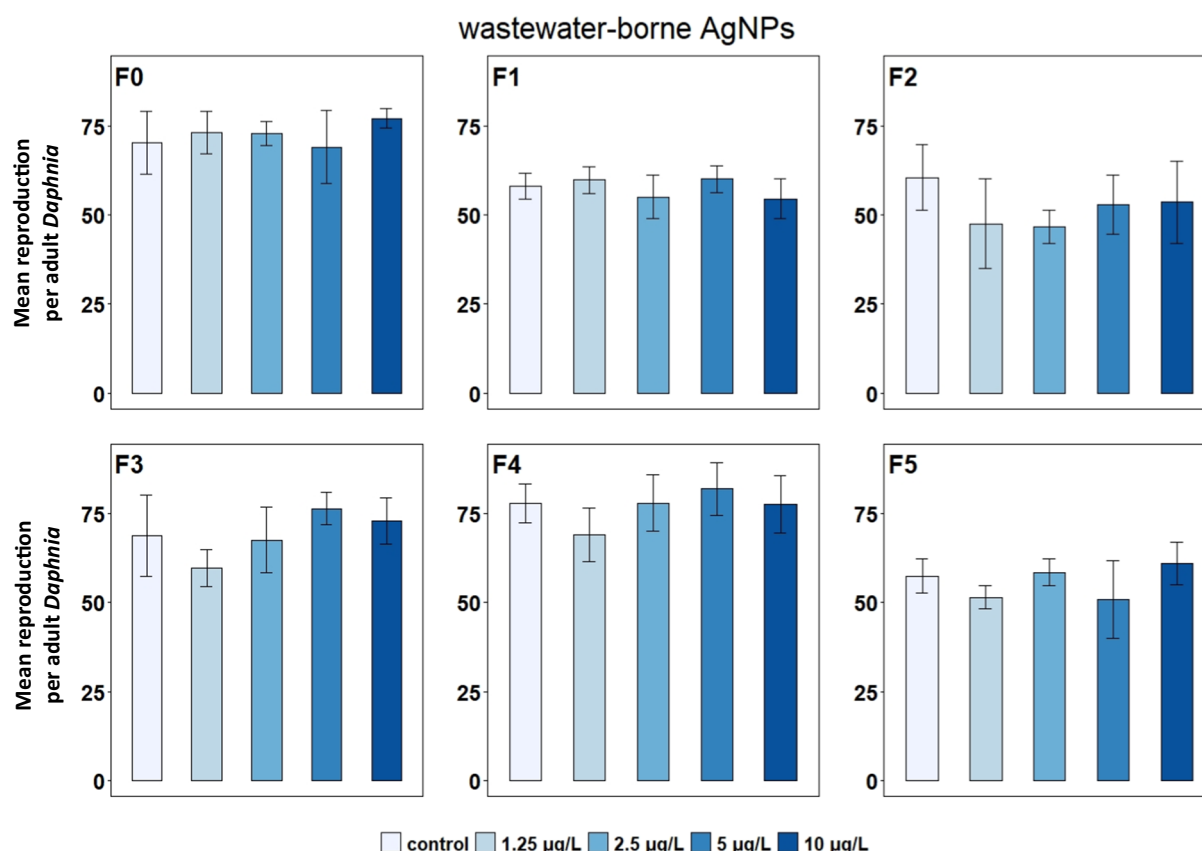


Fig. 5. Cumulative mean reproduction (as mean number of offspring) per adult *Daphnia* ($n = 10$; mean $\pm$ sd) treated with wastewater-borne AgNPs in a multi-generation approach (F0 - F5). Concentrations in treatments are given as nominal concentrations of total Ag in $\mu\text{g/L}$. No significant differences could be detected.

NPs could still not be avoided. However, all control treatments met the validity criteria of the OECD TG 211 (OECD, 2012) and no biologically relevant effects were observed. When testing substances containing metals, it is important to recognise that the properties of the test medium (e.g. hardness, chelating capacity) may affect the toxicity of the test substance. For this reason, OECD TG 211 (OECD, 2012) recommends the use of a fully defined test medium. Test media which are known to be suitable for long-term culture of *D. magna* are Elendt M4 and M7. However, both media contain the chelating agent EDTA, which may reduce the ‘apparent toxicity’ of metals (OECD, 1997). For metal-containing substances it may be, thus, advisable to use an alternative medium such as, for example, ASTM reconstituted hard fresh water (ASTM, 2007), which contains no EDTA and which is suitable for long-term culturing of *D. magna* (Baird et al., 1989). Therefore, our study was carried out with ASTM-medium, which was used to prepare test media containing pristine NPs and to dilute the STP effluents. Ecotoxicological studies with NPs and STP described in the literature are based on a range of different test media. However, care should be taken if results of studies using different fully defined test media are compared because test media can have a significant impact on the bioavailability of NPs (Käkinen et al., 2011).

As described above, NPs may be transformed from their original state through a variety of processes, including aggregation/agglomeration, redox reactions, and dissolution. Exchange of surface moieties and reactions with biomacromolecule or natural organic matter may influence the NP corona. Plasma-based mass spectrometric and microscopic techniques are useful tools to study chemical composition, surface functionalization and to detect NP interactions with cells under pristine and model environmental conditions and should be thus considered to further evaluate the ecotoxicological impact of wastewater-borne NPs on aquatic organisms.

In this study the reproductive success was identified as the most sensitive endpoint. Even at low AgNP-concentrations of pristine NPs we could detect strong effects, which is consistent with previous studies (Antunes et al., 2004; Völker et al., 2013b). However, exposure to wastewater-borne AgNPs and TiO_2 NPs had no significant effect on any key lifecycle parameters. Further investigations are required to elucidate the impact of wastewater-borne NPs on the biochemical level of aquatic organisms. Biomarkers at molecular and cellular level have been proposed as early warning indicators of reduced performance of organisms that may be linked to the effects on higher biological levels (Torres et al., 2008; Valavanidis et al., 2006). To date, a set of effects of NPs at biochemical level in biota were reported, mainly related to the formation of oxygen radicals inducing cytotoxic effects (Yang et al., 2009). Furthermore, effects on oxidative stress, neurological responses, lipid peroxidation, and energy metabolism among others have been reported for several aquatic species (Hackenberg et al., 2011; Klaper et al., 2009; Metzler et al., 2012). Investigations on biomarkers at the molecular and cellular level may help to further elucidate the ecotoxicological impact of wastewater-borne NPs on aquatic organisms (Galhano et al., in preparation).

5. Conclusion

The current study clearly confirms that the exposure scenario is of great importance for a reliable environmental risk assessment of nanoparticles for aquatic invertebrates. In contrast to pristine AgNPs in ASTM-medium, where the reproduction decreased in a dose-response pattern in all tested generations, exposure to wastewater-borne AgNPs in environmentally relevant concentrations (0.7–12 $\mu\text{g/L}$) did not affect key lifecycle parameters in *D. magna* over six continuous generations. No transgenerational effects were observed for both exposure scenarios.

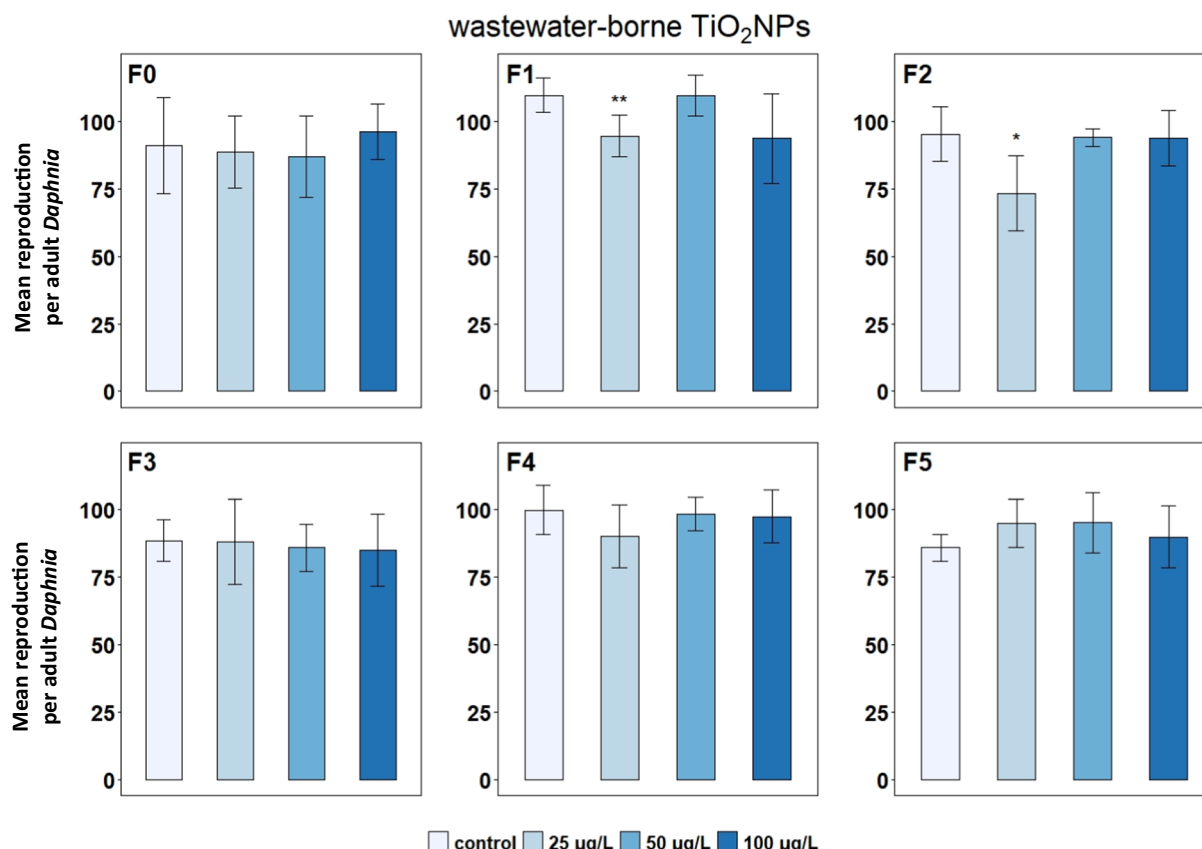


Fig. 6. Cumulative mean reproduction (as mean number of offspring) per adult *Daphnia* ($n = 10$; mean $\pm$ sd) treated with wastewater-borne TiO₂NPs in a multi-generation approach (F0 - F5). Concentrations in treatments are given as nominal concentrations of total Ti in µg/L. Asterisks indicate significant differences compared to the control group: * = $P < 0.05$, ** = $P < 0.01$.

Furthermore, this study shows that the risk of transformed AgNPs present in the aquatic environment, in the form of Ag₂S, is to be classified for aquatic organisms such as *D. magna*, since no negative effects could be determined over six generations. However, further research is needed to e.g. study the bioavailability of Ag₂S to aquatic organisms and potential effects on the biochemical level, which could be shown for the terrestrial isopod *Porcellio scaber* (Kampe et al., 2018). Also, chronic exposure of environmentally relevant concentrations of TiO₂NPs (6–25 µg/L) did not have any effect on *D. magna* independent of the test medium applied and the number of successive generations investigated. Hence, we have provided further evidence that chronic effects of NPs in aquatic organisms reported in the literature tend to overestimate the risk of NPs and care needs to be taken that realistic exposure scenarios and possible involved transformation processes are considered to ensure a reliable environmental risk assessment.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.impact.2019.100163>.

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