Ecotoxicological impacts of wastewater-borne silver and titanium-dioxide nanoparticles on the behaviour, physiology and reproduction of *Daphnia magna* and *Danio rerio* larvae

DISSERTATION

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Author contributions

The experiments described in this thesis were performed by myself and with the help of students from the University of Siegen and the University of Manchester. Kirsten Prenzel and Katharina Müller from the University of Siegen helped to collect the data of Chapter 3, whereby Mrs. Prenzel performed the study with silver nanoparticles and Mrs. Müller those with titanium dioxide nanoparticles. Rebecca Louch from the University of Manchester helped to collect the data of the multi-generational study with silver nanoparticles of Chapter 5. In Chapter 6, Anna Beasley from the University of Manchester helped to collect the data. Finally, the data of the 120 hpf zebrafish larvae of Chapter 7 and of the behaviour study with wastewater-borne AgNPs and zebrafish larvae (Chapter 8) were collected by Anna-Katharina Rauschert from the University of Siegen. All students from the University of Siegen have completed their Master's Thesis under my supervision. The two students for the University of Manchester were Bachelor students and have done their scientific internship in the group of Prof. Dr. Witte under my supervision. The ERASMS+ program of the European Commission funded the exchange. The names of the students are given at the beginning of the respective chapter. I performed the experiments with silver and titanium dioxide nanoparticles of Chapter 5, with 96 hpf zebrafish larvae of Chapter 7 and collected the data of Chapter 8 with pristine AgNPs. For all Chapters, I was responsible for the study design, data collection, handling and visualization of the data as well as the statistical analysis. All students were introduced by myself to laboratory good practice under scientific aspects, in culturing of the test animals, in performing of the experiments and documentation of the results and the test procedure. The project members from the University of Aveiro (Portugal) and the University of Siegen performed the biochemical and analytical parts of this thesis. Victor Galhano and Isabel Lopes from the Department of Biology & Centre for Environmental and Marine Studies (CESAM) from the University of Aveiro did the biochemical analysis of Chapter 3. Dr. Darya Mozhayeva and Prof. Dr. Carsten Engelhard from the Institute for Analytical Chemistry from the University of Siegen provided all the data for SP-ICP-MS and performed the analysis of total silver and titanium content. Benedikt Steinhoff and Prof. Dr. Schönherr from the Institute of Physical Chemistry I from the University of Siegen were responsible for the characterisation of the silver and titanium dioxide nanoparticles. The respective biochemical and analytical parts from the Material and Methods sections of the chapters were wrote by the responsible persons, which are included in the author list of the respective study/chapter. I wrote all other parts.

The results of the experiments were presented in the form of poster presentation and talks on various national and international conferences. For the SETAC Europe Annual Meeting 2018, which took place in Rome, Italy, I received a travel award from the SETAC organisation and for the SETAC Europe Annual Meeting 2019 in Helsinki, Finland one form the DAAD. The Chapter 5 and 7 have been published in two peer-reviewed journals, which are named at the beginning of the chapters and marked italic within the chapters. Some parts of Chapter 3 and 6 were submitted for publication and further manuscript are in preparation.

Chapter 1

General Introduction

1.1 Background

Since the end of the 18th century, the industrial revolution has led to enormous technical, health and economic improvements for the world population. However, technological progress is interfering with global cycles that could lead to negative changes in the environment (Fent, 2013; Parry et al., 2007; Walker et al., 2016). The major environmental problems of our time include climate change, the worldwide extinction of species, destruction of habitats and the ozone layer, and the introduction of chemicals into the environment (Fent, 2013; Parry et al., 2007). Studying the fate and effects of anthropogenic chemicals and nanomaterials on the living environment is the major goal of the scientific discipline of Ecotoxicology. In this field of research, the toxic effects of a substance are considered at all biological levels, from the molecular level to the whole ecosystem. A distinction between direct and indirect effects on organisms and populations are useful in order to take ecological interactions within an ecosystem into account (Fent, 2013). The ecotoxicological potential of a substance depends on its physico-chemical properties, bioavailability, exposure concentration and exposure time (Fent, 2013). Unpredictable chemical accidents, e.g. like the Sandoz catastrophe in 1986 where large quantities of chemical substances flowed into the river Rhine (Giger, 2009), lead to dramatic acute effects. However, most of the chemicals in the environment are present at low concentrations over an extended period wherefore the chronic effects towards organisms and populations are of much higher relevance for the ecotoxicological assessment of a substance (Fent, 2013).

In recent decades, pollution of the aquatic environment has risen to new levels (Borcherding, 2006). One reason for this being the increasing variety and amount of produced chemicals, such as organic and radioactive substances, heavy metals and nanomaterials. These are released into the aquatic environment through anthropogenic sources such as agriculture, industry, household and/or traffic (Kaegi et al., 2011; Fent, 2013). Due to the increasing environmental awareness, laws and regulations have been made in the western countries to protect the environment sustainably and thus reduce the pollution of ecosystems (Fent, 2013). In 2007, the REACH (Registration, Evaluation, Authorisation and Restriction of Chemicals) regulation came into force in the European Union (ECHA, 2019) for a better and uniform risk assessment of chemicals to improve the protection of human health and the environment (ECHA, 2019).

1.1.1 Introduction and definition of Nanomaterials

The word “nano” is deduced from the greek word νᾶνος (nános), meaning “dwarf” (Delay, 2015) and indicates 10^{-9}nm, the scale of 1 billionth of a meter (Frimmel and Delay, 2010). Nanomaterial (NM) is defined as “material having one or more external dimensions in the nanoscale or which is nanostructured” (British Standards Institution (BSI), 2007). Besides NMs with two dimensions like nanofiber, nanorod, nanoplate or nanotubes, nanoparticles (NPs) are defined from the American Society of Testing and Materials (ASTM) as “ultrafine particles with lengths in two or three dimensions greater than 1 nm and smaller than about 100 nm” (ASTM, 2012; Frimmel and Delay, 2010).

Nanoparticles occur naturally in the atmosphere, in soils as well as in water systems (Delay, 2015). There are various natural sources of NPs, for example volcanic dust after a volcanic eruption, weathering of rocks or forest fires (Delay, 2015). However, besides the naturally produced NPs, manmade NPs also known as engineered nanoparticles (ENP), have become an important industrial product over the last few decades. The field of nanoscience and nanotechnology is booming and ENP are used in food production, farming, biology, industry and medicine (Frimmel and Delay, 2010). The National Nanotechnology Initiative (NNI) defines Nanotechnology as “Science, engineering, and technology conducted at the nanoscale, which is about 1 to 100 nanometres”. The small scale of NPs leads to different chemical and physical characteristics compared to the same material in its bulk form. Hence, NPs are of high interest for the above-mentioned application fields, due to their small particle size and the resulting high mass-specific surface area (Delay, 2015; Frimmel and Delay, 2010). The larger area per unit mass of NPs leads to an increase in the reactivity of the material with decreasing particle size, since more of the area (surface) is directly exposed to the environment and more sites for reaction per unit mass are available (Delay, 2015; Rosenkranz, 2010). ENP consist of a core and one or more shells. The core material is responsible for the field of application and can be metallic, like silver (Ag), gold (Au) or iron (Fe); or a metal oxide, as aluminium (Al), silicon (Si), titanium (Ti) or zinc (Zn); or consists of a carbon-based core like carbon nanotubes (CNT), fullerene (C_{60}) or black carbon (Delay, 2015). The difference in the fate between nanoscale and bulk material could be shown very well on the two metallic NPs silver and gold. With a particle size below 100 nm, the colour of these two materials changes to brown-yellowish (silver) and reddish (gold) (Delay, 2015).

Based on the high amount of different core materials, the application area is very wide-ranging. The European Commission estimated the global market for nanomaterials to 11.5 million tons per year and a volume of € 2 trillion in 2015 (European Commission, 2012). For the European Union (EU) the most produced ENPs are titanium-dioxide nanoparticles (TiO_2NPs) with a production volume of 10.000 t/a, followed by zinc oxide NPs with 1600 t/a, CNT with 380 t/a, AgNPs with 30 t/a and fullerene with 20 t/a (Sun et al., 2014). The application area of NPs can be divided into four main fields: food agriculture, industrial, biomedical and environmental industries (Salata, 2004; Zaman et al., 2014). They are used within the food agriculture industry for improving texture, reducing pesticides, food packaging or enhanced nutrient delivery. The environmental area applies NPs as UV protection in cosmetic products, for wastewater treatment or as pollutant scavengers e.g. in water systems (Zaman et al., 2014). In the biomedical area, they are used for cancer therapy and drug delivery, as well as for imaging and are applied in medical products due to antibacterial properties of some NPs. In the industry sector, NPs are used for catalysts, nanopigments like paints, reinforced plastics or tyres (Delay, 2015; Zaman et al., 2014). Because of the numerous applications especially in consumer products, most of the NPs end up in the aquatic environment through the effluent of sewage treatment plants (STPs) (Gottschalk and Nowack, 2011). Common STPs are not able to easily filter out NPs, based on their special properties and having a small size below 100 nm. NPs therefore enter freshwater systems and could lead to harmful effects to aquatic organisms and the environment (Benn and Westerhoff, 2008; Gottschalk and Nowack, 2011).

1.1.1.1 **Silver nanoparticles (AgNP)**

Silver nanoparticles (AgNPs) have been used in many applications due to their known antibacterial, antifungal, antiviral, antiprotozoal and anticancerous activities (Kalantzi et al., 2019; Rai et al., 2014). The Consumer Product Inventory (CPI, 2019) has listed 1814 commercially available consumer products that contain NPs, of which 438 include AgNPs (Kalantzi et al., 2019; Vance et al., 2015). Examples include food containers, sports clothing or nano-washing machines being impregnated with AgNPs in order to reduce bacterial growth and odour (Benn and Westerhoff, 2008). Medical products, such as wound dressings, sanitation devices or bandages also contain AgNPs (Benn and Westerhoff, 2008). Thus, AgNPs are the most widely used nanomaterial in consumer goods (Vance et al., 2015) and have additionally been used as a pesticide in the USA

since the early 50´s (EPA, 1993). Therefore, AgNPs have drawn the attention of scientists and hence are one of the most studied NPs concerning their toxicity to bacteria, algae and aquatic invertebrates, such as *Daphnia magna* and aquatic vertebrates. The mode of action (MoA) concerning the toxicity of AgNPs is not fully understood (Völker et al., 2013) but could be explained by two major mechanisms: increasing the level of reactive oxygen species (ROS) in cells (Carlson et al., 2008; Völker et al., 2013) and the release of ionic silver (Ag^+) from the surface of NPs (Völker et al., 2013; Yang et al., 2012). Although a low level of ROS is necessary for some cellular function, any significant increase damages cell membranes, proteins and DNA and is therefore highly detrimental for the affected organism (Guo et al., 2019; Völker et al., 2013) which could result in apoptosis of the affected cells (Carlson et al., 2008). However, Ag^+ is one of the most toxic compounds for freshwater organisms like the aquatic invertebrate *D. magna* (Bianchini et al., 2002; Ratte, 1999) since it has the same binding structure as Na^+ and K^+ (Bianchini and Wood, 2002). The competitive mimicry from Ag^+ can inhibit the Na^+/K^+/ATPase transport system which leads to failing ion regulation (Bianchini and Wood, 2002; Guo et al., 2019). Various publications have shown that the toxicity of AgNPs is much lower compared to Ag^+ (reviewed by Kalantzi et al. 2019) and that the observed toxic effects were due to the release of Ag^+ (Bundschuh et al., 2016; *Hartmann et al., 2019*; Kühr et al., 2018; Miao et al., 2010; Navarro et al., 2008; Ribeiro et al., 2015; Zhao and Wang, 2011).

1.1.1.2 **Titanium dioxide nanoparticles (TiO_2NP)**

The metal oxide titanium dioxide (TiO_2) is naturally occurring in mineral in the earth´s crust as three different crystalline forms, anatase, rutile and brookite (Menard et al., 2011; Spengler, 2018). The crystalline form rutile is the most frequent form of TiO_2 in nature (EPA, 2010). In comparison to the bulk sized TiO_2, TiO_2NPs have a high particle number per mass unit and the fraction of atoms at the surface is much larger, leading to an increased chemical reactivity (Buzea et al., 2007; Spengler, 2018). Based on these properties, TiO_2NPs are one of the most studied metal oxide nanoparticles because it was the first nanomaterial which was commercially available for research purposes (Cattaneo et al., 2009; Kahru and Dubourguier, 2010; Menard et al., 2011). The anatase phase is mostly used in catalysis and photocatalysis application due to the high photocatalytic activity, whereby the rutile phase is popular for its UV-light absorbing properties as well as a whitener to require a high opacity (Menard et al., 2011; Mueller and Nowack, 2008). The

third crystalline phase, brookite, has a low stability and is therefore not of high interest for the industry and rarely used (Hadjiivanov and Klissurski, 1996; Spengler, 2018). In general, the application area for TiO_2NPs is large and covers environmental remediation, consumer products like hair styling devices, household self-cleaning devices, air filtration devices, electronics, cosmetics like toothpaste or sunscreen, as well as coatings, paints, inks, plastics and UV-protective clothing (CPI, 2019; Menard et al., 2011; Robichaud et al., 2009; Spengler, 2018).

So far, the precise MoA of TiO_2NPs is not fully understood (Griffitt et al., 2008) although oxidative stress has been identified as the major source of TiO_2NPs-mediated toxicity (Bundschuh et al., 2016; Hou et al., 2019). Based on the characteristics of TiO_2NPs, being photoinducible and redox active, they act as a generator for ROS species at its surface, leading to physiological effects like oxidative stress and apoptosis (Bundschuh et al., 2016; Hou et al., 2019; Menard et al., 2011). Oxidative stress induces genotoxicity (Kohen and Nyska, 2002), while damaging cell membranes, lipids, proteins, DNA and the metabolic activity of organisms (Bundschuh et al., 2016; Hou et al., 2019). Especially for aquatic organisms like algae, zooplankton and fish, the mechanical damage and the sorption of TiO_2NPs onto their surface are important mechanisms causing toxicity of TiO_2NPs (Bundschuh et al., 2016; Dabrunz et al., 2011). It has been shown that the adsorption of TiO_2NPs to cells of the algae *Chlorella vulgaris* affected the food uptake of secondary consumers (Bundschuh et al., 2016; Campos et al., 2013). In *D. magna,* moulting is inhibited, resulting in increased mortality, reduced filtering efficiency and decreased swimming speed (Noss et al., 2013a). Furthermore, due to the high accumulation rate of TiO_2NPs in the gut of *D. magna* and an incomplete depuration, the food ingestion and filtration rate is significantly reduced, affecting important life cycle parameters of a key species in the aquatic environment (Zhu et al., 2010). The locomotion behaviour of fish, like the rainbow trout (*Oncorhynchus mykiss*), was significantly reduced by the sorption of TiO_2NPs to the gills of the individuals, causing hypoxia in the blood and in the spleen (Boyle et al., 2013).

1.1.2 State of the Art

In the last decade, the fate and effects of ENPs on aquatic organisms have been investigated on a large scale. However, the focus of research was on the toxicity of the pristine (as provided by the manufacture) NPs, although it is known that AgNPs and

TiO_2NPs undergo several transformation processes before they end up in the aquatic environment. The transformation of NPs during the clarification of a STP could lead to differences in the toxicity of pristine and the so-called wastewater-borne (transformed) NPs.

The main sources for NP contamination of freshwater ecosystems are sewage sludge and effluents of STP (Gottschalk and Nowack, 2011). Most of the NPs end up in sewage sludge while they pass the STP, which is used as fertilizer in agriculture (Fytili and Zabaniotou, 2008; Gottschalk and Nowack, 2011; Kaegi et al., 2011). However, a significant amount of NPs present in the effluent of the STP reaches the aquatic environment (Kaegi et al., 2011) and could lead to harmful effects for organisms, therefore influencing the whole ecosystem. Several studies have shown that consumer products containing nanomaterials are the source of the pathway of NPs into the aquatic environment. Benn and Westerhoff. (2008) and Farkas et al. (2011) demonstrated that socks with antibacterial fibres and the use of a silver releasing washing machine in the household lead to leaching of silver particles into the environment (Benn and Westerhoff, 2008; Farkas et al., 2011). Furthermore, Kaegi et al. (2010) used analytical electron microscopy to show that natural weather conditions resulted in a detachment of TiO_2 particles from facade paints which were subsequently discharged into natural water systems (Kaegi et al., 2010). Adam et al. (2018) calculated that 53 % of the AgNPs in the effluent of a STP are present in a transformed form (mostly Ag_2S), 22 % of the AgNPs are dissolved and only 18 % of the AgNPs are released as pristine particles. In contrast, up to 97 % of TiO_2NPs are still released to the aquatic environment in the pristine form and only 3 % are matrix embedded (Adam et al., 2018). Based on the wide range of application of the products containing AgNPs and TiO_2NPs, high concentrations in STP influents and effluents are expected (Nowack et al., 2012). The predicted environmental concentrations (PECs) in surface waters for AgNPs and TiO_2NPs were calculated as 0.088 to 10.000 ng/L and 0.021 to 10 µg/L, respectively (Gottschalk et al., 2009; Maurer-Jones et al., 2013) and in STP effluent even higher with 0.0164 – 17 µg/L for AgNPs and 1- 100 µg/L for TiO_2NPs (Maurer-Jones et al., 2013). The findings of a recent study from a lake in Austria receiving purified wastewater form a STP indicate the accumulation of silver in waterbodies (Vogt et al., 2019). Here, concentrations of AgNPs were below the limit of quantification (≤ 0.09 µg/l)

in water but high silver concentrations of 2.27 and 2.68 µg/g were detected in the sludge of the lake.

As mentioned before, AgNPs undergo several transformation processes prior to their release into the aquatic environment (Adam et al., 2018; *Hartmann et al., 2019*; Nowack et al., 2012). The major transformation process of AgNPs is the sulfidation into Ag_2S while passing the STP (Georgantzopoulou et al., 2018; *Hartmann et al., 2019*; Kaegi et al., 2011; Levard et al., 2012). The low water solubility and the reduced formation of Ag^+ from Ag_2S might lead to a reduced toxicity to aquatic organisms (Bianchini et al., 2002; *Hartmann et al., 2019*; Kaegi et al., 2011; Levard et al., 2012; Ratte, 1999). So far, only few studies focused on the ecotoxicity of wastewater-borne (transformed) nanoparticles to aquatic organisms (Bruneau et al., 2016; Georgantzopoulou et al., 2018; *Hartmann et al., 2019*; Kühr et al., 2018), although the exposure pathway of nanoparticles to aquatic organisms is mainly due to wastewater effluent (Gottschalk and Nowack, 2011). Muth-Köhne et al. (2013) reported that the toxicity of AgNPs to zebrafish embryos even increased after NPs had passed through a model STP. The estimated 48 h-EC_{50} values of zebrafish embryos malformation were approximately 8 times higher for wastewater-borne AgNPs compared to pristine AgNPs with values of 142 µg Ag/L and 1.09 mg Ag/L, respectively (Muth-Köhne et al., 2013). In contrast, the acute, long-term and the chronic exposure to *Daphnia magna* and to the freshwater amphipod *Hyalella azteca,* with wastewater effluent containing AgNPs showed a reduced toxicity compared to pristine AgNPs (Georgantzopoulou et al., 2018; *Hartmann et al., 2019*; Kühr et al., 2018). For example, long-term exposure with pristine AgNPs at environmentally relevant concentrations in up to six generations of *D. magna* led to a significant reducing reproductive success, while wastewater-borne AgNPs did not affect the offspring rate *(Hartmann et al., 2019).*

The only known transformation of TiO_2NPs happens during incineration where material type is essential for the fate (Adam et al., 2018). In paint debris containing TiO_2NPs, large quantities of calcium are available, while during incineration the TiO_2NPs get transformed into calcium titanate which is present in the ash (Massari et al., 2014). In contrast, no transformation occurs during the incineration of polymers including TiO_2NPs, since the characterisation of the ash show pure TiO_2NPs (Singh et al., 2016). This is also the case by analysing the effluent of a lab-scale STP, where the inlet was spiked with TiO_2NPs, where TiO_2NPs are present as polycrystalline aggregates (Georgantzopoulou et al., 2018;

Hartmann et al., 2019). Georgantzopoulou et al. (2018), however showed that acute exposure with effluent containing TiO_2NPs (concentration range of 0.01 – 10 mg/L) did not have any effect on the mortality of *D. magna*. The chronic exposure over six generations with environmentally relevant concentrations of wastewater-borne TiO_2NPs did not lead to any negative effects on *D. magna* either (*Hartmann et al., 2019).*

Besides transformation process, environmental properties and factors like dissolved organic matter (DOM), pH value, common ligands or UV irradiation could affect the toxicity and the behaviour of NPs (Guo et al., 2019; Ratte, 1999). DOM like humic acids reduce the toxicity of AgNPs, due to the binding of Ag^+, affecting the AgNPs aggregation and the adsorption to the surface of AgNPs (Cedervall et al., 2012; Guo et al., 2019; Kühr et al., 2018). The pH value can strongly influence the stability of AgNPs and the release of Ag^+ (Guo et al., 2019). The combination of a pH value of 8 and 8 mg DOM reduced the toxicity of AgNPs to *D. magna* (Seitz et al., 2015), since a higher pH enhanced the transformation of Ag^+ to AgNPs (Adegboyega et al., 2014). Furthermore, common ligands like phosphate, cysteine, chloride and sulphides, which are naturally present in the environment, could reduce the toxicity of AgNPs through the formation of complexes with Ag^+ (Guo et al., 2019; Xiu et al., 2011). Especially for TiO_2NPs, the presence of UV light is essential for the induced toxicity, since UV light acts as a trigger for the photocatalytic properties of TiO_2NPs due to the formation of ROS (Bhatkhande et al., 2002) which leads to changes of the ecotoxicological potential even under natural UV intensities (Bar-Ilan et al., 2013; Marcone et al., 2012).

Due to the complex fate and the associated toxicity of NPs, ecotoxicological studies under realistic exposure scenarios in both the aquatic and terrestric environments are essential for a reliable environmental risk assessment. So far, there is still a gap in knowledge due to limited and contradictory results of the ecotoxicity of wastewater-borne NPs to aquatic organisms.

1.1.3 Project FENOMENO

The multidisciplinary project FENOMENO (Fate and effect of wastewater-borne manufactured nanomaterials in aquatic ecosystems) aims to understand the fate and effect of wastewater-borne manufactured nanomaterials (MNMs) to organisms in the aquatic environment. The project focused on Ag- and TiO_2NPs as test substances due to

the high application volume in commercially available products and the related high input into STPs (described in Chapter 1.1.1). However, MNMs as Ag- and TiO_2NPs typically undergo several transformation processes before they end up in the aquatic environment and are not found in their pristine form (Figure 1-1). Since there is still a gap of knowledge concerning fate and impact of MNMs after passing a STP, the project FENOMENO focused on (i) the improvement of analytical methodology for the quantification and characterisation of low environmental concentrations of NPs in the effluent from a model STP (lab studies) and in real-world samples and further, (ii) the investigation of the ecotoxicological effects of wastewater-borne NPs in a 3-step model food web, including key species like algae, *Daphnia* and fish.

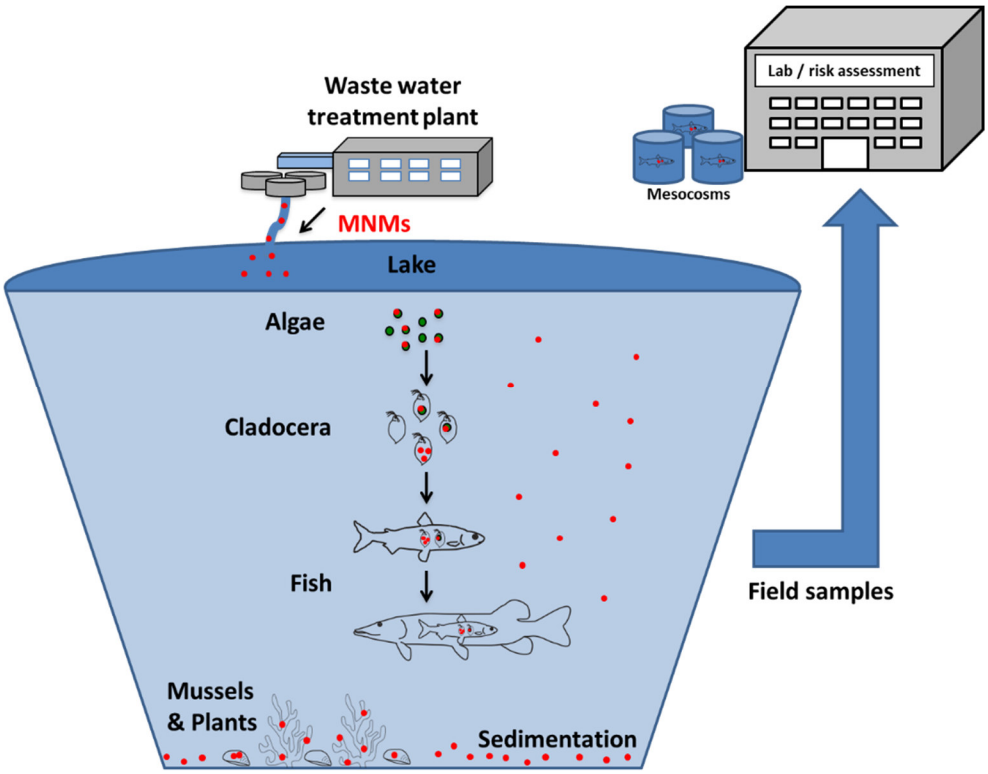


Figure 1-1: Graphical illustration of the project FENOMENO.

The pristine and wastewater-borne Ag- and TiO_2NPs have been quantitatively characterized by determining the NP shape and size as well as the surface functionalization in the presence of the wastewater matrix including novel microscopy approaches and analytical techniques. The uptake and chronic effects of wastewater-borne NPs were investigated on different toxicological endpoints in laboratory studies with the mentioned key species of the aquatic food web. Moreover, bioaccumulation and

bioconcentration of MNMs in the 3-step food web were compared to field samples collected from the lake Mondsee in Austria, where a municipal STP discharged its effluents and from the reference lake Irrsee (Austria). Hence, FENOMENO closed the knowledge gap between lab studies on the one hand and field studies, which reflects the real situation in aquatic ecosystems, on the other. In addition, an early warning system for NP-contaminated water matrices is established, with the detection of behavioural changes in *Daphnia* and fish by using an intelligent tracking-system and the analysis of biochemical markers. Hence, the overall aim of the project FENOMENO was to provide a reliable risk assessment for MNPs use and its safety management for humans and the environment.

1.2 Outline

Despite the huge number of afore-mentioned studies and publications related to the ecotoxicity of AgNPs and TiO_2NPs to aquatic organisms and the aquatic environment, there are still many unanswered questions and hence, many missing approaches in this field of research. My PhD project aims to contribute to an improved understanding of the ecotoxicity of AgNPs and TiO_2NPs to the aquatic organisms *Daphnia magna* and *Danio rerio* by using a more environmentally realistic experimental approach regarding exposure concentrations, transformation process and natural conditions. For instance, although only transformed wastewater-borne NPs are present in a natural aquatic environment, their effects and impacts on common freshwater species such as *D. magna* and *D. rerio* are nearly unknown. Therefore, I aim to elucidate the toxicity and mechanism of transformed NPs to develop a reliable and realistic risk assessment for NPs to protect our environment. Furthermore, besides the environmental research of the ecotoxicity of wastewater-borne AgNPs and TiO_2NPs to an aquatic invertebrate and a vertebrate, this work will provide new insights into the visual perception of light in zebrafish larvae, as an important model species in neuroscience, biology and ecotoxicology. My results and the subsequent discussion will make a valuable contribution to environmental research and protection, striving to open up new scientific issues and perspectives.

First**,** I describe the two model species *D. magna* and *D. rerio* in detail, including their natural way of life and requirements, breeding conditions and handling procedure at the University of Siegen in **Chapter 2**. To quantify potential changes in behaviour of aquatic organisms when being exposed to pristine and wastewater-borne AgNPs and TiO_2NPs, I

used *D. magna* to design and implement a new evaluation method, which I will describe in detail in **Chapter 3** and **Chapter 4**. This third chapter intends to answer the following question: Are there any differences in the response pattern for the tested NPs? Is *D. magna* suitable as a biosensor for NP contamination events? In the fourth chapter I aimed to develop new behavioural-related endpoints and discuss the suitability of those to be used in biological early warning systems (BEWS). **Chapter 5** describes a multigenerational study to investigate the effects of exposure with environmentally relevant concentrations of pristine and wastewater-borne AgNPs and TiO_2NPs to *D. magna.* I investigated three key life cycle parameters (reproduction, body length and extinction rate) over six generations. Unlike single-generation studies, my multigenerational study allows drawing a far more realistic and, therefore, reliable picture of the population structure under NP-influenced conditions of *D. magna* in the aquatic environment. This fifth chapter intends to answer the following question(s): Do wastewater-borne AgNPs and TiO_2NPs influence key life cycle parameters over multiple generations? Does the juvenile *D. magna* of the next generation show transgenerational effects? Subsequently, I analyse the kairomone induced anti-predator defence mechanism in *D. magna* under the impact of pristine AgNPs and TiO_2NPs in **Chapter 6**. This chapter intends to answer the key question: Are *Daphnia* and their offspring able to develop this ecologically important trait under the influence of NP exposure? To investigate the impact of NP exposure on certain behavioural traits, not only of aquatic invertebrates such as *D. magna*, but also of an aquatic vertebrate, the visual perception of *D. rerio* larvae towards near-infrared light (NIR) is analysed. Hence, as a pre-study for using zebrafish larvae in my following ecotoxicological study I aimed to answer the following question in **Chapter 7**: Do zebrafish larvae show a phototactic response towards NIR light with wavelength of 860 nm and 960 nm? In addition, I intended to investigate the general impact of pristine and wastewater-borne AgNPs on the locomotion behaviour of *D. rerio* larvae in **Chapter 8**. More specifically, I examined potential differences in the response pattern towards the two tested AgNPs of *D. rerio* larvae. Finally, I summarise and discuss my results in a broader context in **Chapter 9.**

Chapter 2

Study species

2.1 The water flea *Daphnia magna*

2.1.1 Systematic and Morphology

The water flea *Daphnia magna* Straus, 1820, which I used as test species belongs to the subphylum Crustacea, class Branchiopoda, subclass Diplostraca, infraclass Cladoceromorpha, superorder Cladocera, Order Anomopoda, Family Daphniidae and Genus *Daphnia* O.F. Muller, 1785 after the taxonomic hierarchy of *Daphnia magna* Straus which is reported by the World Register Of Marine Species (WoRMS, 2019).

The anatomy and morphology of the freshwater crustacean *D. magna* (female) is shown in Figure 2-1. *Daphnia* are characterized by an exoskeleton, called carapace, an uncalcified double lobed shell, made of chitin, which covers the body (Smirnov, 2017; Storch and Welsch, 2014). The carapace is often transparent, that makes *Daphnia* to a good model species for a variety of different research areas like e.g. ecology, physiology and ecotoxicology. The body length of female *Daphnia* ranges from 0.5 mm to 6 mm, depending on age and species. *Daphnia* possess two antennae which are located on the head: the first one is much smaller than the second one and is attached to the rostrum and serves as a sensory organ for chemoreception (Storch and Welsch, 2014). The second antennae, one on each side near the middle of the head, are large compared to their body size and serve as an organ for catching food and allowing movements (Ebert, 2005). By the vertical movement of the second antenna *Daphnia* perform the characteristic hops why they are often called water fleas. The head region consists of the compound eye that is enclosed by six eye muscles, the brain and the naupilus eye (integrated in the edge of the brain) which is responsible for the perception of light (Weiss et al., 2012). The mandibles are well developed and directly located at the gut opening, with the function of mechanical food processing. The mouth is located between the two mandibles (Fox, 2009). *Daphnia* are filter-feeding organisms. The filtering apparatus, located inside the thorax, consists of flattened leaf-like legs (phylopods) that produce a permanent water current by moving up- and downwards, in which small, suspended food particles, mostly planktonic algae, are gathered and consumed (Ebert, 2005). Female *Daphnia* possess a paired ovary and a brood pouch. The parthenogenic eggs are extruded from the oviducts and grow into juvenile *Daphnia* (Fox, 2009). *Daphnia* have a posterior apical spine, which may act as a defence mechanism. The abdomen consists of the anus, excretion organ, the abdominal

seatae and a pair of abdominal claws. They serve as a defence mechanism and are responsible for cleaning the filter feeding apparatus by removing algae blockages (Ebert, 2005; Fox, 2009).

Male *Daphnia* differ from females by a smaller body size (2 mm), a shortened more rounded rostrum (Mitchell, 2001), a more developed first antenna which is movable and bear a spine (Ebert, 2005, Mitchell, 2001), modified abdomen and first legs which are equipped with a short chitinised hook used in clasping (Ebert, 2005, Mitchell, 2001).

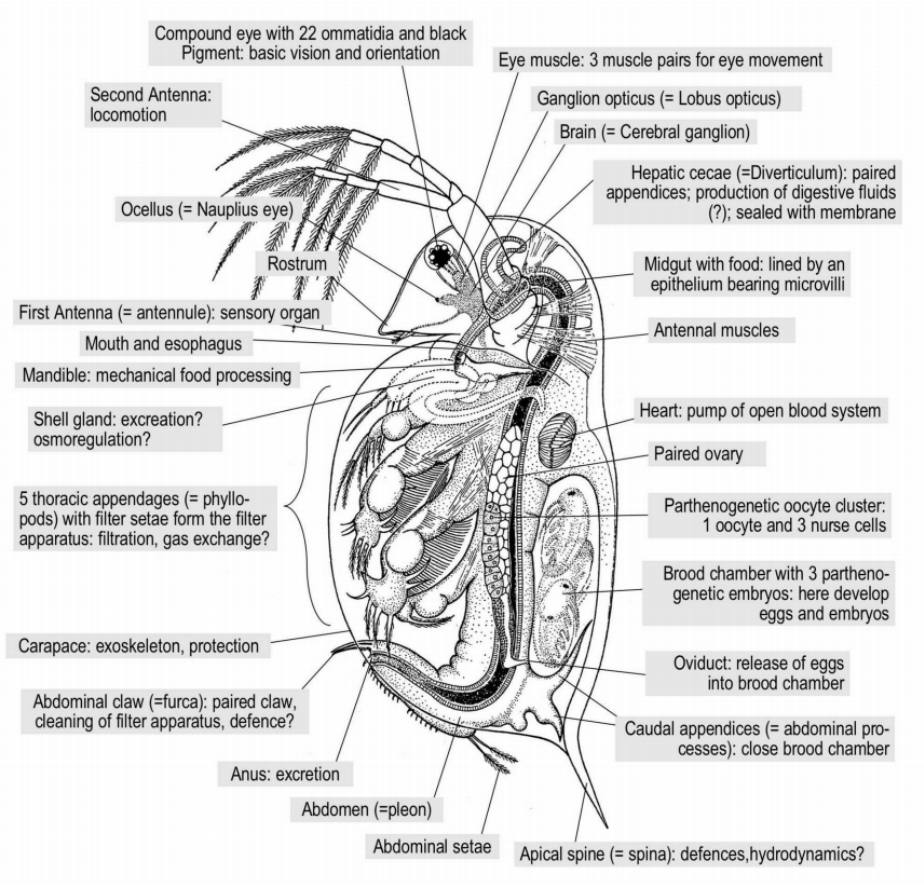


Figure 2-1: The functional anatomy and morphology of female *Daphnia* (from Ebert, 2005).

2.1.2 Life cycle

The life cycle of *Daphnia* is characterized by an asexual (parthenogenetic) and a sexual reproduction phase. Under environmentally good conditions, which are present during the growth season in spring and summer, the parthenogenetic mode of reproduction is dominating (Figure 2-2) (Ebert, 2005). At this time, females compose the natural population of *Daphnia*. They parthenogenetic produce a clutch of diploid (2N) eggs in the ovary which are genetically identical to the mother. The eggs are kept in the brood

chamber of adult *Daphnia,* where they hatch after one day and stay there for two more days for further development (Ebert, 2005). After about three days (20°C), the development is completed and the juvenile *Daphnia* (offspring) are released through ventral flexion of the abdomen (Figure 2-1). After about five to ten days (20 °C) and four to six moulting stages, the offspring themselves become sexually mature and produce pathogenic eggs for the first time. Under excellent laboratory conditions, adult *Daphnia* produce eggs every 3 to 4 days and reach an age of over two months (Ebert, 2005).

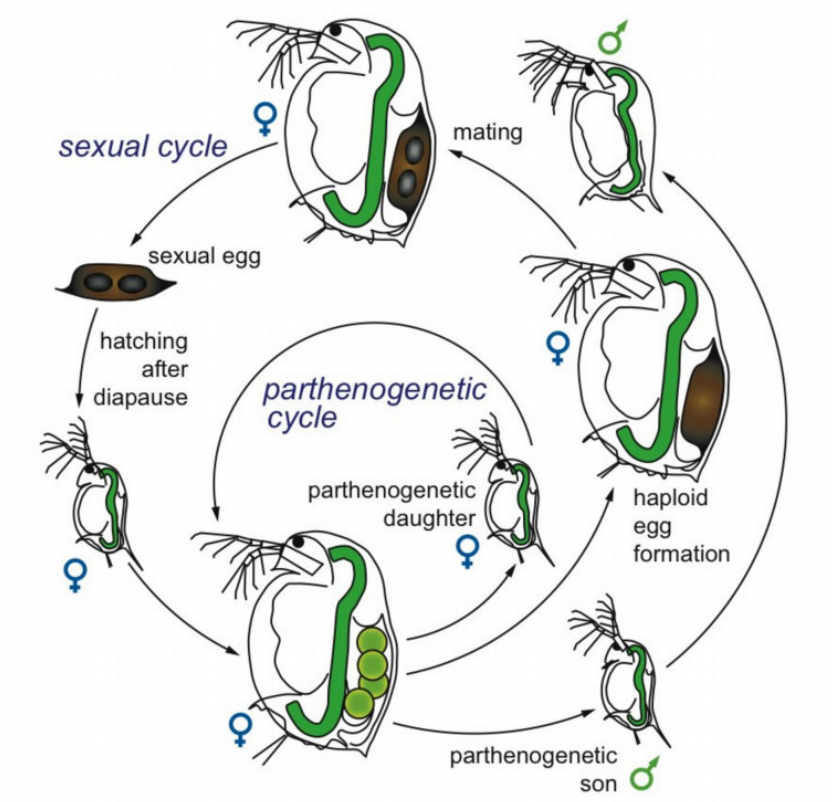


Figure 2-2: Asexual and sexual life cycle of *Daphnia* (from Ebert, 2005).

Environmental triggers, such as reduced food availability, population density or abiotic factors like photoperiod and low temperature, lead to the activation of sexual reproduction (Ebert, 2005; Stross and Hill, 1965). Under such conditions, females begin with the asexual production of diploid (2N) males, which are genetically identical to the mother (Storch and Welsch, 2014). After the release of males, females start to produce, so called resting eggs, two large haploid eggs (1N) which are strongly melanized and protected by a saddlelike structure, the ephippium (Ebert, 2005; Shaw et al., 2008). After copulation haploid resting eggs are internally fertilised in females by haploid sperm (1N) from males (Ebert, 2005; Shaw et al., 2008). Fertilised resting eggs are released into the water by the

next moulting of the mother and sink to the bottom of lakes or disperse with animals or the wind (Ebert, 2005). Once environmental factors shift again to better conditions, e.g. beginning of the next-year spring-time with increasing food availability, females hatch from the resting eggs and start a new generation (Ebert, 2005) and the life cycle begins again. Furthermore, resting eggs can endure long dry periods, a further adaptation to extreme weather events.

2.1.3 Ecological relevance of *Daphnia*

The zooplankton crustacean *Daphnia sp*. can be found all over the world in freshwater and brackish habitats, like lakes and rivers as well as temporary pools. In the aquatic environment, they belong to the trophic level of primary consumers and play a crucial role in the energy transfer from phytoplankton (primary producers) to higher tropic levels (secondary consumers). *Daphnia* are filter-feeding organisms feeding on algae, bacteria and protozoans and were the primary forage for zooplanktivorous vertebrates and invertebrate predators (Shaw et al., 2008). Therefore, *Daphnia* has a high ecological relevance within the aquatic ecosystem and act as an important key species in the food web. *Daphnia* are famous for their diel vertical migration (DVM), a predator avoidance response (Haupt et al., 2009; Lampert, 1989). They spend the daytime in deeper water layers, where it is colder and darker, while at dusk they migrate the water column upwards and spend the night in the upper water layers (Lampert, 1989). This DVM is triggered though the presence of chemical cues (kairomones) released by fish (Loose et al., 1993; Von Elert and Loose, 1996) and through changes in the light intensity around dawn and dusk (Ringelberg, 1991).

2.1.4 In-house breeding

The freshwater cladoceran *Daphnia magna* (clone V, all females) were originally provided by the Federal Environment Agency (Berlin, Germany) and were cultured at Witte lab since November 2013. A detailed plan of the culture room are described in Figure 2-3. The animals were kept in 2 L glass beakers with 1.8 L culture medium and 30 adult female *D. magna* per unit in an air-conditioned room (20 ± 2 °C) with a 16:8 h (light:dark) photoperiod and under continuous aeration (Figure 2-4 A). The glass beakers were placed in a shelving system in the culture room (Figure 2-3) and illuminated with fluorescent lamps (L30W/840 Cool White and L30W/830 Warm White, OSRAM, Munich, Germany). As culture medium

ASTM reconstituted hard freshwater (192 mg/L $NaHCO_3$, 120 mg/L $CaSO_4$*2 H_2O, 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) was used. Water exchange took place once a week and in addition, juveniles were removed three times a week to avoid high density (*Hartmann et al., 2019*). For easier handling during water exchange, *D. magna* were put in a plexiglas cylinder which was placed in the glass beaker (Figure 2-4 A). The *D. magna* culture were fed daily with the green algae *Desmodesmus subspicatus* (Figure 2-4 B).

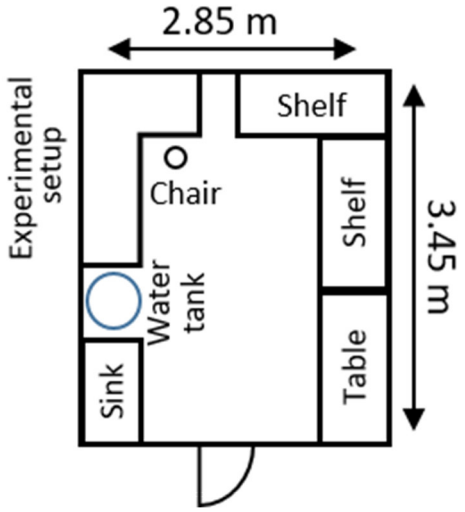


Figure 2-3: Schematic top-view of the culture room of *Daphnia magna*.

Algae were cultured in an air-conditioned room (24 ± 1 °C) with a 16:8 h (light:dark) photoperiod with a defined culture medium based on Bringmann and Kühn (1980) (Bringmann and Kühn, 1980). Fluorescent lamps (L36W/880 Skywhite, OSRAM, Munich, Germany) served as illumination source. The algae suspension was placed on a magnetic stirrer, stirred and aerated continuously and was left to grow for around one week. Before use, the algae stock culture was centrifuged (Centrifuge 5804 R, Eppendorf AG, Hamburg, Germany) and re-suspended in ultra-pure water to provide an appropriate food source.

2.1.5 Model species in ecotoxicology

The zooplankton *Daphnia sp.* is a common model organism for ecotoxicological studies and standard test systems, based on their easy culture conditions in the laboratory and their ecological importance. Based on the ecological relevance of *Daphnia* within the aquatic environment they act as an important key species in the food web. Harmful effects on *Daphnia* could lead to negative effects throughout the entire aquatic ecosystem.

Therefore, *Daphnia* are of high importance for testing the toxicity of pharmaceutical or medicine products, chemicals or microplastic. Laboratory conditions imitate the natural condition for asexual parthenogenic reproduction of *Daphnia*, leading to short reproduction time with a high number of offspring and genetically identical clones and generations. Based on these properties, the species *D. magna* is routinely used in acute and chronic aquatic ecotoxicological studies. International institutions like the European Union, EPA (Environmental Protection Agency of the United States), ISO (International Organization for Standardization) and the OECD (Campos, 2014) officially support ecotoxicological test systems with *D. magna*. The Organisation for Economic Cooperation and Development (OECD) has published various guidelines that use *D. magna* as a test organism to determine the toxicity of anthropogenic manufactured chemicals (Peake et al., 2016). The *Daphnia* immobilization test, OECD no. 202 (OECD, 2004) investigates the short-term (acute) toxicity of a chemical, based on the endpoints lethality, growth and behaviour and the estimation of an EC_{50} (greatest half-maximal effective concentration of a response like immobility) - value. The 21-day reproduction test, OECD no. 211 with *D.magna* (OECD, 2012) analyses the chronic toxicity of a substances by studying the reproductive success of the animals and calculating the LOEC (lowest observed effect concentration) and NOEC (no observed effect concentration) value. The results of such bioassays were used for the environmental risk assessment of all registered chemicals.

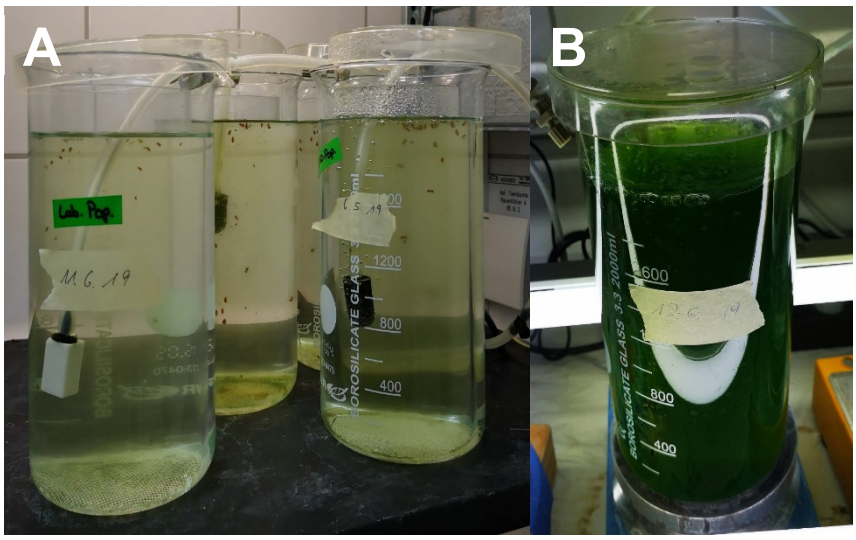


Figure 2-4: Culture of *Daphnia magna* (A) and the green algae *Desmodesmus subspicatus* (B) at Witte lab.

2.2 The zebrafish *Danio rerio*

2.2.1 Systematic and Morphology

The zebrafish is a tropical freshwater fish (Figure 2-5) belonging to the family of minnows (*Cyprinidae*) belongs to subphylum Vertebrata, superclass Gnathostomata, class Actinopterygii, order Cypriniformes, family: Cyprinidae and subfamily: Danioninae Genus: *Danio* Hamilton, 1822 according to the taxonomy classification of the World Register of Marine Species (Froese et al. 2019). The zebrafish *Danio rerio* which I used as model organism is native to South Asia, naturally distributed throughout the river drainages of India, Nepal and Bangladesh (Spence et al., 2008) (Figure 2-6). The zebrafish lives in slow-moving, shallow or stagnant flowing water bodies and is named due to the five uniform, horizontal, blue stripes that run alongside its body reaching to the end of the caudal fin.

Figure 2-5: Male (left) and female (right) adult zebrafish. (Photo taken by S. Hartmann)

They are sexually dimorphic, only after reaching sexual maturity sexes can be easily distinguished from each other. Males have a more slender body shape and an orange tint in the silver parts of the stripes whereas the females have a large whitish belly and a stronger body structure. The mean body length of adult zebrafish ranges from 3 to 5 cm. Zebrafish live in shoals, preferring small schools of 5 – 20 individuals (Harper and Lawrence, 2016). In the natural habitat, zebrafish mainly feed on a wide range of zooplanktonic organisms, insects and consume additionally algae, detritus and other organic material (Harper and Lawrence, 2016; Spence et al., 2006).

2.2.2 In-house breeding

Danio rerio of the wild-type zebrafish strain from West Aquarium GmbH (Bad Lauterberg, Germany) were kept for all experiments in a fish culture room (Figure 2-7) within the Witte lab since February 2016. The fish culture room (Figure 2-7) provided a shelf with home tanks, water tanks, tables and the experimental setup used for the studies. Adult zebrafish

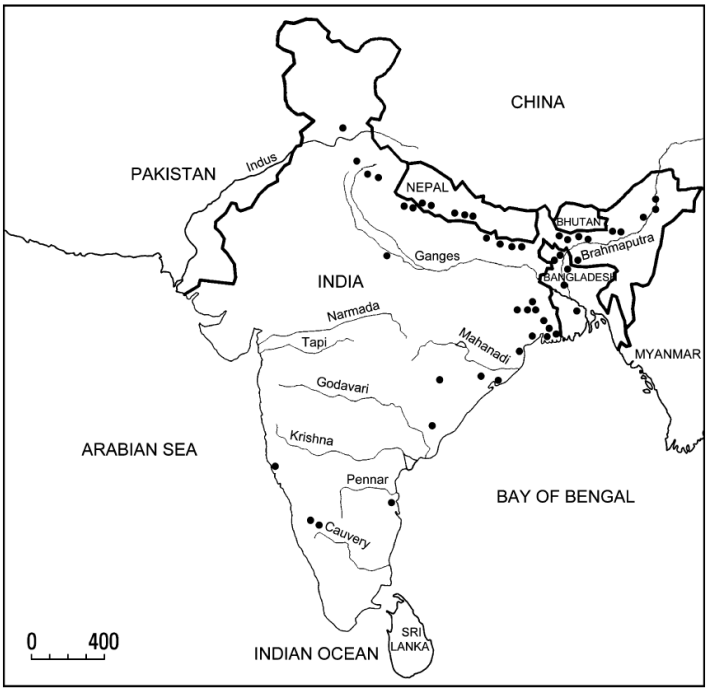


Figure 2-6: Distribution of the zebrafish (*Danio rerio*) in Asia. The black dots indicate recorded occurrences (Map from Spence et al. 2008).

were cultured in 112 L glass tanks (80 x 40 x 35 cm³) in groups of 100 animals with a sex ratio of 50:50 under a light-dark cycle of 14:10 hours. Water had a temperature of 26 ± 1°C, a pH-value of 7-7.5 and a conductivity of 450 µS/cm. Water exchange (40 %) took place once a week and was aerated and filtered continuously. Fluorescent lamp (BIOLUX L 36 W/965, OSRAM, Munich, Germany) serve as illumination and were placed above the home tanks. The animals were fed daily in the morning with dry flake food (JBL GmbH & Co. KG, Germany), and additionally with juvenile *Daphnia magna* to provide a source of environmental enrichment, three times a week in the afternoon (OECD, 2013).

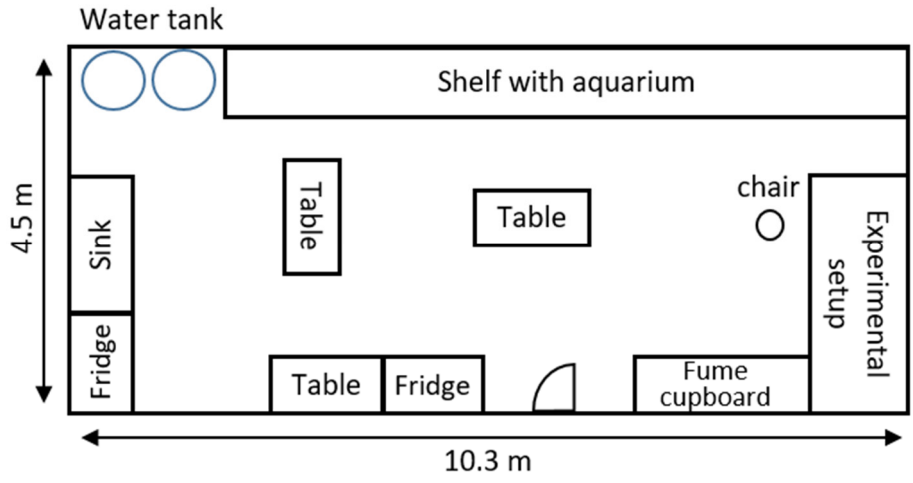


Figure 2-7: Schematic top-view onto the room for keeping fish and the experimental setup.

2.2.2.1 **Collecting eggs**

In the evening a spawning tray (16.8 x 25.7 x 6.2 cm^3) consisting of a flat basin and a metal net with artificial plants (Figure 2-8) was placed in the home tank to stimulate egg laying. Spawning and fertilization takes place in the morning after onset of light. Two hours after the onset of light, the spawning trays were removed, and the collected eggs were rinsed with distilled water and transferred to a petri dish for egg selection. Fertilized and healthy eggs with a cell stage of at least 4 - 8 were selected by using a trinocular stereo-zoom-microscope (hund, Wetzlar, Germany). Further handling information for the experiments are given within the respective chapters.

Figure 2-8: Spawning tray (Photo taken by S. Hartmann)

2.2.3 Zebrafish as a model species in ecotoxicology

In general, fish are the most complex used vertebrate in ecotoxicological studies due to physiological and endocrinological similarities with humans (Peake et al., 2016). The zebrafish is a famous laboratory model species for neurobiology, genetics, molecular and developmental biology as well as in ecology and toxicology (Kimmel et al., 1995; Legradi et al., 2015; Westerfield, 2000; Yang et al., 2009) due to specific characteristics. Zebrafish are cost-effective, small organisms, easily obtainable and easy to culture in the laboratory (Lammer et al., 2009). Furthermore, they grow rapidly and have a short generation time while the maturity is reached at an age of 3 to 4 months. Females provide a large number of eggs with 50 - 300 eggs per day throughout the whole year (Lammer et al., 2009). The eggs are demersal and non-adhesive and protected by a transparent chorion (Harper and Lawrence, 2016). The development of the transparent larvae within the egg and its organs can be easily observed under the microscope (Figure 2-9). The embryos develop and

hatch within 96 hours post fertilization (hpf) and the embryonic development is well described in various publications (Kimmel et al., 1995; Westerfield, 2000). Therefore, any abnormalities triggered by chemical exposure on embryonic or morphological level can be studied and make the zebrafish useful for ecotoxicological research and toxicity testing. Furthermore, in the course of the 3R-principles (Replacement, Reducement, Refinement), research with zebrafish embryos is an approved alternative method (in North Rhine-Westphalia declared as no animal experiment up to 120 hpf) compared to toxicity tests using adult fish, like the acute fish test, OECD no. 203 (OECD, 2019) (Strähle et al., 2012), because it is not restricted by the Animal Protection Law. This resulted in the introduction of the 'OECD Guideline for Testing Chemicals, Test No. 236: Fish Embryo Acute Toxicity (FET) Test' (OECD, 2013) where the acute toxicity of a substance is determined with the calculation of the LC_{50}-value (lethal concentration).

2.2.4 Behaviour assays with *Danio rerio*

A natural behaviour is defined as "the cumulative interaction of a variety of biotic and abiotic factors that represents the animal's response to internal (physiological) and external (environmental, social) factors and relates one organism to another" (Dell'Omo, 2002). Important life cycle characteristics, such as reproduction, predator avoidance or foraging, are controlled and influenced by an undisturbed behaviour and the disturbance of an animal's natural behaviour leads to negative effects on these parameters (Fent, 2013).

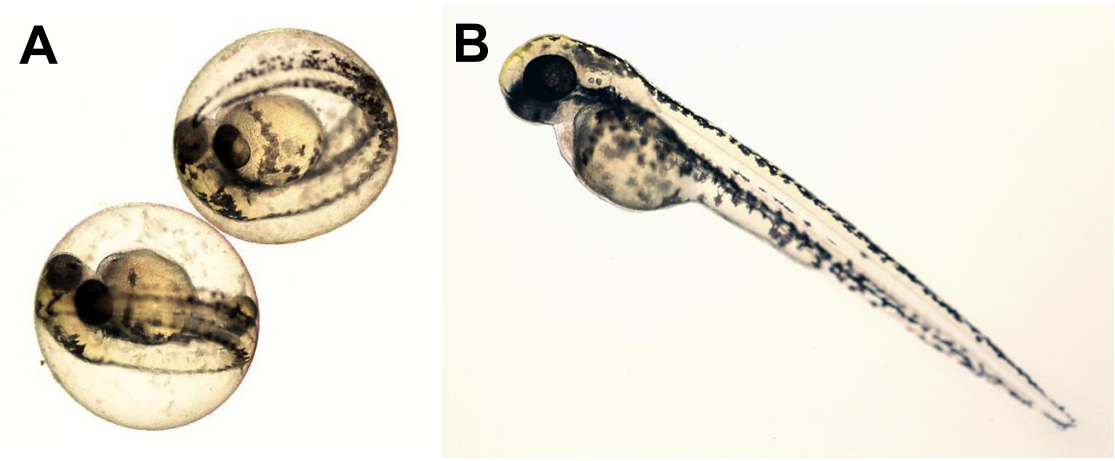


Figure 2-9: Zebrafish larvae within the transparent chorion (A) and hatched larvae (B) (from Zebrafishlab, University of Antwerp)

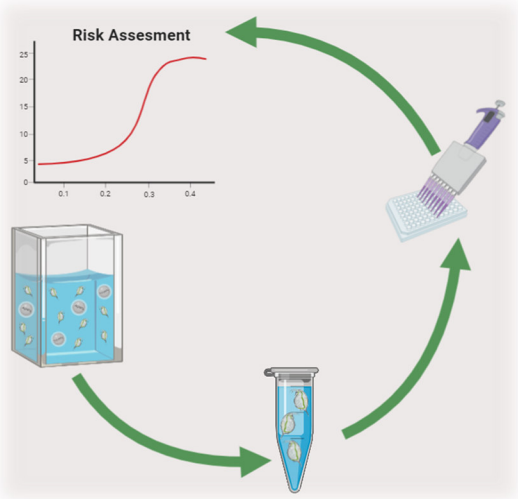
The most detectable response towards the exposure to chemicals or to the contaminated environment is avoidance, however, orientation and migratory movement behaviour are also affected (Fent, 2013). For fish in particular, the thermoregulation, foraging and avoidance behaviour is reliant on an undisturbed behaviour. Behavioural changes triggered by chemical exposure is associated with the reduction of the survival rate of the fish population (Fent, 2013). Changes of the swimming behaviour of fish species show a higher sensitivity compared to the acute toxicity of endpoints like neurotoxicity or lethality and is therefore often used for ecotoxicological studies to evaluate effects of stressors like chemicals to aquatic invertebrates (Fent, 2013; Raley-Susman, 2014; Vignet et al., 2013). Furthermore, behavioural changes act as an early indicator, a so-called biosensor, for toxic effects caused by environmental pollutants (Raley-Susman, 2014).

Over the last years, the organism *Danio rerio* became a popular model species while assessing the behaviour of fish species and seems to be a strong paradigm for research based on many practical advantages (Ahmad et al., 2012; MacPhail et al., 2009; Padilla et al., 2011; Truong et al., 2011). The zebrafish has many qualities that makes it complementary to mammalian models (Ali et al., 2012) and is therefore in the context of the 3R-Principles. The zebrafish genome has remarkable similarity to other high-order vertebrates, especially in signalling processes, anatomy, physiology and cellular structure (Ali et al., 2012; Guo, 2009; Truong et al., 2011). Over 90 % of the human open reading frames (ORF) are similar to genes in fish, which are also involved in behaviour (Ahmad et al., 2012; Kimmel et al., 1995). Besides those characteristics, the favourable life cycle and the good laboratory husbandry conditions with low costs and high-throughput, make the zebrafish reliable as a replacement for higher vertebrate model species and useful for behavioural screens (Ahmad et al., 2012; Ingebretson and Masino, 2013). Zebrafish embryos develop fast and external (outside the brood punch of females) and the embryos show different stages of behaviour during the first few days after the fertilization (Ahmad et al., 2012; Legradi et al., 2015). All major organs are formed within the first day and the first visible response begins at 17 hpf with spontaneous movement of the tail (Ahmad et al., 2012; Legradi et al., 2015). At an age of 24 hpf the different parts of the brain are established and showing a movement behaviour in response to mechanical stimuli demonstrated by rapid tail coils (Ahmad et al., 2012; Legradi et al., 2015). After 27 hpf the behaviour of the embryo becomes more complex by the distinguishment between

independent head and tail movements (Legradi et al., 2015). With 3 dpf (days post fertilisation), the nervous system is fully developed, and the larvae show a startle response, an unconditioned behaviour in response to a visual, tactile or acoustic stimulus (Ahmad et al., 2012; Legradi et al., 2015). At this time point, the embryos hatch into free swimming larvae (Kimmel et al., 1995; Legradi et al., 2015). After 5 - 6 dpf zebrafish larvae have the ability to respond with behavioural changes to light and dark (MacPhail et al., 2009). Hence, monitoring the changes in the behaviour of zebrafish larvae becomes a sensitive method for investigating toxic effects of different compounds using “dark” and light phases (Legradi et al., 2015). For the measurement of behavioural changes systems like DanioVision and ZebraBox, commercially available video tracking systems, proved their effectiveness. Due to the suitable software, changes of the locomotion of the zebrafish larvae can be easily detected, analysed and quantified under specific conditions, like the light/dark transition test of the Photomotor Response test (PMR) (Legradi et al., 2015). Parameters like swimming distance and speed, turning angle, time spent immobile etc. were used as a measurement for the level of locomotor activity of zebrafish larvae (Ahmad et al., 2012).

Chapter 3

Impact of wastewater-borne nanoparticles of silver and titanium dioxide on the swimming behaviour and biochemical markers of *Daphnia magna*: an integrated approach



In this chapter, two different students helped to collect the data. Kirsten Prenzel performed the experiments with AgNPs and Katharina Müller with TiO_2NPs. The project partners Benedikt Steinhoff and Darya Mozhayeva provided the particle characterisation and the analytical data analysed in this chapter and Victor Galhano was responsible for the analysis and discussion of biochemical marker genes. Please note that in this chapter pristine NPs are referred to ASTM-dispersed NPs, unlike in the other chapters.

This chapter has been submitted to the Journal Aquatic Toxicology:

Galhano V, Hartmann S, Monteiro M, Zeumer R, Mozhayeva D, Steinhoff B, Mueller K, Prenzel K, Kunze J, Kuhnert K-D, Schönherr H, Engelhard C, Schlechtriem C, Loureiro S, Soares A, Witte K, Lopes I (Under Review) “Impact of wastewater-borne nanoparticles of silver and titanium dioxide on the swimming behaviour and biochemical markers of *Daphnia magna*: an integrated approach” Aquatic Toxicology

3.1 Abstract

Due to the widespread application of silver (Ag) and titanium dioxide (TiO_2) nanoparticles (NPs), these NPs have been potentially discharged into aquatic environments also via sewage treatment plants (STPs). Even though NPs are mostly found in biosolids, a small fraction is still available in STP effluents and may induce toxic effects to aquatic biota. This study aimed to assess the effects of wastewater-borne AgNPs (NM-300K; 15.5 ± 2.4 nm; 25-125 µg L) and TiO_2NPs (NM-105; 23.1 ± 6.2 nm; 12.5-100 µg L) from a laboratory-scale STP, on the filter-feeding cladoceran *Daphnia magna*, at individual and subcellular levels. For effect comparison, *Daphnia* were also exposed to ASTM-dispersed NPs at the same nominal concentrations. State-of-the-art electron microscopy and newly modified SP-ICP-MS (with µs time resolution) were used for particle characterization. The behaviour of *D. magna* was evaluated through monitoring swimming height and allocation time for preferred zones at the beginning (0 h; to measure immediate responses) and after a 96-h bioassay. Biochemical markers of neurotransmission, anaerobic metabolism, biotransformation, and oxidative stress were subsequently determined. Whereas ASTM-dispersed AgNPs resulted in a 96-h EC_{50} (immobilization) of 113.8 µg/L, no EC_{50} could be calculated for the other three NP-treatments (immobilization ≤ 4%). Wastewater-borne TiO_2NPs at 12.5, 75 and 100 µg/L caused immediate (0-h) reductions of 39, 26 and 25% of swimming height, respectively. Allocation time analyses showed that animals exposed to ASTM-dispersed AgNPs spent more time on the surface and bottom at 0 h, and in the middle and bottom at 96 h. This pattern was neither observed in the ASTM-dispersed TiO_2NPs nor with wastewater-borne AgNPs or TiO_2NPs. At the biochemical level, the more pronounced effects were observed at high concentrations of wastewater-borne AgNPs, *e.g.* decrease of 35% of acetylcholinesterase activity at 75 µg/L; increase of 129 and 180% of lactate dehydrogenase activity at 50 and 125 µg/L, respectively; and decrease of 69 and 65% of catalase activity at 50 and 75 µg/L, respectively.

This work showed that: (i) the behavioural and biochemical response-patterns are distinct in *Daphnia* exposed to wastewater-borne and ASTM-dispersed NPs; (ii) the most pronounced effects on allocation time are achieved with ASTM-dispersed AgNPs; and (iii) at the subcellular level, wastewater-borne AgNPs are more toxic than wastewater-borne TiO_2NPs. The integrated approach of swimming behaviour and biochemical markers can,

therefore, provide relevant information for a comprehensive environmental risk assessment on the effects of wastewater-borne NPs in aquatic environments.

3.2 Introduction

Engineered nanomaterials (ENMs) are increasingly used in various applications and commercial products, ranging from common household items (*e.g.* textiles, paints, sunscreens, cosmetics) to novel medical technologies (Kahru and Ivask, 2013). However, important environmental concerns regarding ENMs still persist nowadays, because they can enter the environment from a multitude of sources either: (i) directly, *e.g.* during runoff of washed nanoparticles (NPs) from swimming sunscreens and house wall paints after heavy rain (Kiser et al., 2009); or (ii) indirectly, *e.g.* through effluents from sewage treatment plants (STPs) (Lazareva and Keller, 2014; OECD, 2016). Regardless of their provenience, the ENMs are likely to enter both surface water and groundwater systems, thereby posing risks to aquatic biota and affecting the drinking water resources (Gartiser et al., 2014; Georgantzopoulou et al., 2018). In this regard, the EU-2020 legislative framework entails restrictive effluent standards for protected areas and severe restrictions on STP-effluent properties, which can potentially contain ENMs (Lai et al., 2018; Svanström et al., 2014). Therefore, according to the precautionary principle adopted by the current legislation contemplated in the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH), and in the context of the continuous increase of the sustainable nanotechnology industry at the global scale, a consistent and well-founded prospective environmental risk assessment (ERA) strategy for safe implementation of ENMs in wastewater management programmes still needs to be adopted (Lai et al., 2018; Roco et al., 2011). Although few reports claim that there is no clear evidence of damage towards aquatic biota with regard to the current low discharge levels of ENMs (measured or measure-based predicted) (Coll et al., 2016), it is well accepted that there is a gap of knowledge regarding the fate and effect of wastewater-borne manufactured nanomaterials on dynamic and complex STP-associated environments. Effectively, despite the recommendations of international policies for a critical need of ERA on wastewater-borne ENMs, comprehensive knowledge on their ecotoxicological effects on aquatic biota is still very poor.

Even though different types of NPs have been detected in receiving effluents from STPs (Brar et al., 2010; Kiser et al., 2009), their ecotoxicological effects on aquatic biota have been scarcely addressed in this matrix (Gartiser et al., 2014; Wu et al., 2018). So far, few studies (Georgantzopoulou et al., 2018; *Hartmann et al., 2019*; Kühr et al., 2018; Muth-Köhne et al., 2013) reported the toxicity effects of silver NPs (AgNPs) and titanium dioxide NPs (TiO_2NPs), two commonly used NPs in several manufactured products (Kahru and Ivask, 2013; Roco et al., 2011; Zhang et al., 2018), on aquatic biota and after passing through STP compartments. These findings indicated that, despite the very low concentrations (in the µg/L range) found in STP effluents (Lazareva and Keller, 2014; Maurer-Jones et al., 2013), the so-called wastewater-borne NPs could be potentially very harmful to aquatic organisms (Muth-Köhne et al., 2013). The composition and properties of wastewater effluents show big differences and present additional substances compared to those of natural water, such as type and quantity of metal ions, composition of dissolved organic matter (DOM; with humic and fulvic acids), colloidal substances, electrolytes, and several types of soluble microbial products derived from the metabolism of the microbiome (Mahlalela et al., 2017; Zhou et al., 2015). Once present in influent, the NPs can therefore undergo several transformation processes (e.g. dissolution, sulphidation, aggregation, coating with DOM, adsorption to biological surfaces, and deposition/sedimentation), which may ultimately influence their speciation in the effluent and, thereafter, affect their fate, transport, bioavailability and toxicity to aquatic organisms (Adam et al., 2018; Zhang et al., 2018). Therefore, in order to assess the toxicological effects of wastewater-borne AgNPs and TiO_2NPs on non-targeted biota, new approaches must be developed and optimized accordingly, taking into account the intrinsic complexity of these particular matrices. Ecotoxicological tests with different model organisms have been recently performed to get insight into the toxicity mechanisms associated with AgNPs and TiO_2NPs present in STP effluents (Georgantzopoulou et al., 2018; *Hartmann et al., 2019*; Kühr et al., 2018; Muth-Köhne et al., 2013). Besides, the fate and ecotoxicity of both AgNPs and TiO_2NPs were already investigated in aquatic compartments at defined concentrations (Ribeiro et al., 2017; Sharma, 2009). Based on prospective models, predicted environmental concentrations (PECs) of NPs for several compartments in STPs, both in influents and effluents, are available. For example, in effluents, Maurer-Jones et al. (2013) summarized PECs of 0.0164-17 and 1-100 µg/L for AgNPs and TiO_2NPs, respectively. These values

were subsequently updated for STP effluents from the EU countries by Sun et al. (2014), which specifically pointed to integer PECs of 0.17 ng L and 16 µg/L for AgNPs and TiO_2NPs, respectively. Among the studies mentioned above, the few works on the environmental impact of AgNPs and TiO_2NPs from STPs on aquatic biota have been mainly focused on their toxicological effect at only one level of biological organisation, normally considering survival and growth as endpoints. As far as we are concerned, no studies are currently available regarding the toxicity of both types of wastewater-borne NPs through following a consistent and integrated approach at different levels of biological organisation. Therefore, since the responses at the lower level can act as early warning signals for the effects on higher level, the main objective of this study was to generate, integrate and add valuable knowledge on the toxicity evaluation profile of wastewater-borne AgNPs and TiO_2NPs, to the water flea *Daphnia magna*, a global keystone species of freshwater ecosystems, at both behavioural and biochemical levels.

Specifically, this study aimed at: (i) evaluating the toxicity of wastewater-borne effluents containing AgNPs (originally NM-300K) and TiO_2NPs (originally NM-105) to the freshwater cladoceran *D. magna*; and (ii) infer on its prediction through simple spiked media with the same original particles. This approach will be carried out through the assessment of behavioural and biochemical endpoints, in order to (a) elucidate the respective modes/mechanisms of action of each NP type by linking the effects at both levels of organisation, and (b) provide insight and establish interrelationships between the early warning responses at the biochemical level to be translated at the behavioural level. This new combined approach should, therefore, contribute to a better comprehension of the effects caused by NPs on aquatic organisms and provide input towards a more realistic risk assessment.

3.3 Materials and methods

3.3.1 Silver and titanium dioxide nanoparticles

The aqueous dispersions of AgNPs (test material: NM-300K) were obtained from the OECD Working Party on Manufactured Nanomaterials (WPMN) sponsorship programme. The dispersions of NM-300K NPs have a stated nominal silver (Ag) concentration of 10% (w/w), primary particle size of 15.5 ± 2.4 nm (measured in-house by TEM in ASTM medium) and are stabilized with NM-300K DIS, a mixture of non-ionic dispersing agents

containing 4% (w/v) of each polyoxyethylene glycerol trioleate (trade name: Tagat® TO) and polyoxyethylene (20) sorbitan monolaurate (trade name: Tween® 20). Also obtained from the OECD WPMN programme, the NM-105 reference material was used for the preparation of the TiO_2NPs stock dispersions. Presented as a dry white powder, the uncoated TiO_2NPs consisted of anatase and rutile (86:14 ratio) individual particles with a primary particle size of 23.1 ± 6.2 nm (measured in-house by TEM in ASTM medium).

3.3.2 Model sewage treatment plant (STP)

By following the test guideline (TG) 303A (OECD, 2001), up to six units of a lab-scale STP (behrotest® Laboratory Sewage Plant KLD 4N, behr Labor-Technik GmbH, Düsseldorf, Germany) were set up and conducted as previously described (*Hartmann et al., 2019*). The details about the constitution and functioning of STP and the obtention of NP-containing effluents for exposure experiments (Section 2.4) are provided in Materials and Methods of Supporting Information (SI). In two independent experiments, namely 1A and 2A for AgNPs and TiO_2NPs, respectively (Table 3-1), the NPs were added to the influent at the denitrification reactor. The concentrations of NPs in the influent were chosen in order to achieve, after proper dilution, environmentally relevant concentrations in the obtained effluent, which may potentially result in quantifiable effects. In total, six and four STP units ran with AgNPs and TiO_2NPs, respectively, including the respective effluent controls without NPs. Concentrations of total silver (Ag) and total titanium (Ti) were measured in the collected effluents after their passage through the STP units (Ag: after 26 days; Ti: directly after system operation). Inductively coupled plasma mass spectrometry (ICP-MS) and inductively coupled plasma-optical emission spectrometry (ICP-OES) were used for the determination of total Ag and total Ti concentrations, respectively. The analytical protocols and main instrumental parameters are detailed in SI including Table S3-1.

3.3.3 Test species and culture conditions

The freshwater crustacean *Daphnia magna* Strauss (clone V) was used as model species. The culture conditions of the test organism *D. magna* and of the food source, *Desmodesmus subspicatus* are described in Chapter 2.2.1.

Table 3-1: Exposure experiments and respective treatments (test media + NPs), including controls, showing the nominal concentrations of NPs (in mg/L) supplemented to the STP influent and the dilution factors used for the preparation of tested concentrations in effluent (in µg/L) of total Ag and total Ti. EFF, CT, and DA are the controls (without NPs) composed by the effluent, ASTM medium, and dispersing agent, respectively. See text for further details.

Experiments	Treatments	STP: nominal sewage inlet concentrations (mg/L)	STP: total concentrations in the effluent (µg/L) ♦	Effluent dilution factors (parts of effluent per parts of ASTM)	Nominal concentrations of NPs in the test media (µg/L)
1A	EFF *	0	n/d	1:1.2	0
	Wastewater-borne AgNPs	1	54 ± 3	1:2.1	25
		2.5	65 ± 2	1:1.2	50
		3.5	141 ± 3	1:1.8	75
		5	193 ± 3	1:1.9	100
		6.5	239 ± 4	1:1.9	125
1B	CT	n/a	n/a	n/a	0
	DA (ASTM medium + dispersing agent)	n/a	n/a	n/a	0
	ASTM-dispersed AgNPs	n/a	n/a	n/a	25 50 75 100 125
2A	EFF *	0	< LOD	1:3.7	0
	Wastewater-borne TiO_2NPs	1#	104.3 ±2	1:13.9	12.5
		1#	104.3 ± 2	1:6.9	25
		2.5	114.1 ± 1	1:3.7	50
		5#	464 ± 5	1:10.3	75
		5#	464 ± 5	1:7.7	100
2B	CT	n/a	n/a	n/a	0
	ASTM-dispersed TiO_2NPs	n/a	n/a	n/a	12.5 25 50 75 100

* The EFF controls served as stock solutions for the correspondent EFF controls used in the exposure experiments after dilution with ASTM medium. # In inlet with 1 and 5 mg/L TiO_2NPs, two dilutions were prepared in order to achieve four different concentrations in the obtained effluents. In short, 6 + 4 STP units lead to 6 + 6 treatments, including controls, for exposure experiments with AgNPs and TiO_2NPs, respectively. ♦ Measured concentrations of NPs in collected effluents (n = 1 ± standard deviation of 10 and 3 internal replicates for total Ag and total Ti, respectively). n/d – not determined; n/a – not applicable; LOD – limit of detection.

3.3.4 Exposure experiments

3.3.4.1 Controls and treatments

Three NP-free controls were used in exposure experiments (Table 3-1), namely an effluent control (EFF; in 1A and 2A with wastewater-borne NPs), an ASTM medium control (CT; in 1B and 2B with ASTM-dispersed NPs) and a dispersant agent control (DA; in 1B with ASTM-dispersed AgNPs).

For exposure experiments with wastewater-borne AgNPs (1A; Table 3-1) and wastewater-borne TiO_2NPs (2A; Table 3-1), the collected effluents from the model STP were manually shaken for 2 min in order to generate homogeneous dispersions, which served as stocks for further dilutions. Based on the total concentrations of NPs measured in the effluent, proper dilutions were performed with ASTM medium in order to achieve the respective nominal concentrations of 25-125 µg/L of total Ag and 12.5-100 µg/L of total Ti. The concentration range of AgNPs was chosen according to Völker et al. (2013a), which determined a nominal 48-h EC_{50} (immobilization) of 121 µg/L AgNPs in *D. magna*. The concentration range of TiO_2NPs was selected taking into account the nominal PECs of 1-100 µg/L TiO_2NPs (Maurer-Jones et al., 2013). The dilution factors (parts of effluent per parts of ASTM medium) and information about the preparation of effluent-related treatments are detailed in Table 3-1.To compare the effects of wastewater-borne NPs with the effects of ASTM-dispersed NPs, exposures were also carried out in the ASTM medium containing AgNPs or TiO_2NPs at the same nominal concentrations. These treatments were prepared in two additional experiments, namely 1B and 2B for ASTM-dispersed AgNPs and ASTM-dispersed TiO_2NPs, respectively (Table 3-1). In 1B, the original glass vial with AgNPs was sonicated in an ultrasonic bath (Bransonic 221 ultrasonic cleaner, Branson Ultrasonic, USA) for 10 minutes to disperse agglomerates and avoid air bubbles. Afterwards, the AgNPs were diluted to 1 g/L of total Ag with ASTM medium. This dispersion served as basis for subsequent dilutions, in order to achieve the final NP concentrations in the test medium (Table 3-1). Also, in 1B, proportional dilutions were prepared with the dispersing agent to check for potential effects of the solubilizing agent in the treatments containing ASTM-dispersed AgNPs. For this purpose, the solutions were prepared by mixing the aqueous solution of NM-300K DIS in the ASTM medium at the same concentration used for the preparation of the highest AgNP concentration, *i.e.* 125 µg/L.

To obtain the nominal concentrations of ASTM-dispersed TiO_2NPs (2B; Table 3-1), the NP powder was dispersed in PP vials containing ASTM medium (VWR International, Darmstadt, Germany) at 500 mg/L of total Ti. A homogeneous dispersion was obtained afterwards by sonicating the stock in an ultrasonic homogenizer (Bandelin SONOPLUS HD2200, Berlin, Germany; 13 mm MS 72 horn, 40% amplitude) for 16 min. This dispersion served as basis for further dilutions in order to achieve the respective final concentrations of TiO_2NPs in the tested media (Table 3-1). As a standard endpoint in ecotoxicity testing, the NP concentration that led to 50% immobilization after 96 h [96-h EC_{50}; greatest half-maximal effective concentration (OECD, 2004)] was derived whenever possible.

All treatments described above served as test media for the subsequent behavioural (section 2.4.2) and biochemical (section 2.4.3) assays.

3.3.4.2 **Behavioural assays**

The behavioural assays were performed in a temperature-controlled room (20 ± 2 °C) under constant conditions. Pools of randomly selected 10-day old *D. magna* (~3.1 ± 0.3 mm) were used. A 100 mL glass vessel (97 × 44 × 34.5 mm^3; model type 740-OG, Hellma Analytics, Müllheim, Germany) served as test vessel (Figure S1, B-C). For each assay, five replicates per concentration plus controls, with ten organisms each, were tested. A computer vision system was used to monitor the swimming behaviour of *D. magna* in real-time (Kunze et al., 2016). Briefly, the animal's behaviour was recorded with a custom-built 2D-dimensional tracking system in a test chamber, which was completely covered with black polyvinyl chloride (PVC) plates to avoid light (Figure S1, A). To record movements of animals in darkness, a CVI STAR BL-LED backlight source (Stemmer Imaging AG, Puchheim, Germany), with a wavelength of 850 nm, was placed near the test vessel (Figure S1, B-C). This background illumination was chosen because it cannot be seen by *D. magna*, thereby not affecting their motion. Contrary to the record of animals at visible light, the darkness set up, with only a single background light, presents the advantage of avoiding possible additional stress, phototaxis and/or resulting artefacts/biases.

Immediately before the behaviour assays, the glass vessel was filled up with 100 mL of the respective exposure treatment (Table 3-1). Then, adult animals were rapidly and carefully transferred to the vessel by means of a fine mesh to minimize stress and avoid media dilution. For each trial, animals were randomly selected from the four culture

beakers and immediately placed inside the vessel. In order to minimize water movements within the vessel, a period of 10 min was accomplished before recording. Afterwards, the onset of recording (time point 0 h) was triggered by the operator and swimming behaviour was registered in real-time. The animals were not fed during the recording process and only those with continuous swimming behaviour were considered. A minimal threshold of 100 s was set in order to exclude reflections or crossings of/by organisms within analyses. The behaviour was recorded in the test vessel for 2 min [time period long enough to detect behavioural responses (Bownik, 2017) and references therein]. The 2-min recording process was done at two precise time points, *viz.* immediately at the beginning of experiments (0 h), to measure the short-term effects of NPs on the behaviour, and after 96 h, to assess long-term responses. The 96 h exposure time was chosen because it was shown that *D. magna* exhibited an increased sensitivity to NPs during this period of time, as evaluated through the respective immobilization EC_{50}'s [*e.g.* for TiO_2NPs: 96-h EC_{50} = 0.73 mg/L; 72-h EC_{50} = 3.8 mg/L; Dabrunz et al. (2011)].

The average swimming height (defined as the vertical path length, from the bottom to the top of the test vessel; in mm) and allocation time (defined as the time that animals spent in a specific zone within the test vessel; in s) were assessed as behavioural-related markers. For allocation time evaluation, the total volume of the test vessel (Figure S3-1 B + C) was horizontally divided into three same sized swimming zones, named 1 (top), 2 (centre) and 3 (bottom). Normally, zooplankton organisms belonging to the genus *Daphnia* present randomly swimming trajectories in defined volumes of water when stimuli (*e.g.* predator cues, matting, light) are absent, like in our experimental setup (Uttieri et al., 2004; Uttieri et al., 2005). Once organisms detect a chemical signal (*e.g.* dispersed NPs), their swimming behaviour become more coherent and deterministic, thus reflecting a change in the resulting trajectories [see *e.g.* Noss et al. (2013a) for TiO_2NPs]. These observations served as the rationale for the selection of the swimming zones within the test vessel, thereby assuming zone 2 as the preferred one. The pH, temperature and dissolved oxygen were monitored with a digital precision meter (WTW Multi 3430, WTW GmbH, Weilheim, Germany) in all treatments and respective controls, both directly after media preparation (0 h) and after 96 h (Table S3-2). All parameters fulfilled the TG criteria (OECD, 2004).

3.3.4.3 **Biochemical assays**

After the 96 h of exposure required for the behavioural assays, the animals were removed from the test vessel and appropriately maintained on ice until storage. For this purpose, the organisms in each replicate ($N_{number\ of\ replicates}$ = 3-4; 9-10 animals each; 14-d old) were pooled together to yield enough biomass for the determination of total protein content and biochemical markers. Aiming at a complete removal of NPs from the outer carapace of *D. magna*, each pool of organisms was carefully rinsed thrice with 0.1 M phosphate buffer saline (PBS, pH 7.4) to prevent potential bias caused by *in vitro* interactions. The organisms were then immediately transferred to 1.5 mL microtubes, filled with ice-cold 0.1 M PBS (pH 7.4) at 100 µL/organism, snap-frozen in liquid nitrogen and stored at -80 ºC until processing.

A battery of biochemical markers was performed, *viz.* determination of the enzymatic activities of acetylcholinesterase (AChE), lactate dehydrogenase (LDH), glutathione *S*-transferase (GST), catalase (CAT); and determination of lipid peroxidation (LPO) levels. On the day of the analyses, samples were thawed on ice and homogenized with an ultrasonic homogenizer (Sonifier 250A, Branson Ultrasonics; pulse intensity and duration adjusted accordingly). An aliquot of 150 µL of the resulting homogenate was placed in a microtube with 4 µL 2,6-Di-*tert*-butyl-4-methylphenol for LPO determination. The remaining volume was centrifuged at 10,000 ×*g* for 20 min at 4 ºC to separate the post-mitochondrial supernatant (PMS). The PMS fraction was divided into aliquots for the subsequent quantification of each enzymatic activity and further diluted in 0.1 M PBS (pH 7.4) whenever necessary. All reactions were performed in 96-wells microplates at 25 ºC and determined spectrophotometrically in a microplate reader (Multiskan Spectrum, Thermo Fisher Scientific, Waltham, USA) by following the methodologies briefly described below.

The AChE activity was measured by using acetylthiocholine iodide as substrate, according to Ellman *et al.* (1961) and adapted to microplate by Guilhermino et al. (1996, through monitoring the absorbance (414 nm) of complexes formed by the thiol reagent 5,5′-dithiobis(2-nitrobenzoic acid) and thiocholine, every 20 s, during 5 min (ε_{414} = 1.36 × 10^4 M/cm). The AChE activity was expressed as nmol of hydrolysed substrate per min per mg of protein. The LDH activity was evaluated by monitoring, at 340 nm (every 20 s, during 5 min), the decrease of NADH (ε_{340} = 6.3 mM/cm) due to its oxidation, as per Vassault (1983,

adapted to microplate (Diamantino et al., 2001), and expressed as nmol of hydrolysed substrate per min per mg of protein. The GST activity was measured according to the method of Habig et al. (1974), adapted to microplate, and monitored every 20 s during 5 min at 340 nm; it was expressed as nmol of the conjugated substrate (2,4-dinitrochlorobenzene plus reduced glutathione – GSH) per min per mg protein (ε_{340} = 9.6 × 10^3 M/cm). The CAT activity was assayed by following the decrease of absorbance at 240 nm due to H_2O_2 consumption (ε_{240} = 40 M/cm), recorded every 10 s during 2 min, and expressed as µmol of hydrolysed H_2O_2 per min per mg protein (Clairborne, 1985). The LPO levels were quantified according to the thiobarbituric acid (TBA) reactive substances (TBARS) assay (Ohkawa et al., 1979), by measuring the amount of malondialdehyde-TBA complex (ε_{535} = 1.56 × 10^5 M/cm), and expressed as nmol of hydrolysed TBARS per mg of protein. All biochemical marker measurements were repeated 2-4 times and normalized to protein concentration. Protein was determined at 595 nm (Bradford, 1976), with the Bio-Rad® dye-binding micro-assay method adapted for 96-well microplates. The bovine γ-globulin was used as standard.

All reagents for the determination of enzymatic activities, lipid peroxidation, and protein assays were of the highest available analytical grade quality (≥ 99%) and were purchased from Merck KGaA (Darmstadt, Germany), with the exception of Bradford reagent (Bio-Rad, Munich, Germany). Ultra-pure water was prepared by using a Milli-Q mod. Academic water purification system (Merck KGaA, Darmstadt, Germany).

3.3.5 Characterisation of nanoparticles

The Scanning Transmission Electron Microscopy (STEM) was performed on a FEI Talos F200X electron microscope with an acceleration voltage of 200 kV. A high-angle annular dark-field (HAADF) detector was used for a better contrast of NPs containing heavy elements (Ag and Ti) within an otherwise organic background. Energy-dispersive x-ray analysis (EDX) was performed with a Super-X EDX detector to obtain spatially resolved elemental information. Fresh stock suspensions of AgNPs and TiO_2NPs at 100 µg/L (nominal) were prepared in ASTM medium. At the beginning of the exposure (time point 0 h; see Section 3.3.4), two aliquots from stocks (40 mL each) were sampled, immediately frozen in liquid nitrogen in polypropylene (PP) centrifuge tubes (VWR International, Darmstadt, Germany) and stored at -20 °C until use. The remaining dispersions (170 mL each) were transferred into sterile glass bottles and submitted to a 16/8 h (light/dark)

photoperiod, at 20 ± 2 °C, for 96 h. Afterwards, the dispersions were homogenized by manual shaking and particles isolated from respective media via cloud point extraction (Hartmann et al., 2013). In parallel, previously frozen samples of ASTM medium and effluents (see Section 3.3.2) were thawed in a water bath at 30 °C before preparation. All of the extracts proceeding from ASTM medium and effluent matrices were centrifuged onto an amorphous carbon-coated copper grid (200 mesh, Plano, Wetzlar, Germany). Organic residues were removed by depositing small droplets of absolute ethanol (≥ 99.8%, VWR, Germany) on the copper grid, which were absorbed by underlying paper tissue.

The kinetic determination of particle size distribution (PSD) was performed by single particle inductively coupled plasma mass spectrometry (SP-ICP-MS) in ASTM medium aliquots withdrawn from a glass vessel in which organisms were exposed (Section 3.3.4). Contrary to TEM analysis, the medium in the test vessel was not homogenized before sampling in SP-ICP-MS determinations because of the experimental design followed in the behavioural assays (see Section 3.3.4.2. for details). Accordingly, aliquots with a defined volume were taken from the middle zone of the test vessel at 0, 3, 6, 12, 24, 48, 72 and 96 h. A model iCAP Qc (Thermo Fischer Instrument, Bremen, Germany) quadrupole ICP-MS was used. For characterisation of NM-300K NPs, the size calibration was done with 20 nm AgNPs (ECP1374, nanoComposix, San Diego, CA, USA), with a mean particle size of 18.5 ± 3.4 nm (TEM provided by the manufacturer). For characterisation of NM-105 NPs, the size calibration was done with TiO_2NPs (IoLiTec Ionic Liquids Technologies GmbH, Heilbronn, Germany), with a mean particle size of 41.5 ± 9.9 nm (in-house TEM measurements). Prior to SP-ICP-MS measurements, samples were diluted with double distilled water in PP centrifuge tubes (VWR International, Darmstadt, Germany) to ensure the detection of individual particles and were analysed right after dilution. A total consumption microflow DS- 5 nebulizer (Teledyne CETAC Technologies, Omaha, NE, USA), operating at a self-aspiration rate of ~ 5 µL/min and a low-volume spray chamber, was used for the analysis of AgNPs. A MicroFlow PFA-50 nebulizer (Thermo Fisher Scientific, Bremen, Germany), with a self-aspiration rate of ~ 65 µL/min at 1 L/min of argon (according to the manufacturer instructions), and a Peltier-cooled cyclonic quartz spray chamber, held at 3 °C, were used for the analysis of TiO_2NPs. An additional roughing pump (Sogevac SV40 BI, Leybold, Cologne, Germany) was connected to the instrument to decrease the interface pressure. Due to the principal limitation of the quadrupole mass

analyser, only one isotope ($^{107}Ag^+$ or $^{49}Ti^+$) was monitored at a time to get continuous time-resolved measurements. A prototype data acquisition system (Strenge and Engelhard, 2016), which was applied earlier for AgNP (Mozhayeva and Engelhard, 2017; Mozhayeva et al., 2017) and AuNP (Franze et al., 2017) characterization, was used to continuously acquire data with 5 µs time resolution. The SP-ICP-MS measurements were performed during 10 and 3 min for AgNPs and TiO_2NPs, respectively. The main SP-ICP-MS instrumental parameters are presented in Table S3-3.

3.3.6 Statistical analysis

Each data set of swimming height and biochemical markers was tested beforehand for normal distribution (Shapiro-Wilk test) and homoscedasticity (Brown-Forsythe test) prior to further analysis (all variables met the required assumptions). For swimming height analysis, if not otherwise stated, a two-way analysis of variance (ANOVA), followed by Tukey's HSD *post-hoc* test was used to determine the interactive effect of each treatment (test medium with NPs; including controls) and the respective time point (0 or 96 h). For each particular time point and treatment, the differences within each NP concentration and respective controls (EFF, DA or CT) were determined by one-way ANOVA followed by Dunnett´s *post-hoc* test. The differences between means of the same concentration of each NP type (AgNPs or TiO_2NPs) dispersed in each exposure medium (effluent or ASTM medium) were determined by Student's paired t-test with a two-tailed test of significance. For the evaluation of immobilization, an appropriate dose-response model was adopted for each treatment and each particular time point. The model was selected based on Akaike's information criterion for each dataset of immobile organisms, thus allowing the calculation of the respective median effective concentrations (96-h EC_{50}'s) by using the *'drc'* extension package (Ritz et al., 2016).

For the evaluation of allocation time, the mean ± standard error (SE) of animals distributed in each zone (1 – top; 2 – centre; and/or 3 – bottom) of the test vessel was determined. To analyse allocation time in the Experiments, we used linear mixed effect (LME) models with the *lmer* function of the '*lmerTest*' package (Kuznetsova et al., 2017). The effect of each treatment assessed at each particular time point was analysed separately in a different model with allocation time as the dependent variable. All models were computed to check if the allocation time of organisms followed a concentration-response pattern, taking into account all treatments and respective controls (EFF, DA or CT) wherefore the

concentrations and controls were included into the models as are numerical variables (fixed effects). Additionally, to test differences between DA and CT in the experiment with ASTM-dispersed AgNPs (Experiment 1B; Table 1) another model was performed with allocation time as the dependent variable (as above), and DA and CT as categorical variables (fixed factors). The same settings of variables and factors as just described, were applied for two additional models to compare the CT of Experiment 1B and Experiment 2B to the EFF of Experiment 1A and Experiment 2A, respectively (Table 3-1). The identity of animals nested in the test vessel was included as a random effect in all models. The verification of the assumptions of the models by visual inspection of residual plots (Q/Q, residuals against adjusted values and normality of residues) showed no clear deviations from normality and homoscedasticity. Conditional plots were made using the '*visreg'* package (Breheny and Burchett, 2016). For the analysis of biochemical markers**,** a one-way ANOVA followed by Dunnett's *post-hoc* test was used to check the differences between treatments and controls. The differences between means of the same concentration of NPs dispersed in different tested media were assessed with t-tests as above. The analysis of swimming behavioural-related markers and calculation of 96-h EC_{50} were done using R for Windows (version 3.2.4). All other analyses were performed with SigmaPlot for Windows, v. 14 (Systat Software, Inc., San Jose, CA, USA) and Statistica 64, v. 12 (StatSoft Inc., Tulsa, OK, USA). Results are expressed as mean ± SE and α-level set at 0.05.

3.4 Results

3.4.1 Particle characterisation and size distribution

As revealed by TEM, the ASTM-dispersed AgNPs possessed a modal diameter of 15.5 ± 2.4 nm at 0 h, which did not change over the exposure time (15.6 ± 2.2 nm after 96 h; Figure S2). The ASTM-dispersed TiO_2NPs formed agglomerates, comprising primary TiO_2 particles with a diameter of 23.1 ± 6.2 nm at 0 h, which also remained constant after 96 h (26.0 ± 5.8 nm; Figure S3). The EDX analysis of wastewater-borne AgNPs showed that silver was always co-localized with substantial amounts of sulphur, indicating a change in their chemical composition, from pristine silver towards silver sulphide (Ag_2S). This was supported by the atomic Ag/S ratio of ~ 2:1 (Figure 3-1 A). It is therefore assumed that AgNPs were completely sulphidized in the effluent matrix. By contrast, the ASTM-

dispersed AgNPs were not chemically affected over the 96-h period (Figure 3-1 B). In comparison to the sulphidized NPs, the amount of sulphur in ASTM-dispersed AgNPs was found to be substantially lower (atomic Ag/S ratio of ~ 23:1) and most likely stemmed from sulphate ions present in the ASTM medium (Figure 3-1 B). For TiO_2NPs, the particles present in effluent and ASTM medium did not undergo any transformation because of their chemical inertness. In both matrices, the small amounts of sulphur detected by EDX were exclusively attached to the organic matrix, as observed in the elemental maps (Figure 3-2 A + B). This was supported by the homogeneous distribution of sulphur on TiO_2 particles compared to locally concentrated signals of titanium and oxygen. Other elemental signals are either attributed contaminations (Si), spurious X-rays from the support grid (Cu), organic residues from the AgNP stabilizer and/or the cloud point extraction surfactant (C, O), or even residues (N, Na, Ca, Mg, P) from wastewater effluent or the ASTM medium (Figure 3-1 + 3-2).

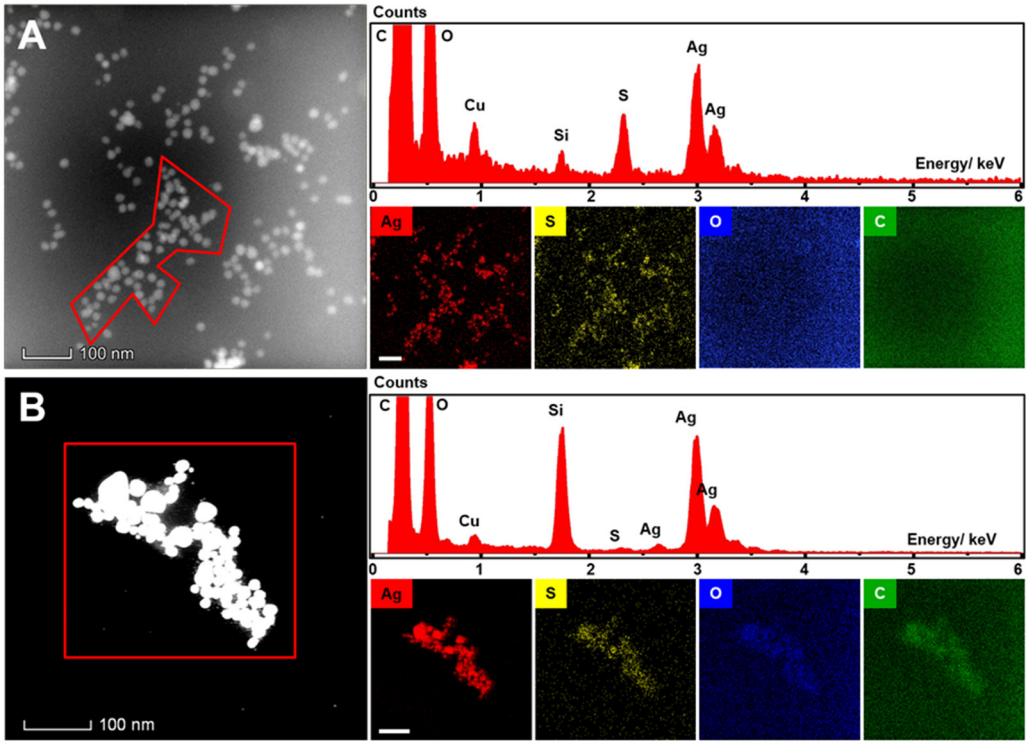


Figure 3-1: HAADF-STEM images and elemental maps of (A) wastewater-borne AgNPs and (B) ASTM-dispersed AgNPs. The EDX spectra correspond to the highlighted regions of interest.

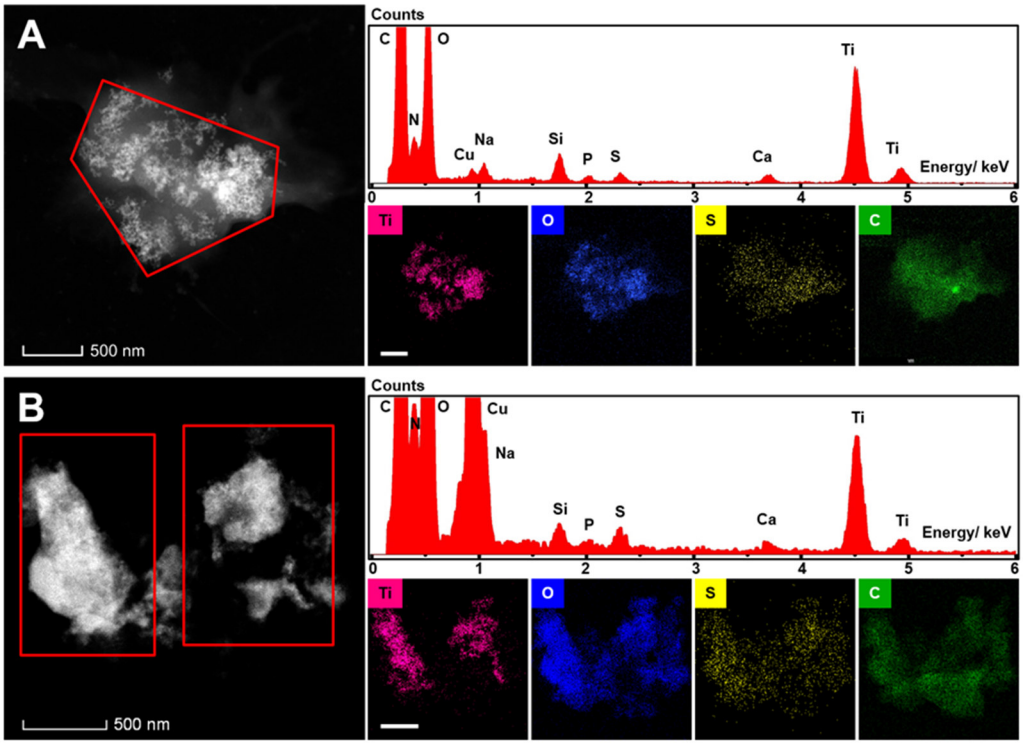


Figure 3-2: HAADF-STEM images and elemental maps of (A) wastewater-borne TiO_2NPs and (B) ASTM-dispersed TiO_2NPs. The EDX spectra correspond to the highlighted regions of interest.

The mean particle size evolution over time of ASTM-dispersed NPs obtained by SP-ICP-MS is shown in Figure 3-3. The size detection limit for both types of NPs was 10 nm (using Poisson statistics for data handling, with 5% false positive and 5% false negative error tolerances) (Mozhayeva and Engelhard, 2019). A Gaussian distribution was used for fitting PSDs. Due to the dispersing agent, all silver particles remained practically stable over 96 h in all dispersions and the mean particle size did not change to a high extent among all tested concentrations (Figure 3-3 A). Assuming a spherical shape of TiO_2 particles at all tested concentrations, the mean particle size decreased by 47.0 ± 6.7% until 12 h and then slightly decreased of up to 51.7 ± 6.7% of their initial average size after 96 h (in absolute: from 34 ± 1 nm at 0 h, to 17.2 ± 0.5 nm at 12 h, and to 15.6 ± 0.2 nm at 96 h; Figure 3-3 B).

3.4.2 Effects of wastewater-borne and ASTM-dispersed NPs

3.4.2.1 Effects on immobilization

No 96-h EC_{50} could be determined for *D. magna* exposed to wastewater-borne AgNPs since all tested concentrations led to 0% immobilization (Experiment 1A; Table S3-4). The exposure of animals to ASTM-dispersed AgNPs resulted in a 96-h EC_{50} for the

immobilization of 113.8 µg/L AgNPs (Experiment 1B; Table S3-4). Wastewater-borne TiO_2NPs (Experiment 2A; Table S3-4) and ASTM-dispersed TiO_2NPs (Experiment 2B; Table S3-4) caused low immobilization (< 5%) to animals and hence the respective 96-h EC_{50}'s could not be computed.

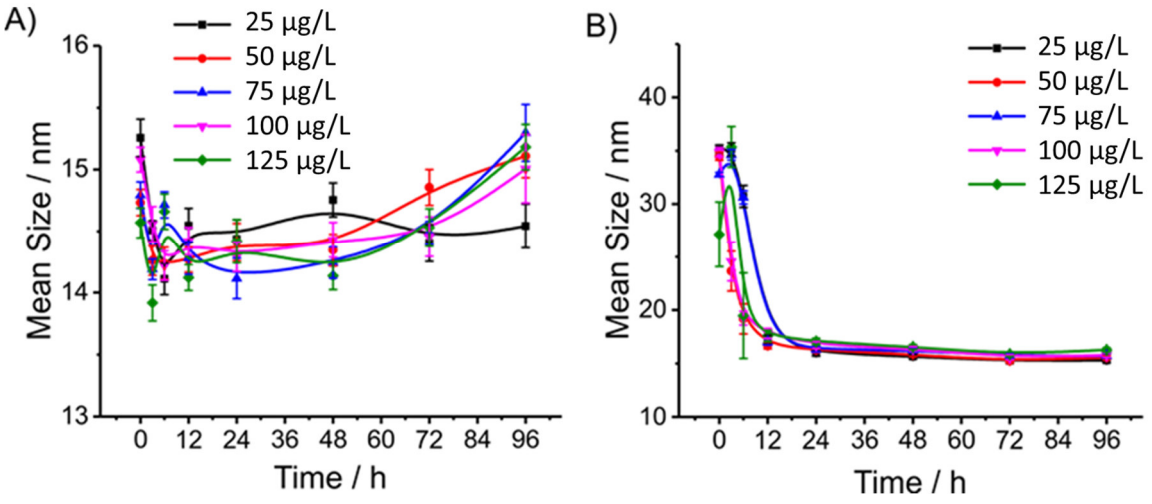


Figure 3-3: Mean particle size ± SE of the Gaussian distribution of (A) AgNPs and (B) TiO_2NPs dispersed in ASTM medium at the tested concentrations and monitored over 96 h. Results of SP-ICP-MS with 5 µs time resolution.

3.4.2.2 Effects on behaviour

3.4.2.2.1 Swimming height

At 0 h, the *D. magna* exposed to 25 µg/L of ASTM-dispersed AgNPs showed a 65% higher swimming height than animals exposed to the DA control (Dunnett's test, $p < 0.05$; Figure 3-4). At 96 h, *Daphnia* exposed to DA presented a lower swimming height of 66% compared to animals exposed to the negative CT (Dunnett's test, $p < 0.05$; Figure 3-4). Also, at 96 h, *Daphnia* showed a higher swimming height of 65% when exposed to 100 µg/L of ASTM-dispersed AgNPs compared to DA exposed animals (Dunnett's test, $p < 0.05$; Figure 3-4). Due to 100% immobilization of animals with 125 µg/L of ASTM-dispersed AgNPs at 96 h (Table S3-4), the respective treatment was omitted from Figure 3-4 and the statistic. Over time, *i.e.* from 0 to 96 h, an increase in swimming height of 67 and 53% was observed in *Daphnia* exposed to 25 and 50 µg/L of ASTM-dispersed AgNPs, respectively (Tukey's test, $p < 0.05$; Figure 3-4). At 0 h, *Daphnia* exposed to 25, 75 and 100 µg/L of wastewater-borne AgNPs had a lower swimming height of 101, 59 and 71% compared to animals respectively exposed to ASTM-dispersed AgNPs at the same tested concentrations (t-test, $p < 0.05$; Figure 3-4).

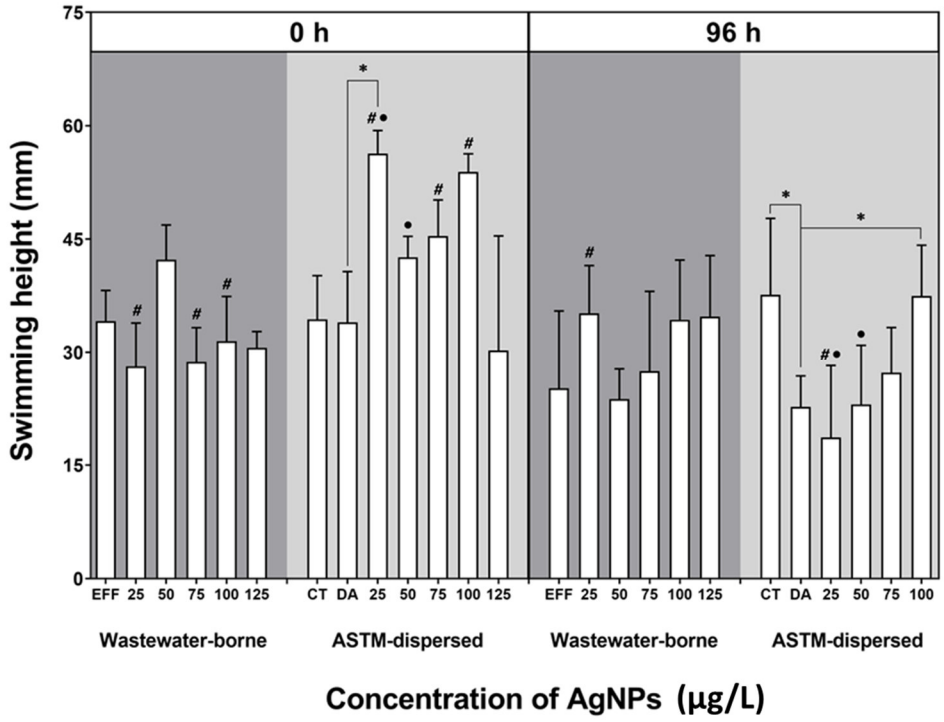


Figure 3-4 Swimming height (mean ± SE) of *Daphnia magna* ($N_{number\ of\ replicates}$ = 3-4 with 9-10 animals each) exposed to wastewater-borne and ASTM-dispersed AgNPs (nominal concentrations; expressed as µg/L of total Ag) in the beginning (0 h) and end (96 h) of the experiment. EFF, DA, and CT are the controls (without NPs) for the experiments with effluent (EFF) and ASTM medium (DA – dispersing agent control; CT – negative control, with only ASTM medium). * denotes significant differences of each NP concentration in a treatment (test medium + NPs), compared to the respective control (EFF or DA) or within CT and DA controls at a certain time-point, according to the one-way ANOVA followed by Dunnett's post-hoc test. • indicates significant differences between identical controls or identical NP concentrations dispersed in the same media at different time points (two-way ANOVA followed by Tukey's post-hoc test). # represents significant differences between identical NP concentrations or between different controls tested in different exposure media (effluent or ASTM medium) at a particular time point (t-test). $p < 0.05$.

At 96 h, *Daphnia* showed a higher swimming height of 47% with 25 µg/L of wastewater-borne AgNPs compared to animals exposed to ASTM-dispersed AgNPs at the same tested concentration (t-test, $p < 0.05$; Figure 3-4). On the contrary, at 0 h, the animals exposed to 12.5, 75 and 100 µg/L of wastewater-borne TiO_2NPs presented a swimming height reduction of 39, 26 and 25% compared to EFF, respectively (Dunnett's test, $p < 0.05$; Figure 3-5). Over time, *Daphnia* exposed to EFF presented a swimming height reduction of 38% (Tukey's test, $p < 0.05$; Figure 3-5) and animals exposed to 25 µg/L of wastewater-borne TiO_2NPs and 12.5 µg/L of ASTM-dispersed TiO_2NPs showed a swimming height reduction of 74 and 45%, respectively (Tukey's test, $p < 0.05$;

Figure 3- 5). The animals exposed to 12.5 µg/L of wastewater-borne TiO_2NPs at 0 h, and to 25 and 75 µg/L of wastewater-borne TiO_2NPs at 96 h, respectively showed a lower swimming height of 86, 55 and 50% compared to animals exposed to ASTM-dispersed TiO_2NPs at the same tested concentrations and corresponding time points (t-test, $p < 0.05$; Figure 3-5). At 96 h, animals exposed to 25 and 75 µg/L of wastewater-borne TiO_2NPs showed a lower swimming height of 51% compared to animals exposed to ASTM-dispersed TiO_2NPs at identical concentrations (Tukey's test, $p < 0.05$; Figure 3-5).

3.4.2.2.2 Allocation time

In experiments with AgNPs, at both time points, *D. magna* exposed to DA spent more time in zone 1 (top) compared to animals in CT, (LMER, estimates: 0 h – 12.43, 96 h – 44.38, $p \leq 0.03$; Table S3-5, Figure 3-6 A + B). At both time points, animals in EFF spent more time in zone 1 (LMER, estimates: 0 h – 17.80, 96 h – 22.67, $p \leq 0.02$) and zone 3 (LMER, estimates: 0 h – 16.97, 96 h – 10.50, $p \leq 0.01$) compared to animals in CT (Table S3-5; Figure 3-6 C + D). The allocation time per zone of *D. magna* was not affected by wastewater-borne AgNPs at both time points (LMER, $p > 0.05$; Table S3-5, Figure 3-6 E + F). Notwithstanding, at 0 h, animals treated with ASTM-dispersed AgNPs spent more time in zone 1 (LMER, estimate: 3.63, $p < 0.005$) and zone 3 (LMER, estimate: 2.59, $p = 0.009$), and less time in zone 2 (LMER, estimate: -2.23, $p = 0.004$) (Table S3-5; Figure 3-6 G). At 96 h, animals treated with ASTM-dispersed AgNPs spent more time in zones 2 (LMER, estimate: 3.76, $p = 0.006$) and zone 3 (LMER, estimate: 3.29, $p < 0.001$), and less time in zone 1 (LMER, estimate: -8.85, $p < 0.001$) (Table S3-5; Figure 3-6 H). Based on 100% immobilization found after 96 h of exposure to 125 µg/L of ASTM-dispersed AgNPs (Table S3-4), this treatment was omitted from Figure 3-6 H. The effects of TiO_2NPs on the allocation time of *D. magna* are depicted in Figure 3-7. At 0 h, compared to CT, the EFF exposed animals spent more time in zone 3 (LMER, estimate: 22.05, $p < 0.001$) and less time in zone 2 (LMER, estimate: -11.10, $p = 0.034$; Table S3-5; Figure 3-7 A). At 96 h, compared to CT, the EFF exposed animals spent less time in zone 1 (LMER, estimate: -15.70, $p = 0.034$) and more time in zone 3 (LMER, estimate: 15.39, $p < 0.001$). At 0 h, the animals exposed to increasing concentrations of wastewater-borne TiO_2NPs spent less time in zone 2 (LMER, estimate: -1.82, $p = 0.047$). At 96 h, animals spent more time in zone 1 (LMER, estimate: 32.00, $p < 0.001$) and less time in zone 3 (LMER, estimate: -0.76, $p = 0.017$) with increasing concentrations of wastewater-borne TiO_2NPs. In a

different way, at 0 h, animals treated with increasing concentrations of ASTM-dispersed TiO_2NPs spent more time in zone 1 (LMER, estimate: 3.36, $p < 0.001$) and less time in zone 2 (LMER, estimate: -3.90, $p < 0.001$) (Table S3-5; Figure 3-7 E). At 96 h, with increasing concentrations of ASTM-dispersed TiO_2NPs, animals spent less time (LMER, estimate: -4.72, $p < 0.001$) and more time (LMER, estimate: 5.67, $p < 0.001$) in zones 2 and 3, respectively (Table S3-5; Figure 3-7 F).

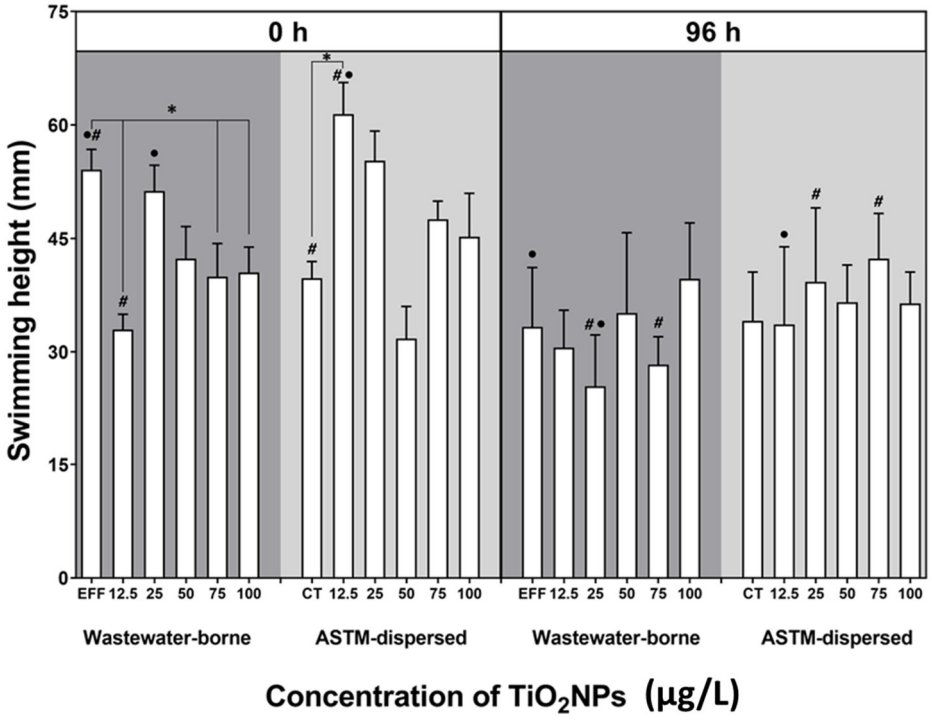


Figure 3-5: Swimming height (mean ± SE) of *Daphnia magna* ($N_{number\ of\ replicates}$ = 3-4 with 9-10 animals each) exposed to wastewater-borne and ASTM-dispersed TiO_2NPs (nominal concentrations; expressed as µg/L of total Ti) in the beginning (0 h) and end (96 h) of the experiment. EFF and CT are the controls (without NPs) for the experiments with effluent (EFF) and ASTM medium (CT), respectively.) * denotes significant differences of each NP concentration in a treatment (test medium + NPs compared to the respective control (EFF or CT) at a certain time-point according to the one-way ANOVA followed by Dunnett's *post-hoc* test. • indicates significant differences between identical controls or identical NP concentrations dispersed in the same media at different time points (two-way ANOVA followed by Tukey's *post-hoc* test). # represents significant differences between identical NP concentrations or between different controls tested in different exposure media (effluent or ASTM medium) at a particular time point (t-test). $p < 0.05$.

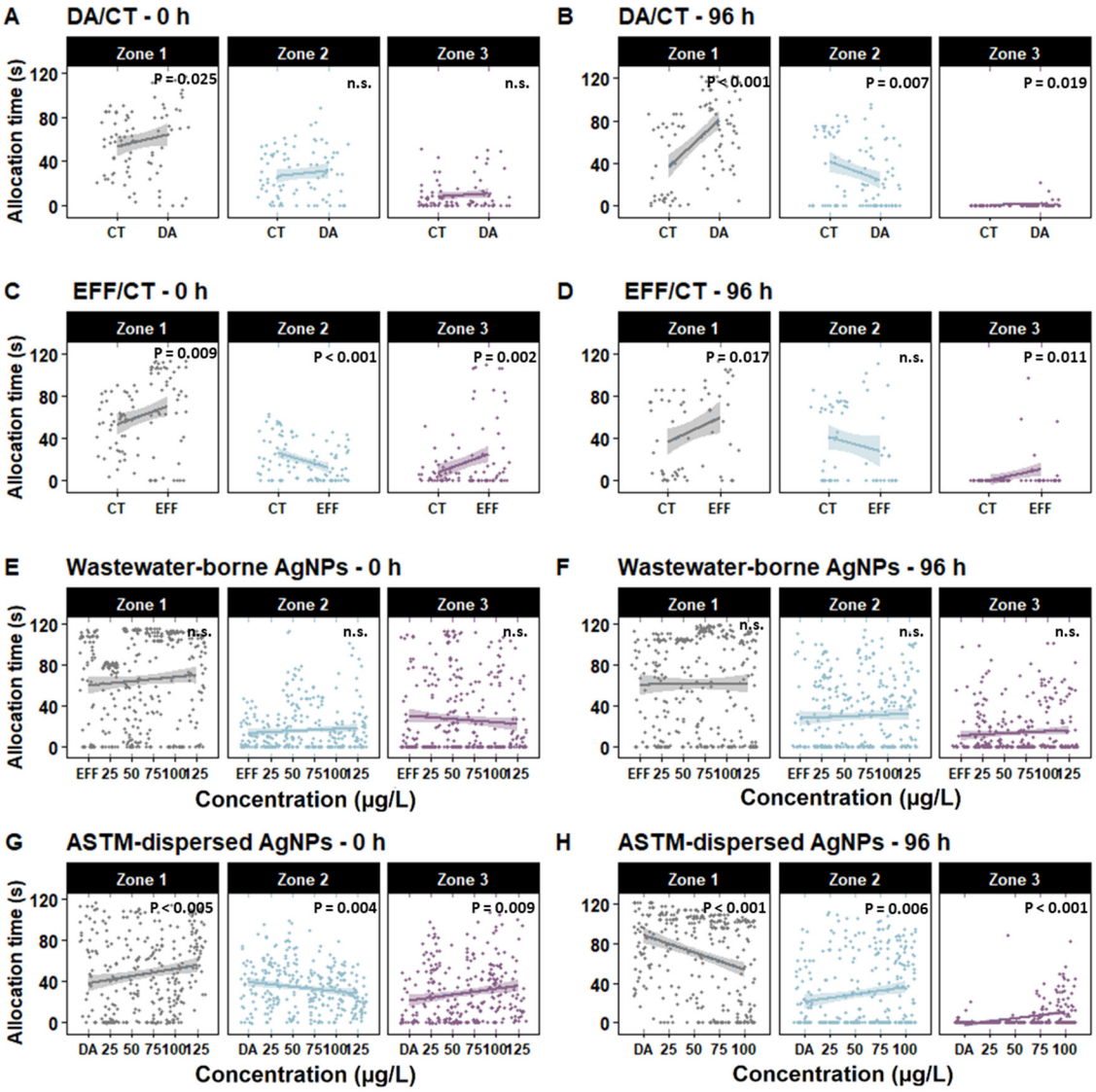


Figure 3-6: Conditional plots for the allocation time of *Daphnia magna* exposed to different AgNP treatments (test media + NPs) at 0 h (A, C, E, G) and 96 h (B, D, F, H). In each zone of the vessel (1, top; 2, central, or 3, bottom), the allocation time (s) was predicted for controls (0 h: A, C; 96 h: B, D) and concentration gradient of AgNPs (0 h: E, G; 96 h: F, H). Each panel illustrates the effects between: (A, B) dispersing agent (DA) and ASTM medium (CT) controls at 0 and 96 h, respectively; (C, D) effluent (EFF) and ASTM medium (CT) controls at 0 and 96 h, respectively; (E, F) wastewater-borne AgNPs at 0 and 96 h, respectively; and (G, H) ASTM-dispersed AgNPs at 0 and 96 h, respectively. In treatments, the NP concentrations are given as total Ag (in µg/L; nominal). Plots were calculated from linear mixed effect models, with 'allocation time' as the dependent variable and interaction between 'zone' and 'concentration' as fixed factors. Dots represent partial residuals; lines with shaded zones are regression lines and respective confidence intervals.

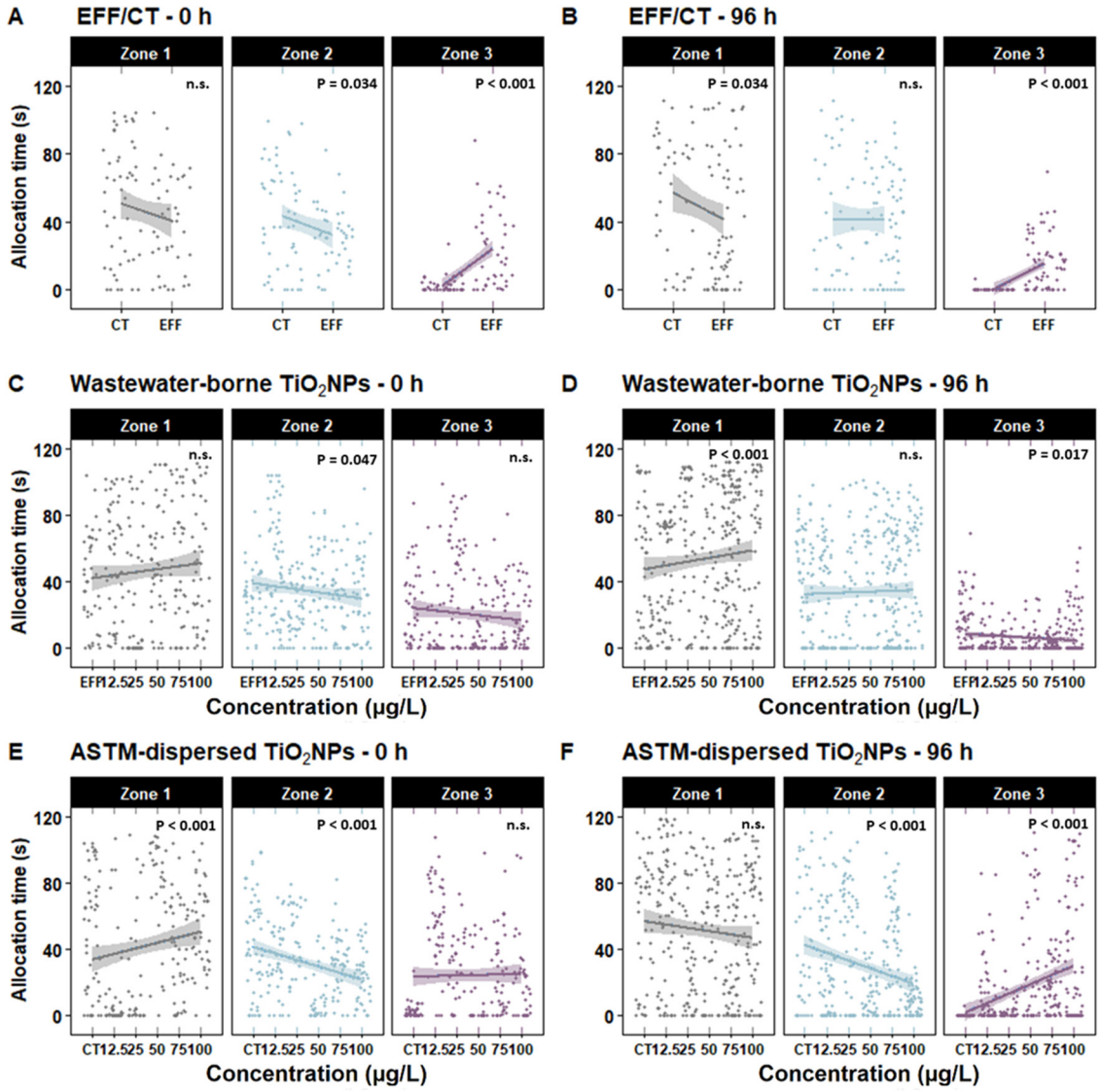


Figure 3-7: Conditional plots for the allocation time of *Daphnia magna* exposed to different TiO_2NP treatments (test media + NPs) at 0 h (A, C, E) and 96 h (B, D, F). In each zone of the vessel (1, top; 2, central, or 3, bottom), the allocation time (s) was predicted for controls (0 h: A; 96 h: B) and concentration gradient of TiO_2NPs (0 h: C, E; 96 h: D, F). Each panel illustrates the effects between (A, B) effluent (EFF) and ASTM medium (CT) controls at 0 and 96 h, respectively; (C, D) wastewater-borne TiO_2NPs at 0 and 96 h, respectively; and (E, F) ASTM-dispersed TiO_2NPs at 0 and 96 h, respectively. In treatments, the NP concentrations are given as total Ti (in µg/L; nominal). Plots were calculated from linear mixed effect models, with 'allocation time' as the dependent variable and interaction between 'zone' and 'concentration' as fixed factors. Dots represent partial residuals; lines with shaded zones are regression lines and respective confidence intervals.

3.4.2.2.3 Effects on biochemical markers

Compared to CT exposed *D. magna*, the DA exposed animals respectively showed a significant reduction of 37 and 21% of AChE and GST activities, and also an increase of

33% of LDH activity (Dunnett's test, $p \leq 0.03$; Figure 3-8). Compared to CT exposed Daphnia, the CAT activity significantly increased by 32 and 229% in EFF exposed animals used in AgNPs and TiO_2NPs experiments, respectively (t-test; $p \leq 0.02$; Figure 3-8 G + H). Besides, in TiO_2NP experiments, the EFF exposure caused a decrease of 64% and an increment of 44% on AChE and GST activities, respectively (t-test, $p = 0.01$; Figure 3- 8 B+F).

In experiments with AgNPs, the AChE was only affected by the 75 µg/L exposure to wastewater-borne AgNPs and the respective activity decreased 35% (Dunnett's test, $p = 0.03$; Figure 3-8 A). The AChE activity was 25% higher in 100 µg/L of wastewater-borne AgNPs compared to ASTM-dispersed AgNPs at the same tested concentration (t-test; $p = 0.01$; Figure 3-8 A). Compared to EFF, the animals exposed to wastewater-borne AgNPs presented an increase of LDH activity of 129 and 180% at 50 and 125 µg/L, respectively (Dunnett's test, $p < 0.001$; Figure 3-8 C). Compared to *Daphnia* exposed to ASTM-dispersed AgNPs at the same concentrations, the animals treated with 25 and 50 µg/L of wastewater-borne AgNPs presented a higher LDH activity of 26 and 48%, respectively (t-test, $p = 0.01$; Figure 3-8 C). Compared to EFF exposed *Daphnia*, there was an increase of GST activity of 120% in animals exposed to 100 µg/L of wastewater-borne AgNPs (Dunnett's test, $p = 0.01$; Figure 3-8 E). Furthermore, the GST activity was 19% higher at 100 µg/L of wastewater-borne AgNPs compared to ASTM-dispersed AgNPs at the same concentration (t-test, $p = 0.01$; Figure 3-8 E). Compared to controls, organisms exposed to wastewater-borne AgNPs showed a reduction on CAT activity of 63, 69 and 65% with 25, 50 and 75 µg/L AgNPs, respectively (Dunnett's test, $p \leq 0.01$; Figure 3-8 G). Besides, the CAT activity was 14 and 40% higher in organisms respectively exposed to 25 and 100 µg/L of wastewater-borne AgNPs compared to organisms exposed to ASTM-dispersed AgNPs at identical concentrations (t-test, $p \leq 0.03$; Figure 3-8 G). Animals exposed to 75 µg/L of wastewater-borne AgNPs presented 22% higher LPO levels than animals exposed to ASTM-dispersed AgNPs at the same concentration (t-test; $p = 0.03$; Figure 3-8 I).

In experiments with wastewater-borne TiO_2NPs, the only concentration responsible for a significant effect on AChE activity of *Daphnia* was the highest tested (100 µg/L), and the respective value raised by 297% compared to EFF (Dunnett's test, $p = 0.001$; Figure 3- 8 B). On the contrary, the AChE activity was reduced by 36 and 34% at 12.5 and 25 µg/L of ASTM-dispersed TiO_2NPs, respectively (Dunnett's test, $p \leq 0.03$; Figure 3-8 B).

Organisms exposed to 50 µg/L of wastewater-borne TiO_2NPs presented a decrease on AChE activity of 68% compared to those exposed to ASTM-dispersed NPs at the same concentration (t-test; p = 0.002; Figure 3-8 B). The LDH activity was 118% lower in *Daphnia* exposed to 100 µg/L of wastewater-borne TiO_2NPs compared to animals exposed to ASTM-dispersed TiO_2NPs at the same concentration (t-test, p = 0.04; Figure 3-8 D). The only significant effect of wastewater-borne TiO_2NPs on GST was achieved at 25 µg/L, which caused a reduction of 49% on GST activity (Dunnett's test, p = 0.006; Figure 3-8 F). Although wastewater-borne TiO_2NPs caused no significant effects on *Daphnia´*s CAT 12.5 µg/L of ASTM-dispersed TiO_2NPs led to an increase of 449% of its activity (Dunnett's test, p = 0.03; Figure 3-8 H). At last, 25 µg/L of ASTM-dispersed TiO_2NPs was the only concentration responsible for a significant change of the LPO levels in *Daphnia*, with a 52% reduction below controls (Dunnett's test, p = 0.001; Figure 3-8 J).

3.5 Discussion

In this study, we investigated the effects of environmentally relevant concentrations of wastewater-borne AgNPs and TiO_2NPs on behavioural and biochemical markers of *D. magna* in comparison to effects induced by the same type of NPs dispersed in ASTM medium.

3.5.1 Size characterisation of NPs

As shown by TEM and SP-ICP-MS, the average size of Ag particles was stable over the exposure time while the PSD of TiO_2NPs, under an assumption of spherical particles, showed a decrease in the primary particle size over time with all tested concentrations The particle number concentration was not assessed with SP-ICP-MS due to the lack of standards with quantified particle number concentration and the kinetic nature of the study. Our results are different from Jacobasch et al. (2014), which observed a rapid increase of particle size with increasing concentrations (from 1.19 to 6 mg/L; nominal) of TiO_2NPs (Evonik Aeroxide® P25; anatase-rutile, 21 nm) immediately upon dispersion in Elendt M4 medium. Possible explanations for such a difference may be related to the different sampling procedures adopted and the media used in both studies. In the present work, the decrease of TiO_2 particle size after 12 h can be explained by the absence of a proper dispersing agent, with probable sedimentation of the formed TiO_2 agglomerates. Notwithstanding, the primary particle size of TiO_2NPs determined by TEM was found to be

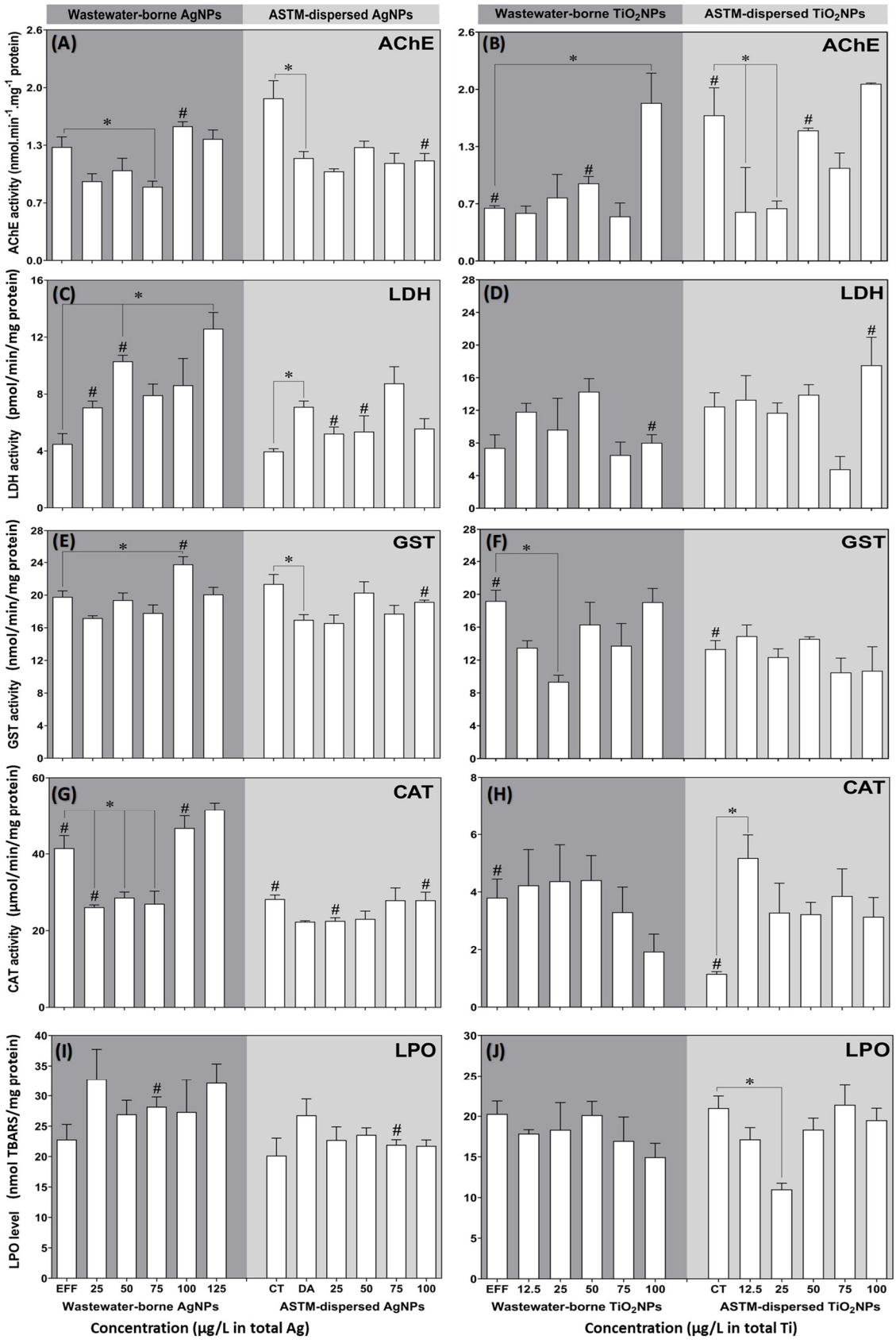


Figure 3-8: Enzymatic activities of (A, B) acetylcholinesterase (AChE), (C, D) lactate dehydrogenase (LDH), (E, F) glutathione S-transferase, (G, H) catalase, and (I, J) lipid peroxidation (LPO) levels of *Daphnia magna* after a 96 h exposure to AgNPs (A, C, E, G, I)

and TiO_2NPs (B, D, F, H, J) presented in the effluent (i.e. wastewater-borne) and dispersed in ASTM medium. Concentrations are nominal and expressed as µg/L of total Ag and total Ti. EFF, CT, and DA are NP-free controls, constituted by the effluent, ASTM medium, and dispersant agent, respectively. Bars are means of 2-4 independent replicates (with 9-10 organisms each) ± SE. The * represents significant differences ($p < 0.05$) relatively to controls in independent t-test or one-way ANOVA followed by Dunnett's post-hoc test. # represents significant differences (t-test; $p < 0.05$) between identical NP concentrations or between different controls within different matrices (effluent or ASTM medium).

relatively constant (between 23 and 26 nm) over the 96-h period. This can be explained by the overall lower number of TiO_2 particles (compared to Ag particles) analysed by TEM, which most likely had an impact on the accuracy of PSD determination, thus causing a minor deviation in their modal value over time. The diameter of TiO_2 particles represents the equivalent circle diameter since these particles are irregularly shaped within the agglomerates. Still, the different findings obtained with both analytical techniques herein applied could be explained by different sampling procedures in different media. Effectively, TEM samples were obtained from homogenized dispersions and therefore all particles and agglomerates present in suspension were considered for analysis. This contrasts with the sampling procedure adopted in SP-ICP-MS analysis, *i.e*. without homogenization. Most likely, the agglomeration had already occurred in the medium as well as during the sampling deposition (as a drying artefact), which might have compromised the primary particle size analysis to a certain degree. In summary, the effects of both types of NPs described below cannot be simply explained by the variation of PSD over time.

3.5.2 Effects on immobilization

The 96-h EC_{50} for *D. magna* exposed to ASTM-dispersed AgNPs was 113.8 µg/L of nominal Ag, while no immobilization was observed in other treatments. This value is in accordance with Völker et al. (2013a), who obtained an immobilization-based 48-h EC_{50} of 121 µg Ag/L (nominal) with AgNPs (NM-300K) dispersed in M4 medium. However, Muth-Köhne et al. (2013) reported that 0.7 and 5.5 mg/L (nominal) wastewater-borne AgNPs induced toxicity to post-fertilized zebrafish larvae after 48 h. Effectively, we found that wastewater-borne AgNPs did not affect *Daphnia* mobility, which is most likely explained by chemical transformations of AgNPs during the STP processing. The obtained STEM images showed that AgNPs were sulphidized to Ag_2S to a great extent after their passage through the STP compartments. It is recognized that one important mode of

action of AgNPs is the release of Ag^+ from the surface of particles, which is toxic to aquatic organisms upon uptake (Ratte, 1999; Völker et al., 2013b). The Ag_2S has low water solubility [solubility product (K_{sp}) = 6 × 10^{-51} M^3, at 25 °C], thus resulting in reduced bioavailability and toxicity of the AgNPs after sulphidation and/or complexation with other effluent ligands (Kaegi et al., 2011; Ratte, 1999). Our results are in agreement with Georgantzopoulou et al. (2018) and *Hartmann et al. (2019)*, which respectively showed that environmentally relevant concentrations of wastewater-borne AgNPs caused a significant reduction in toxicity to *D. magna* in acute and chronic experiments. Irrespective of their dissolution capacity and the negative impact at the biochemical level (see below), the assumption that wastewater-borne AgNPs are probably associated with the effluent solids that settled on the bottom of the test vessel in the form of precipitates (with reduction of direct exposure and ingestion by *Daphnia*), should also be considered (Georgantzopoulou et al., 2018).

Regarding the effects of TiO_2NPs on *D. magna* immobilization, Lovern and Klaper (2006) reported a nominal 48-h EC_{50} of 5.5 mg/L TiO_2NPs, while Dabrunz et al. (2011) found a lower nominal EC_{50} of 0.73 mg/L TiO_2NPs with a 96-h test duration. However, the effects of such high concentrations were not considered herein, since experimental designs where based on available PECs (Maurer-Jones et al., 2013). *Hartmann et al. (2019)* found that 25-100 µg/L (nominal) of wastewater-borne and ASTM-dispersed TiO_2NPs did not affect *D. magna* reproduction in up to six generations. In our study, the high mobility of *Daphnia* exposed to wastewater-borne TiO_2NPs could be justified by the absence of transformation processes of the TiO_2 particles during their passage throughout the STP compartments. This was confirmed by STEM analysis. On average, ~ 97% of engineered TiO_2NPs are released into surface waters in the non-transformed form and the only known transformation process that occurred during the wastewater treatment is the formation of calcium titanate (Adam et al., 2018).

3.5.3 Effects of the dispersing agent

In our study, the AgNPs were sterically stabilized with a lipophilic mixture of two non-ionic synthetic dispersants in order to improve the dispersibility of particles, thereby preventing their settling and agglomeration. It is known that dispersing agents may sometimes increase NP toxicity, mainly through improved dispersibility, thus promoting the interaction between NPs and cell surfaces (Deng et al., 2017). Effectively, dispersing agents with

lipophilic properties can interact with the lipid bilayer of the cell membrane, thereby facilitating the unintentional entry of NPs into the cell (Deng et al., 2017; Handy et al., 2012). Therefore, the potential effects of dispersant agents cannot be excluded from laboratory experiments, since they may have a direct effect on the toxicity mechanism caused by NPs (Handy et al., 2012). Our results support evidences of DA toxicity to *Daphnia*, since, after 96 h: (i) there was a decrease in the swimming height of DA exposed animals compared to the CT exposed ones; and (ii) the allocation time of DA exposed animals was affected in the three zones of the test vessel, being particularly evident at the top, thus indicating an avoidance response. The lipophilic mixture of surfactants was probably responsible for the induced effects, not only at the individual level but also at the biochemical level, since the DA caused inhibition of both AChE and GST activity, and induction of LDH activity.

Very few studies take into account the effects of dispersing agents in ecotoxicological tests with aquatic biota. For example, 10 µg/L of the same DA caused no significant effects on length, survival rate and body burden of the freshwater amphipod *Hyalella azteca* (Kühr et al., 2018). Notwithstanding, Pettersson et al. (2000) confirmed that some non-ionic surfactants (26 detergents and 5 softeners) presented acute toxicity to *D. magna*, and the resulting toxicity mechanism increased after 48 h with the increasing number of carbon atoms in the carbon chain of each surfactant molecule.

3.5.4 Effects of the effluent without NPs

The results of EFF at individual and biochemical levels suggest that the background effluent used in TiO_2NP experiments induced neurotoxicity and oxidative stress in exposed animals. In *Daphnia* subjected to this type of effluents, the neurotoxicological responses obtained at the biochemical level had also implications at the individual level, since the allocation time at the bottom of the vessel were significantly reduced over time.

It is well accepted that STP effluents comprise a complex mixture of substances which include, among others, soluble microbial products derived from the metabolism of different types of bacteria consortia normally present in synthetic sewage, various types of DOM, colloidal substances, electrolytes, pharmaceutical products, drugs and potentially harmful contaminants not eliminated by STPs (Kim and Farnazo, 2017; Mahlalela et al., 2017; Ren et al., 2017; Zhou et al., 2015). This amalgam of compounds was probably responsible for

the observed effects on *D. magna* exposed to each effluent type. The different ages of effluents at the beginning of the exposure assays (4-week and 3-month in AgNP and TiO_2NP experiments, respectively) may also have contributed to the observed effects. It has been shown that ageing and properties of the exposure matrix may change not only its intrinsic toxicity but also the respective NP-associated toxicity (Cupi et al., 2015; Seitz et al., 2015). Consequently, the obtained effects caused by all NP-containing treatments at both behavioural level and biochemical level should be interpreted in the light of the effect range of the respective background effluents (Georgantzopoulou et al., 2018).

3.5.5 Behavioural effects of wastewater-borne and ASTM-dispersed NPs

The swimming height of *D. magna* was not affected by wastewater-borne AgNPs at both time points and minor effects were observed with ASTM-dispersed. On the contrary, at 0 h, the swimming height was significantly reduced at high concentrations of wastewater-borne TiO_2NPs, thus showing an immediate response of animals exposed to this treatment. Pokhrel and Dubey (2012) showed that vertical migration of *D. magna* was not affected by 2 µg/L of citrate-capped AgNPs (55.9 ± 14.6 nm), but the combination of citrate-AgNPs and a predator cue (dragonfly nymph, *Anax junius*) led to a significant vertical upward response compared to the predator treatment alone. Furthermore, Brausch et al. (2011) found that the vertical migration of *D. magna* was not affected by fullerene (C_{60}; 117 ± 50.6 nm) and functionalized C_{60} NPs (1040 ± 0.06 nm). However, the swimming height of animals increased after 45 min when 545 mg/L of C_{60} NPs were administered together with food particles. The obtained differences were explained as an exclusive response of the animals to food particles instead of the dispersed NPs themselves (Brausch et al., 2011). On this regard, Wiklund et al. (2012) showed that *D. magna* placed in a medium with sucralose present an increased swimming height comparatively to controls. Therefore, it seems that other factors like *e.g.* light, predator cues and/or food availability may have more influence on the *Daphnia*'s swimming height than the NPs themselves. Taking these pieces of evidence into account, further investigations are therefore necessary to assess the suitability of this behavioural marker in future investigations.

The analysis of allocation time at 0 h revealed dissimilarities in the behaviour pattern of *Daphnia* exposed to AgNPs dispersed in both matrices. Whereas the allocation time was not affected with wastewater-borne AgNPs (*i.e. Daphnia* showed a similar distribution

within the three regions of the vessel), the animals exposed to ASTM-dispersed AgNPs spent more time at the top and at the bottom of the test vessel. This is in accordance with the respective 96-h EC_{50}'s discussed above. Indeed, contrary to ASTM-dispersed AgNPs, the wastewater-borne AgNPs did neither affect the animal's mobility nor had any influence on their allocation time. The lack of changes in allocation time with wastewater-borne AgNPs could be justified by the chemical transformations of AgNPs into other lesser toxic species (*e.g.* Ag_2S) in the effluent (Kaegi et al., 2011). This might have contributed to the absence of effects within this treatment, at least at the behavioural level, since a significant inhibition of AChE activity was observed with 75 µg/L of wastewater-borne AgNPs.

The irregular swimming behaviour of animals exposed to ASTM-dispersed AgNPs, could be indicative of the impairment of the nervous system with a consequent loss of orientation. Poynton et al. (2012) showed that 10-day old *D. magna* exposed to $AgNO_3$ presented a downregulation of several gene sets involved in nervous system function, locomotion, behaviour and developmental processes. In agreement with these findings, important downregulated mechanisms related to the nervous system functioning were probably disturbed at the tested concentrations of ASTM-dispersed AgNPs, thus contributing to the abnormal swimming behaviour of animals. Nevertheless, such mechanisms are not directly related to AChE, since no alterations in the activity of this enzyme were observed at all tested concentrations of ASTM-dispersed AgNPs. Besides, the increased tendency of the animals to swim towards the vessel surface at increasing concentrations of ASTM-dispersed AgNPs, at 0 h, may be interpreted as a rapid avoidance response, and probably based on a chemoreception mechanism. However, this response was lost after 96 h due to a sedimentation process of the NPs or a yet unknown adaptation process. A similar swimming behaviour was already observed in the freshwater snail *Physa acuta* exposed to 30 µg/L of carboxy-functionalized AgNPs (1-10 nm) (Justice and Bernot, 2014). According to these authors, this behaviour is considered as an attempt of the animals to crawl out of the water phase, which is regarded as an avoidance response based on unknown chemical perception mechanisms.

The effects of TiO_2NPs on *D. magna* behaviour were studied by Noss et al. (2013a), which found a concentration-dependent aggregation of animals in the central region of a vessel immediately upon exposure to 1, 5 and 20 mg/L of TiO_2NPs (Aeroxide® P25; 21 nm). This aggregation phenomenon was interpreted as a kind of swarming behaviour, a well-known

response of *Daphnia* towards predator cues, abiotic factors, and neuro-active drugs (Čolović et al., 2013; Noss et al., 2013a; Szulkin et al., 2006). However, Noss et al. (2013a) also noticed that the swarming behaviour of TiO_2NP exposed animals disappeared after 24 h at all tested concentrations. In contrast, the *Daphnia´s* swarms of our study immediately exposed to wastewater-borne and ASTM-dispersed TiO_2NPs spent less time in the centre of the vessel. Still, this response was lost after 96 h with wastewater-borne TiO_2NPs but it was maintained with ASTM-dispersed TiO_2NPs. Since TiO_2NPs are rarely transformed during WWTP processing (Adam et al., 2018), a similar behavioural response would be expected in both TiO_2NP matrices, which did not effectively occur, thus indicating that the two distinct matrices led to distinct effects over time. Despite these observations and taking into account the available literature, this is the first study who shows that environmentally relevant concentrations of wastewater-borne TiO_2NPs (Sun et al., 2014) had a significant impact on the allocation time of *D. magna*. Therefore, this parameter should be considered as a good behavioural marker for an effective assessment of the effects caused by TiO_2NPs.

3.5.6 Biochemical effects of wastewater-borne and ASTM-dispersed NPs

At the biochemical level, the negative impacts were generally more observed in the wastewater-borne NPs' exposure compared with the ASTM-dispersed NPs. This is particularly evident for high concentrations of wastewater-borne AgNPs, which caused a significant increase in the enzymatic activity of both GST and CAT. These differences can be interpreted in the light of the particular responses of animals against the two tested matrices with different degrees of physicochemical complexity, together with the dispersed NPs. Probably, the wastewater-borne NPs induced the formation of reactive oxygen species (ROS) that enhanced particular toxicity mechanisms (Li et al., 2018; Liu and Wang, 2017). On the other hand, the likely formation of a DOM-protein eco-corona coating around wastewater-borne TiO_2NPs might have contributed to the limited irradiation of particle surfaces in the effluent, thus contributing to the potential scavenging of harmful ROS that could have been formed under prevalent irradiance conditions (Seitz et al., 2015; Shakiba et al., 2018). Regardless of the exposure matrices, the less significant effects of TiO_2NPs compared to AgNPs on the generality of the studied biochemical markers might have been due to the capability of the former to form stable agglomerates, thus becoming less available to *Daphnia* (Sharma, 2009; Zhou et al., 2015).

3.5.6.1 **Effects on AChE activity**

In our study, there was an inhibition of AChE activity with 75 µg/L of wastewater-borne AgNPs and with 12.5-25 µg/L of ASTM-dispersed TiO_2NPs, thus suggesting neurotoxicity of both types of NPs, but not in a dose-related way. The available literature on the effects of metals, metal oxides and respective nano-counterparts on AChE activity revealed contradictory information due to the absence of standardization in experimental protocols, such as *e.g.* different animal species, different administration routes, dosing, sizes, shapes and crystal forms of NPs (Šinko et al., 2014; Ulm et al., 2015). For example, our results are contrary to Ulm et al. (2015), which showed a concentration-dependent increase of AChE activity in *D. magna* neonates with 1-10 µg/L of citrate-coated AgNPs (18.2 ± 10.1 nm) dispersed in standard culture medium for 48 h. Yet, our findings are concordant with Katuli et al. (2014), which observed an inhibition of AChE activity in zebrafish (*Danio rerio*) erythrocytes with 2 and 4 mg/L of AgNPs (25-100 nm) after 21 d. In the same way, it was also demonstrated that AChE activity can be inhibited by TiO_2NPs. For example, Khalil (2015) observed that 10, 50 and 100 µg/kg of TiO_2NPs (anatase-rutile; 20-40 nm) inhibited the AChE activity of the earthworm *Pheretima hawayana*, in a concentration-dependent manner, after 28 days. Guan et al. (2018) reported that the AChE activity was inhibited in the blood clam (*Tegillarca granosa*) exposed to 0.1-10 mg/L of TiO_2NPs (anatase; 35 ± 5 nm) for 96 h, thereby suggesting the occurrence of neurotoxicity associated mechanisms. Through following a modification of the colorimetric Ellman's assay, Wang et al. (2009) showed that the adsorption of 800 mg/L (final concentration) of each rutile-DJ3 (50 nm) and anatase-HR3 (5-10 nm) TiO_2NPs to AChE inhibited the activity of this enzyme through the sorption of NPs to the protein after 3 min. Although these authors did not test any type of AgNPs, it was demonstrated that TiO_2NPs (and other oxide-type NPs) caused a lower reduction of AChE activity compared to other metal-type NPs. Although the inhibitory mechanism of AChE activity by AgNPs remains unclear, it has been suggested that the long-term activity inhibition of the highly purified human AChE is due to the released Ag^+, which binds to the enzymatic complex and cause its irreversible inactivation through loss of protein structure caused by the reaction of charged Ag ions with the amino acids of the enzyme (Vrček and Šinko, 2013). This inhibitory response could be effectively explained by the adsorption of AChE onto the NP surface, with the subsequent inactivation of the enzyme due to conformational changes after surface coverage and ion release (Šinko et

al., 2014; Vrček and Šinko, 2013; Wang et al., 2009). On the contrary, the significant induction of AChE activity with 100 µg/L of wastewater-borne TiO_2NPs could be explained by a *de novo* synthesis of the enzyme to cope with the stress imposed by the highest concentration of TiO_2NPs. A similar compensatory mechanism was already reported in *D. magna* exposed to insecticides (Ren et al., 2017). Likewise, this adaptive mechanism was also observed in the gill and digestive gland of the marine scallop (*Chlamys farreri*) treated with 1 mg/L of TiO_2NPs (anatase-rutile; 21 nm) for 14 days (Xia et al., 2017). Still, the detailed mechanism by which NPs inhibited or stimulated the AChE activity remains unclear in terms of the specific binding interactions between the enzyme and each particular type of NPs.

3.5.6.2 **Effects on anaerobic metabolism**

Lactate is a key metabolite of the anaerobic metabolism and when the energy demand by tissues and/or organisms cannot be met only by aerobic respiration, an increase in lactate concentration will occur (Rathee et al., 2016). The LDH is an oxidoreductase enzyme with a high catalytic activity, which reversibly converts pyruvate into lactate with the conversion of NADH to NAD^+ (Diamantino et al., 2001; Rathee et al., 2016). Therefore, the evolution of LDH activity can function as a good biochemical marker of the anaerobic metabolism triggered by contaminants or other high-energy demanding factors. Although TiO_2NPs did not cause any effects on *Daphnia*'s LDH activity, there was a significant induction in the activity of this enzyme with 50 and 125 µg/L of wastewater-borne AgNPs, thus suggesting an increase of the anaerobic metabolism.

3.5.6.3 **Oxidative stress responses**

Together with superoxide dismutase, the CAT represents the earliest line of protection against ROS and both enzymes are pivotal for the antioxidant defence system of the cells (Halliwell and Gutteridge, 2015). On the other hand, the GST is a member of a large family of multifunctional enzymes involved in the cellular detoxification of many xenobiotics through phase II of the biotransformation process, having also an important role in the oxidative stress response (Halliwell and Gutteridge, 2015). Accordingly, an induction in the activity of GST and/or CAT indicate an instigation of the detoxification processes used to either catalyse the conjugation of GSH with a xenobiotic or a particular ROS species (in the case of induction of GST activity) and/or a H_2O_2 reduction during oxidative stress (in

the case of induction of CAT activity) (Klaper et al., 2009). In our study, it appears that ASTM-dispersed AgNPs did not cause any oxidative stress to *D. magna*. On the contrary, the wastewater-borne AgNPs seem to induce oxidative stress to the animals, since significant changes in the activity of both GST (induction with 100 µg/L of total Ag) and CAT (impairment with 25-75 µg/L of total Ag) were registered. Despite these alterations, the exposure to AgNPs resulted in null oxidative damage to *Daphnia*, since the LPO levels were neither affected with wastewater-borne AgNPs nor with ASTM-dispersed AgNPs; in general, a similar response occurred in animals exposed to wastewater-borne TiO_2NPs and ASTM-dispersed TiO_2NPs. As pointed by Xiong et al. (2011), this might be explained by the extremely low concentrations of both types of NPs, which were incapable of generating enough ROS to trigger oxidative damage on lipids.

The inhibition of CAT activity with wastewater-borne AgNPs could be explained by the build-up of H_2O_2 and other ROS inside the cells, thus contributing to an imbalance between oxidative stress and the antioxidant defence system through a process of enzymatic denaturation, with a consequent loss of enzymatic activity. A similar inhibitory effect on CAT activity was obtained in the brain tissues of two freshwater fish, *viz.* Nile tilapia (*Oreochromis niloticus*) and redbelly tilapia (*Tilapia zillii*) exposed to 4 mg/L of AgNPs (< 100 nm) dispersed in deionized water, thus suggesting an over-accumulation of ROS which exceeded the scavenging ability of the antioxidant defence system, yet without any oxidative damage (Afifi et al., 2016). However, different results were obtained by Ulm et al. (2015), which observed an increase of CAT activity in *D. magna* neonates submitted to 0.5-10 µg/L of citrate-coated AgNPs (18.2 ± 10.1 nm) and 0.01-0.3 µg/L of Ag^+ for 48 h. These authors suggested that the increment of CAT activity with increasing concentrations of AgNPs are indicative of ROS production, while the induction and posterior decrease of CAT activity with increasing Ag^+ concentrations was due to the increased production of hydroxyl radicals by Ag^+, which, in turn, leads to the rapid inactivation of enzymatic activity caused by high concentrations of H_2O_2. As above-mentioned, the Ag^+ is known to be released from AgNPs during oxidation processes, which requires both dissolved O_2 and protons (H^+), and this reaction culminates with the release of injurious peroxide intermediates, thereby leading to oxidative stress (Liu and Hurt, 2010).

Also relevant to our study was the increment of CAT activity with all tested concentrations of ASTM-dispersed TiO_2NPs, albeit only significant at the lowest ones. These findings are

substantiated by Klaper et al. (2009), which showed that the CAT activity of *Daphnia pulex* was enhanced with 75-500 mg/L of TiO_2NPs (anatase, < 25 nm) dispersed in moderately hard reconstituted water for 24 h, thus reflecting oxidative stress. Canesi et al. (2010) showed that the activity of CAT and GST increased in the digestive glands of the bivalve *Mytilus galloprovincialis* exposed to 1-5 mg/L of TiO_2NPs (Degussa/Evonik Aeroxide® P25; anatase-rutile; 22 nm) dispersed in artificial seawater for 24 h. Nevertheless, in our study, the GST activity was unchanged with ASTM-dispersed TiO_2NPs, which means that the imposed chemical stress within the range of tested concentrations was not strong enough to instigate other pathways involved in the antioxidant system (*e.g.* those associated to GST) after an apparent induction of CAT activity. Since the primary outcome of the antioxidant defence system after a cascade of reactions due to NP exposure is the activation of phase II detoxification enzymes, our results are in agreement with the hierarchical oxidative stress hypothesis initially proposed by Nel et al. (2006) to describe the three-tiered mechanism for NP-mediated oxidative stress.

3.6 Conclusion

In the present study, the integrated approach at both behavioural and biochemical levels clearly offered the advantage of allowing for an immediate (0 h) and early (96 h) detection of the ecotoxicological status of *D. magna* before the occurrence of more severe effects after longer exposure periods. Aligned with the published literature on the fate and effects of wastewater-borne NPs to aquatic biota, our approach and findings went further than the state of the art and, for *D. magna* exposed to environmentally relevant concentrations of NPs, demonstrated that: (i) the wastewater-borne TiO_2NPs are prone to induce disturbances on *D. magna* swimming behaviour, particularly upon their prompt exposure; and (ii) even though wastewater-borne AgNPs caused minor effects on the swimming behaviour of *Daphnia* over 96 h, it is clear that the antioxidant machinery of animals was affected by wastewater-borne AgNPs. In brief, the chosen behavioural-related parameters proved to be suitable for the assessment of toxic effects caused by wastewater-borne NPs in *Daphnia*. Besides, the battery of selected biochemical markers can effectively function as important warning indicators for the detection of adverse effects caused by this type of xenobiotics. Accordingly, this behavioural-biochemical integrative approach can therefore provide essential and early warning background information to the environmental

policymakers and stakeholders enrolled in the environmental risk assessment of NPs present in WWTP effluents.

Though the toxicological effects induced by wastewater-borne NPs may vary due to different factors, like *e.g.* type of NPs, exposure medium, and dispersing agent, our findings added relevant information to the current topic. Regarding the way by which wastewater-borne NPs interact with biotic (*e.g.* molecules, cells, individuals) and/or abiotic (*e.g.* pH, ionic strength, DOM) variables within the effluent, it is likely that their mechanism of action could be much more complex compared to the corresponding one after dispersion in less elaborate matrices, like *e.g.* ASTM medium. Besides NPs, the presence of other additional substances and unknown xenobiotics in the effluent should also be considered. In the forthcoming investigations, it will be therefore advisable to gather additional data about the interaction of biotic and abiotic factors regarding each NP-matrix interface in order to better comprehend the associated toxicological mechanisms. Also important is the choice of suitable dispersion agents during NP manufacturing, since some detrimental effects were observed with the chemicals used for AgNP solubilisation. This highlights the need for a deeper understanding of the chemical composition of the dispersion agent in order to better comprehend the potential resulting side-effects within a particular dispersion, not only in standardized laboratory media but also in more complex matrices. In this regard, the aggregation state of wastewater-borne NPs could inevitably change in complex environments and there is still a gap in the discussion about the use of suitable solubilizing agents in the view of their environmental relevance. Arguably, the use of natural compounds, like *e.g.* DOM would be of greater relevance since they occur in the real environment. Bearing these environmental implications in mind, future integrated approaches should contemplate longer periods of time, like those followed in chronic and multi-generational studies. Concluding, the usual ecotoxicity tests carried out in the laboratory to evaluate NPs' toxicity from wastewater effluents should involve other than the routinely used synthetic waters as they may underestimate the toxicity of NPs.

3.7 Supporting Information

Model wastewater treatment plant (STP)

Each lab-scale STP consisted of three reactors, *viz.* denitrification, nitrification, and a second clarifier, which were fed with active sewage sludge (2.5 g dry mass/L, sieved at ≤ 2 mm) from a municipal STP (51°09'N 8°16'E, Schmallenberg, Germany). All lab-scale STPs ran in a temperature-controlled room (20-25 °C) and were continuously fed with artificial wastewater with a defined composition (OECD, 2001). After an adaptation phase of 5-6 days, the STPs reached a stable condition, namely by presenting elimination rates of dissolved organic carbon (DOC) > 80% and constant concentrations of ammonium, nitrate, and nitrite, in accordance with the OECD (2001) standards. The AgNP dispersions and artificial wastewater stock solutions were concentrated 10-fold higher than the respective nominal inlet concentrations, stored at 4 °C and freshly prepared every 3-4 days. Via a tube system (PLP 33; SP04/3.5 K, behr Labor-Technik), both suspensions were diluted at 1:10 with tap water and pumped into the denitrification reactor of the STPs (flow: 750 mL/h; retention time: 6 h). The dispersions of wastewater-borne TiO_2NPs were prepared daily (concentration of stock dispersion equal to inlet concentration) and pipetted manually into the STP units to avoid sedimentation. The pH, O_2 saturation, ammonium, nitrate, and nitrite were monitored regularly in all effluents and kept constant within the recommended values (OECD, 2001). After an operation time of 6-10 days, the effluents were collected in polyethylene containers (Züchner GmbH, Köln, Germany) and stored (AgNPs: four weeks; TiO_2NPs: three months) at 4 °C until used as working matrices for exposure experiments.

Determination of total silver (Ag) and total titanium (Ti) concentrations in the effluents

Measurement of total Ag:

Inductively coupled plasma mass spectrometry (ICP-MS; iCAP Qc, Thermo Fisher Scientific, Bremen, Germany) was used for the determination of total Ag content in STP effluents. Before analyses, samples were taken out of the fridge and shaken for 30 minutes with a shaking machine (Edmund Bühler, Bodelshausen, Germany). The samples for total Ag analysis were digested with concentrated nitric acid (> 68%, Trace Analysis Grade, Fisher Scientific, Loughborough, UK) for 90 min and diluted 100 times afterwards to obtain

a concentration of 2.85% (w/v) HNO_3. Instrument calibration was done on the same day with an Ag^+ standard solution (Inorganic Ventures, Christiansburg, VA, USA). All aqueous samples were measured 10 times and quantified using the isotope $^{107}Ag^+$. Indium (Inorganic Ventures, Christiansburg, VA, USA) served as the internal standard. All concentrations were calculated from calibration curves using the internal standard correction. For total Ag measurements, samples were diluted by a factor of 100 and each sample was measured 10 times with a dwell time of 10 ms. The limit of detection (LOD) and limit of quantification (LOQ) for $^{107}Ag^+$ were 0.06 and 0.19 µg/L, respectively.

Measurement of total Ti:

For total Ti analysis, an effluent aliquot of 4 mL was placed in a Teflon vial, acidified with 0.8 mL of nitric acid (69%, Suprapur®, Carl Roth, Germany) plus 0.2 mL of hydrofluoric acid (40%, Suprapur®, Merck, Germany), and digested in an UltraClave II (MLS GmbH, Germany) microwave (25 min heating up to 220 °C, 30 min on 220 °C, maximum pressure 80 bar). For the complexation of hydrofluoric acid, 1 mL of 4% boric acid (Merck, Germany) was added after the digestion and samples filled up to 15 mL with ultrapure water. The determination of total Ti concentrations was performed by inductively coupled plasma optical emission spectrometry (ICP-OES; Agilent 720, Agilent Technologies, Waldbronn, Germany) set on 338.377 nm. Commercially available 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. Linear regression was used by the ICP-OES software to calculate function and LOD (calculated as three times the standard deviation (SD) of blank samples divided by the regression line slope). The LOQ was calculated as three times the LOD. To validate calibration, certified aqueous reference materials (TMDA 70.2, Environment Canada) and quality control samples were independently prepared from calibration samples (*e.g.* from multielement standard Merck IV, Merck, Darmstadt, Germany) and measured in parallel. For total Ti measurements, samples were diluted by a factor of 19. The LOD and LOQ for Ti measurements were 0.5 and 1.4 µg/L, respectively. Water was purified using an ELGA Pure Lab Ultra water purification system (> 18 MΩcm). All samples were measured in triplicate (internal triplicate measurement).

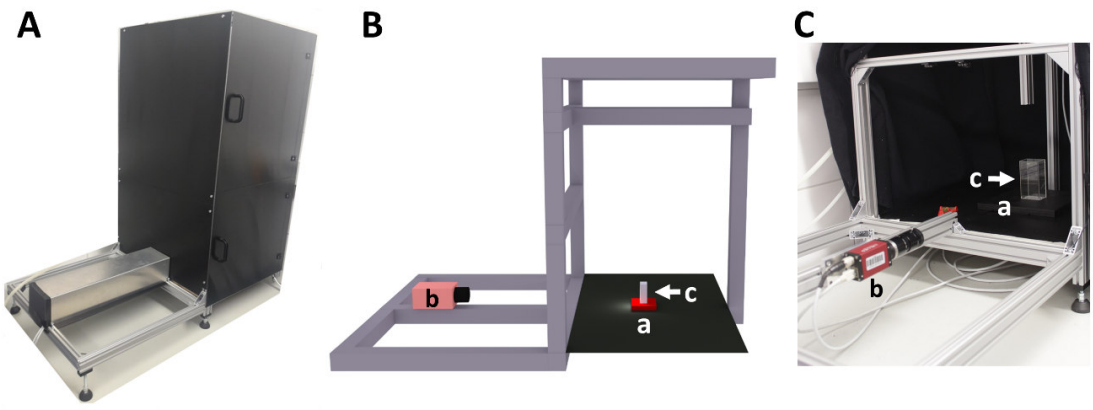


Figure S3-1. Detailed illustrations of the test chamber used for the simultaneous recording of the swimming behaviour of *D. magna* in real-time. (A) Picture of the computer vision system, covered with black PVC plates in order to avoid light from the outside. (B, C) Detailed view of the inside of the custom-built computer vision system, showing the (a) background infrared (IR) illumination source with 850 nm, and the (b) IR-sensitive camera, placed 45 cm away from the test vessel (c) during the recording process. The tracking system camera was a monochrome Manta G-223B NIR (Allied Vision Tech. GmbH, Stadtroda, Germany), with a maximum framerate of 53.7 fps, two megapixels resolution, focal length 50 mm, maximum range 20 mm and a GigE Vision interface. The camera was connected to a desktop PC with the custom-built software (Institute of Real-Time Learning Systems, University of Siegen, Germany) for setting properties, starting each experiment and generating the required datasets for subsequent data analyses (Kunze et al., 2016).

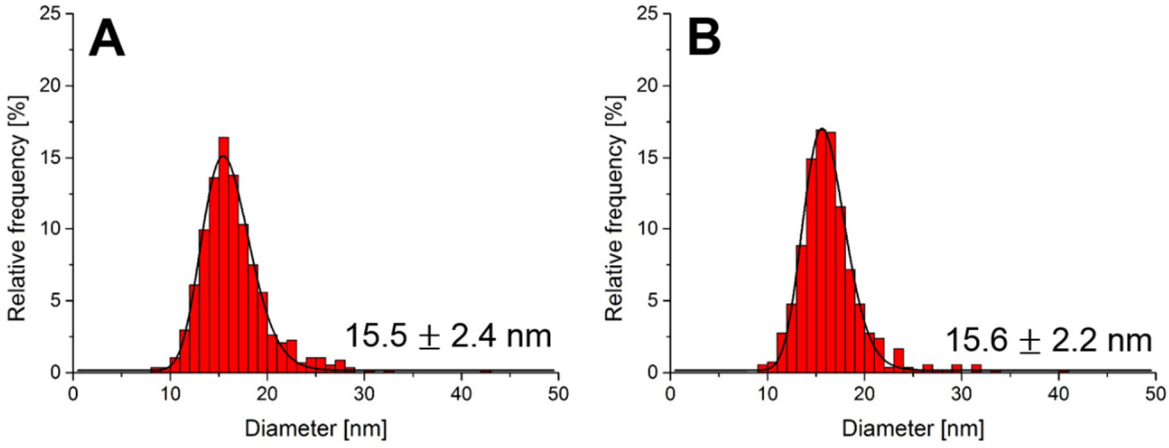


Figure S3-2. Size distribution of ASTM-dispersed AgNPs at 0 h (A, n = 573) and 96 h (B, n = 543). Particle diameters were based on STEM data and were determined with ImageJ (Version 1.50i). Histograms were fitted with a log-normal function and the results are given as mode ± SD.

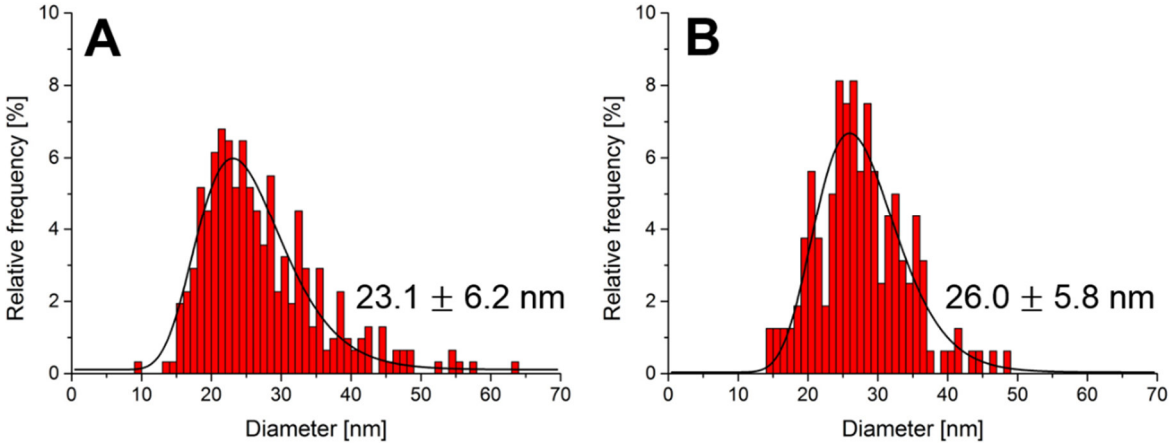


Figure S3-3. Size distribution of ASTM-dispersed TiO_2NPs at 0 h (A, n = 309) and 96 h (B, n = 160). Particle diameters were based on STEM data and were determined with ImageJ (Version 1.50i) and are to be considered as equivalent circle diameters due to the non-spherical shape of particles. Histograms were fitted with a log-normal function and the results are given as mode ± SD.

Table S3-1. Main instrumental parameters of inductively coupled plasma mass spectrometry (ICP-MS) and inductively coupled plasma optical emission spectrometry (ICP-OES) used for the determination of total Ag and total Ti in STP effluents, respectively.

Used for	Total Ag measurement	Total Ti measurement
	ICP-MS iCAp Qc (Thermo Fisher Scientific, Bremen, Germany)	ICP-OES 720, Agilent Technologies (axial plasma view)
Nebulizer	Pneumatic PFA µ-FLOW nebulizer	SeaSpray nebulizer
Spray chamber	Peltier-cooled cyclonic quartz	IsoMist Programmable Temperature Spray Chamber
Radio-frequency power (W)	1400	1200
Torch injector inner diameter (mm)	2.5	2.4
Cooling flow (L/min)	14	15
Auxiliary flow (L/min)	0.8	1.5
Nebulizer flow (L/min)	1.0	0.75
Sampling position (mm)	4	n/a

* n/a – not applicable.

Table S3-2. Physicochemical parameters of the treatments (test medium with NPs) used in exposure experiments. Each value corresponds to a single measure in one vessel, assessed at 0 and 96 h. EFF, CT, and DA are effluent, ASTM medium and dispersing agent controls, respectively.

Experiments and treatments	Nominal concentrations of NPs, including controls (µg/L)	0 h			96 h		
		pH	Temperature (°C)	Dissolved O_2 (mg/L)	pH	Temperature (°C)	Dissolved O_2 (mg/L)
1A: Wastewater-borne AgNPs	EFF (0)	7.1	19.3	9.7	7.9	21.3	8.3
	25	7.2	19.7	10.8	7.1	20.3	7.9
	50	7.0	18.9	10.5	6.7	20.4	7.5
	75	7.1	19.3	10.2	6.7	19.9	7.9
	100	7.4	19.8	9.8	7.4	20.1	8.2
	125	7.4	18.7	8.9	8.2	24.2	7.6
1B: ASTM-dispersed AgNPs	CT (0)	7.1	20.3	7.4	6.9	19.8	7.4
	DA (0)	7.9	19.4	8.8	8.1	18.7	8.6
	25	8.0	19.4	8.7	8.1	18.6	8.4
	50	8.3	20.1	10.0	8.3	19.2	9.4
	75	8.0	19.0	8.9	8.0	19.5	8.1
	100	7.9	18.8	8.8	8.2	19.3	8.2
	125	7.9	18.7	9.0	7.9	20.4	8.6
2A: Wastewater-borne TiO_2NPs	EFF (0)	6.8	20.4	8.6	6.8	21.3	8.4
	12.5	7.1	21.1	8.6	7.1	21.3	7.5
	25	7.1	20.7	8.6	7.2	22.5	7.8
	50	7.3	20.7	8.6	7.3	20.5	8.5
	75	8.7	20.0	8.8	6.7	21.2	7.4
	100	6.8	21.2	8.7	6.7	21.0	7.9
2B: ASTM-dispersed TiO_2NPs	CT (0)	6.4	21.1	8.6	6.4	21.4	7.9
	12.5	6.9	20.8	8.5	6.8	21.1	8.1
	25	6.4	20.9	8.6	6.6	20.7	8.2
	50	7.4	19.9	8.9	6.6	20.9	8.3
	75	6.7	19.9	9.0	6.6	20.1	7.8
	100	6.8	20.0	8.8	6.5	21.2	7.3

Table S3-3. Main instrumental parameters of single particle inductively coupled mass spectrometry (SP-ICP-MS) for the characterization of the particle size distribution of AgNPs and TiO_2NPs.

	$^{107}Ag^+$	$^{49}Ti^+$
Radio frequency power (W)	1450	1300
Torch injector inner diameter (mm)	1.5	1.0
Cooling flow (L/min)	14	14
Auxiliary flow (L/min)	0.8	0.8
Nebulizer flow (L/min)	1.0	0.5
Sampling position (mm)	3.5	1.0

Table S3-4. Percentual immobilization of 14-day old *Daphnia magna* (n = 4-5; 9-10 animals per replicate) after 96-h of exposure to the tested treatments. EFF, CT, and DA are the controls (without NPs) for the effluent, ASTM medium, and dispersant agent, respectively.

Experiments and treatments	Controls and nominal concentrations of NPs (µg/L)	Immobilization (%)
1A: Wastewater-borne AgNPs	EFF (0)	0
	25	0
	50	0
	75	0
	100	0
	125	0
1B: ASTM-dispersed AgNPs	DA (0)	0
	CT (0)	0
	25	0
	50	0
	75	0
	100	0
	112.5*	32
	125	100
	150*	100
2A: Wastewater-borne TiO_2NPs	EFF (0)	2
	12.5	0
	25	0
	50	2
	75	0
	100	4
2B: ASTM-dispersed TiO_2NPs	CT (0)	0
	12.5	0
	25	2
	50	0
	75	0
	100	2

* Performed additionally to assess the EC_{50} (not used in the behaviour and biochemical assays).

Table S3-5. Results of the linear mixed effect modelling of 'zone × treatment' interaction on the allocation time of *Daphnia magna*, following the 2-min exposure to different treatments and assessed at 0 and 96 h. The coefficient estimates, standard errors (SE), degrees of freedom (*df*), *t*-values and *p*-values for fixed effects are shown. The significant differences ($p < 0.05$) within controls, and within NP concentrations and controls are marked in bold. EFF, CT, and DA are the controls (without NPs) for the effluent, ASTM medium, and dispersant agent, respectively. The marginal R^2 refers to the % of variance explained by fixed effects and the conditional R^2 represents the % of variance explained by the entire model, including both fixed and random effects (Nakagawa et al., 2017; Nakagawa and Schielzeth, 2013).

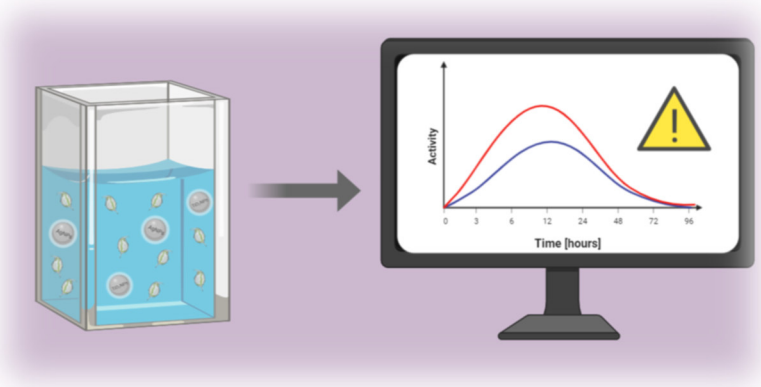
Experiments and treatments	Fixed effects	Time point	Estimate	SE	*df*	*t*	*p*	Marginal R^2 (%)	Conditional R^2 (%)
1A: EFF-CT	(Intercept)	0	35.03	11.62	57.51	3.01	**0.004**		
		96	13.99	13.54	39.77	1.03	0.307		
	Zone 1	0	17.80	6.69	93.21	2.66	**0.009**	6	14
		96	22.67	9.08	35.50	2.50	**0.017**	9	22
	(Intercept)	0	40.48	5.77	69.31	7.01	**< 0.001**		
		96	54.44	14.16	51.77	3.84	**< 0.001**		
	Zone 2	0	-14.30	3.42	93.27	-4.18	**< 0.001**	15	19
		96	-13.52	9.21	56.41	-1.47	0.147	3	9
	(Intercept)	0	-9.08	9.43	53.80	-0.96	0.339		
		96	-10.50	6.00	60.00	-1.75	**0.085**		
	Zone 3	0	16.97	5.37	93.19	3.16	**0.002**	9	17
		96	10.50	4.05	60.00	2.59	**0.011**	10	10
1A: Wastewater-borne AgNPs	(Intercept)	0	56.81	6.00	37.28	9.48	**< 0.001**		
		96	60.40	6.47	333.00	9.33	**< 0.001**		
	Zone 1	0	2.18	1.38	338.55	1.59	0.113	7	3
		96	0.20	1.62	333.00	0.12	0.904	5	5
	(Intercept)	0	11.97	2.97	55.05	40.27	**< 0.001**		
		96	26.81	4.87	92.14	5.50	**< 0.001**		
	Zone 2	0	1.00	0.72	329.27	1.39	0.165	5	2
		96	0.86	1.18	329.64	0.73	0.469	1	7
	(Intercept)	0	31.56	5.16	29.79	6.12	**< 0.001**		
		96	9.63	3.31	66.22	2.91	**< 0.001**		
	Zone 3	0	-1.68	1.15	344.49	-1.46	0.145	5	3
		96	1.05	0.78	329.18	1.35	0.177	5	2
1B: DA-CT	(Intercept)	0	39.98	12.04	14.65	3.32	**0.004**		
		96	-7.85	11.89	86.48	-0.66	0.511		
	Zone 1	0	12.43	5.47	80.35	2.27	**0.025**	4	40
		96	44.38	7.00	87.25	6.34	**< 0.001**	31	61
	(Intercept)	0	22.30	8.06	24.72	2.77	**0.010**		
		96	58.54	10.83	89.19	5.41	**< 0.001**		
	Zone 2	0	4.05	4.24	80.65	0.95	0.343	1	24
		96	-17.54	6.44	87.68	-2.72	**0.007**	8	8
	(Intercept)	0	6.29	5.59	8.52	1.13	0.291		
		96	0.14	0.39	86.08	0.35	0.724		
	Zone 3	0	1.81	1.89	80.15	0.96	0.342	0	61
		96	0.33	0.13	16.51	2.59	**0.019**	0	98

Table S3-5: Continued.

1B: ASTM-dispersed AgNPs	(Intercept)	0	34.14	5.88	25.09	5.81	**< 0.001**		
		96	97.64	6.10	51.19	16.01	**< 0.001**		
	Zone 1	0	3.63	1.27	301.13	2.86	**< 0.005**	3	7
		96	-8.85	1.60	310.12	-5.53	**< 0.001**	8	10
	(Intercept)	0	41.36	3.25	48.60	12.71	**< 0.001**		
		96	17.10	5.05	73.18	3.39	**< 0.001**		
	Zone 2	0	-2.23	0.78	295.56	-2.87	**0.004**	3	4
		96	3.76	1.38	310.81	2.72	**0.006**	2	3
	(Intercept)	0	19.18	5.34	11.30	3.59	**0.004**		
		96	-5.70	1.79	32.68	-3.18	**0.003**		
	Zone 3	0	2.59	0.98	279.38	2.63	**0.009**	2	11
		96	3.29	0.44	309.27	7.40	**< 0.001**	15	17
2A: EFF-CT	(Intercept)	0	60.55	10.57	76.08	5.73	**< 0.001**		
		96	72.77	12.37	92.04	5.89	**< 0.001**		
	Zone 1	0	-10.17	6.73	87.04	-1.51	0.135	3	5
		96	-15.70	7.32	83.68	-2.15	**0.034**	5	15
	(Intercept)	0	54.47	8.15	74.61	6.68	**< 0.001**		
		96	41.17	11.08	67.80	3.72	**< 0.001**		
	Zone 2	0	-11.10	5.18	87.08	-2.15	**0.034**	45	8
		96	-0.62	6.25	80.31	-0.10	0.921	0	15
	(Intercept)	0	-19.85	4.83	92.00	-4.11	**< 0.001**		
		96	-15.06	4.23	75.34	-3.56	**< 0.001**		
	Zone 3	0	22.05	3.16	92.00	6.98	**< 0.001**	35	35
		96	15.39	2.38	101.05	6.47	**< 0.001**	28	32
2A: Wastewater-borne TiO_2NPs	(Intercept)	0	40.33	5.51	41.38	7.32	**< 0.001**		
		96	44.68	4.56	400.61	9.80	**< 0.001**		
	Zone 1	0	1.78	1.30	244.78	1.37	0.171	1	7
		96	32.00	3.78	400.07	8.47	**< 0.001**	1	7
	(Intercept)	0	40.82	3.47	82.41	11.76	**< 0.001**		
		96	32.00	3.78	400.07	8.41	**< 0.001**		
	Zone 2	0	-1.82	0.92	267.05	-1.99	**0.047**	2	2
		96	0.48	0.93	376.06	0.52	0.603	1	2
	(Intercept)	0	25.42	3.61	75.33	7.05	**< 0.001**		
		96	9.22	13.66	57.32	6.75	**< 0.001**		
	Zone 3	0	-1.46	0.91	249.93	-1.61	0.109	1	2
		96	-0.76	0.32	398.54	-2.40	**0.017**	2	3
2B: ASTM-dispersed TiO_2NPs	(Intercept)	0	30.52	4.91	236.00	6.22	**< 0.001**		
		96	58.89	4.87	339.00	12.09	**< 0.001**		
	Zone 1	0	3.36	1.28	236.00	2.63	**< 0.001**	3	3
		96	-1.99	1.21	339.00	-1.65	0.100	1	1
	(Intercept)	0	45.32	3.11	236.00	14.56	**< 0.001**		
		96	47.19	4.27	42.34	11.04	**< 0.001**		
	Zone 2	0	-3.90	0.81	236.00	-4.81	**< 0.001**	8	9
		96	-4.72	0.96	335.20	-4.90	**< 0.001**	7	8
	(Intercept)	0	23.20	3.73	236.00	6.22	**< 0.001**		
		96	-0.36	3.38	100.04	-1.08	0.283		
	Zone 3	0	0.32	0.97	236.00	0.33	0.744	0	0
		96	5.67	0.83	333.30	6.69	**< 0.001**	12	13

Chapter 4

The development of new behavioural-related endpoints for using *Daphnia magna* as a biosensor for nanoparticles in water systems



This chapter is based on the experiments performed in Chapter 3. Therefore, some parts of the material and methods section of this chapter are related to the previous one. The evaluation of the data is based on a different concept, which will be described and discussed within this chapter.

4.1 Abstract

The protection of the aquatic environment against pollution is an important topic, as unpredicted chemical accidents can have dramatic consequences towards the aquatic ecosystems including their organisms. Nowadays, biological early warning systems (BEWS) are applied to monitor the water quality and to detect chemical contaminants of water systems to protect the environment and the human population. A sensitive biosensor, a representative organism of an aquatic tropic level, is essential to sense a range of different contaminations, like heavy metals, pesticides or nanoparticles (NPs). Due to the rising production of NPs, the risk of environmental pollution is increasing since NPs end up in the aquatic environment and may cause negative effects on the ecosystem. Thus, the monitoring of nanoparticle contamination events for water system is an important topic in the environmental protection. Hence, the aim of this study is to develop new behavioural-related endpoints that show a high sensitivity and a fast response time towards nanoparticle contamination in water systems by using the model species *Daphnia magna* and two common nanoparticles, AgNPs (NM-300K) and TiO_2NPs (NM 105). The Grid Count approach is used to evaluate the endpoints “crossings”, “cross backs” and “swimming direction” as a tool for a BEWS. We showed, that the parameter “cross backs” indicates behavioural changes of *D. magna* exposed to low concentrations of ASTM-dispersed AgNPs and ASTM-dispersed TiO_2NPs directly after the exposure and after 3 h of exposure, respectively. The parameter “crossings” was also sensitive towards ASTM-dispersed AgNPs and ASTM-dispersed TiO_2NPs, but responded only after 3 h, therefore slower than “cross backs”. The parameters “crossings” and “cross backs” did not indicate behavioural changes neither for wastewater-borne AgNPs nor for wastewater-borne TiO_2NPs. The newly-developed endpoint “swimming direction” was the most inefficient endpoint. In this study, “cross backs” was the most sensitive endpoint with the best response time towards NPs contamination. The results provided in this study, by using “crossings” and “cross backs” as behavioural-related endpoints, fitted well with those obtained in the study of Chapter 3 regarding the behavioural toxicity of ASTM-dispersed AgNPs and TiO_2NPs. Hence, the developed endpoints in this study are be reliable and can be used as parameters in a BEWS to detect low concentrations of NPs with a high sensitivity and a fast response time. With this study we provided further tools to enhance

the efficiency and safety of water monitoring to protect the aquatic environment and the human health by using BEWS.

4.2 Introduction

Monitoring of surface, ground, effluent and drinking water quality is crucial for a prompt detection and containment of chemical contaminants to protect the aquatic environment and human health (Bae and Park, 2014). So far, chemical monitoring techniques have been used to assess and control water quality but their range of application is limited by high costs and specificity (Borcherding, 2006; Gruber et al., 1994). The technological advancements in computer hard- and software allow developing new monitoring systems to detect changes in aquatic ecosystems (Bae and Park, 2014). Biological early warning systems (BEWS) track the physiological responses of whole organisms to identify abnormal behavioural patterns of individuals, the so-called “biosensors” (Gerhardt, 2007; Van der Schalie et al., 2001). The behaviour of an organism comprises its response to internal (physiological) and external (environmental and social) factors (Dell'Omo, 2002; Gerhardt, 2007; Hellou, 2011). Behavioural-related parameters show a 10 – 100 times higher sensitivity than e.g. mortality data (Gerhardt, 2007; Hellou et al., 2008), thus serving as reliable endpoints in ecotoxicological risk assessment of chemicals, (Jeon et al., 2008; Tahedl and Häder, 2001). To be eligible as a biosensor, an organism has to react very sensitively and reliably to an unknown chemical and with absolute minimum of false alarms (Gruber et al., 1994). All levels of the aquatic food chain, namely bacteria, algae, invertebrates like *Daphnia* or mussels and fish are used as biosensors in various BEWS with different, species-specific endpoints (Gruber et al., 1994). For instance, the Microtox® assay uses the bioluminescent bacteria *Photobacterium phosphoreum* to detect pesticide contamination in wastewater effluents (Somasundaram et al., 1990), the *Dreissena* Monitor identifies toxic compounds in wastewater effluents by measuring the vale movement of the zebra mussel *Dreissena polymorpha* (Borcherding, 2006), and the ToxProtect® system (bbe Moldaenke, Germany) assesses the acute toxicity of drinking water by monitoring the swimming activity of fish (Storey et al., 2011).

BEWS systems are not able to identify specific substances or to detect all concentrations of various chemical compounds, but they can indicate changes in the water quality in real time (Bae and Park, 2014; Gerhardt, 2007). The species *Daphnia* is known for its

sensitivity towards changing environmental conditions like temperature or pH but also towards environmental pollutions like pesticides, herbicides or heavy metals (Ebert, 2005; Jeon et al., 2008). Under natural conditions the behaviour of *Daphnia* follows a regular movement pattern that changes under the exposure of chemical toxicants with increasing swimming activity (hyperactivity) (Bae and Park, 2014; Jeon et al., 2008). Monitoring the swimming activity of *D. magna* could therefore be used to identify abnormal behaviour due to toxic substances (Jeon et al., 2008) and therefore assess the ecotoxicological impact of wastewater-borne nanomaterials. To our knowledge, so far no tool exists that is able to identify changes in swimming behaviour of *D. magna,* which indicates NP contaminations in water systems.

Therefore, the aim of this study was to (i) develop new behavioural-related endpoints that serve as parameters for a BEWS, to (ii) assess the sensitivity of *D. magna* towards environmentally relevant concentrations of wastewater-borne and ASTM-dispersed AgNPs and TiO_2NPs and to (iii) investigate the reliability of *D. magna* as a biosensor for nanoparticles in STP effluents. This chapter is based on the methods of the previous chapters but will go one step further than the current existing state-of-the-art.

4.3 Materials and Methods

4.3.1 Preparation of Ag and TiO_2 nanoparticle dispersions

The preparation of AgNPs and TiO_2NPs dispersions is described in Chapter 3.3.1.

4.3.2 Lab-scale wastewater treatment plant (STP)

The performance and the conditions of the lab-scale STP are described in detail in Chapter 3.3.2 and the corresponding Supporting Information.

4.3.3 *Daphnia magna* as test organism

The culture conditions of the test organism *D. magna* and of the food source, *Desmodesmus subspicatus* are described in Chapter 2.2.1.

4.3.4 Experimental exposure

4.3.4.1 Experiments

The used treatments and the used control were the same as described in Chapter 3.3.4.1.

4.3.4.2 Behavioural assay

The experimental setup and the computer vision system, which is described in Chapter 3.3.4.2, were used to monitor the swimming behaviour of *D. magna*.

In addition, to the method of the Grid Count approach (Jeon et al. 2008), we performed the 2-min recording additionally at time point 3 h, 6 h, 12 h, 24 h, 48 h and 72 h, to gain a detailed overview of the behavioural pattern of treated *D. magna.* We used a software coded in C^{++}, to analyse the data of the Grid Count approach that was developed by the Institute of Real-time Learning Systems of the University of Siegen. According to the method described in Jeon et al. (2018), a cell consisting of a matrix array of 68 x 68 cells (resulting in 456 square cells in total) was virtually placed over the test vessel to analyse the number of events (cell changes). An event was counted, when the centre of *D. magna* crosses the line of the cell (Jeon et al., 2008). Using this method, the following behavioural-related endpoints were created: (i) the number of events of cell changes, named "crossings", (ii) the number of "cross backs" to the previous cell and (iii) the "swimming direction". Therefore, we have created a 3 x 3 matrix of cells around a centre that contains the *Daphnia* (Figure 4-1) to follow the swimming movement and count the number of cells through which the *Daphnia* swam. For example, if the *Daphnia* swam straight upwards, the cell "U" was counted. At the end of the recording time, the counts for each cell per *Daphnia* were converted into percentages relative to the total number of cell changes (100 %). We then used this data to analyse the "swimming direction" and to compare between the treatments. These three endpoints served as a further tool to measure the activity of *D. magna,* while a higher number of events reflected a higher swimming activity (Jeon et al., 2008). For this analysis, the same threshold (min. recording time of 100 s) was set as for the allocation time approach (see Chapter 3.3.6).

4.3.5 Statistical analysis

The behavioural-related endpoints "crossings" and "cross backs" were analysed using a generalized linear mixed model (GLMM) via the *glmer* function of the '*lmerTest*' package (Kuznetsova et al., 2017). The effects of treatments of each experiment (1 A- wastewater borne AgNPs, 1 B- ASTM-dispersed AgNPs, 2 A-wastewater borne TiO_2NPs and 2 B-ASTM-dispersed TiO_2NPs) were analysed separately in different models. The differences between the controls (CT, DA, EFF) of the experiments were also analysed separately in

own models. For each model we first tested the complete time period (0 h – 96 h) and thereafter we tested different parts of the time periods, namely 0 h, 0 h – 3h, 0 h – 6 h, 0 h – 12 h, 0 h – 24 h and 0 h – 48 h. This was done to receive a realistic picture of the response of *D. magna* to a treatment and to test, whether *Daphnia* is a reliable biosensor for nanoparticle contaminations. The dependent variables ("crossings" and "cross backs") were count data and therefore poisson-distributed data. "Treatment" and "Time" were z-transformed to follow the same scale and were included as a fixed factor into the model. "Treatment" as a numeric variable served as random effect. The overdispersion of the model was assessed with the function *dispersion_glmer* of the package '*Blmenco'* (Korner-Nievergelt et al., 2015a). The model was fitted with an observation level as additional random factor to correct for overdispersion (Korner-Nievergelt et al., 2015b). The model assumptions were checked visually as described in Chapter 3.3.6.

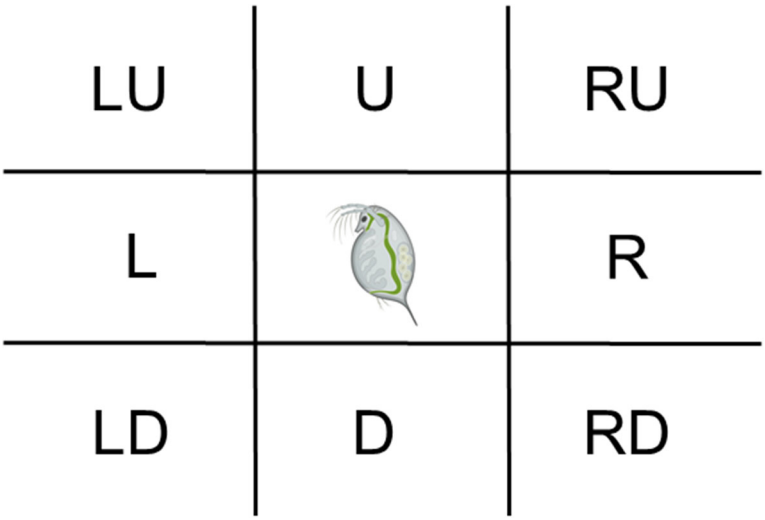


Figure 4-1: The 3 x 3 matrix, with the *Daphnia* in the centre, for analysing the "swimming direction".

For the analysis of the behavioural-related endpoint "swimming direction", the relative number of cell changes of each direction were analysed separately for every time point and treatment by using the chi-squared test due to categorical data of the dependent variable. For this endpoint, the direction "U", "R", "D" and "L" and the time points 0 h, 3 h, 6 h and 12 h are of highest interest and were analysed to investigate the suitability of *D. magna* as a reliable biosensor for nanoparticle contaminations. All analyses were done with the program R for windows (version 3.5.0). Significant level was set to 0.05 and all p-values are two-tailed.

4.4 Results

4.4.1 Crossings and Cross backs

In experiments with AgNPs, the animals of the control group CT (ASTM medium) had a significantly higher number of “crossings” and “cross backs” over the whole exposure period of 96 h compared to animals of control group DA (dispersant agent NM-300 K) (GLMM, for statistic details see Table S4-1 and S4-2). The EFF (control effluent without NPs) exposed animals showed a significantly lower number of “crossings” and “cross backs” compared to CT (GLMM, for statistic details see Table S4-1 and S4-2).

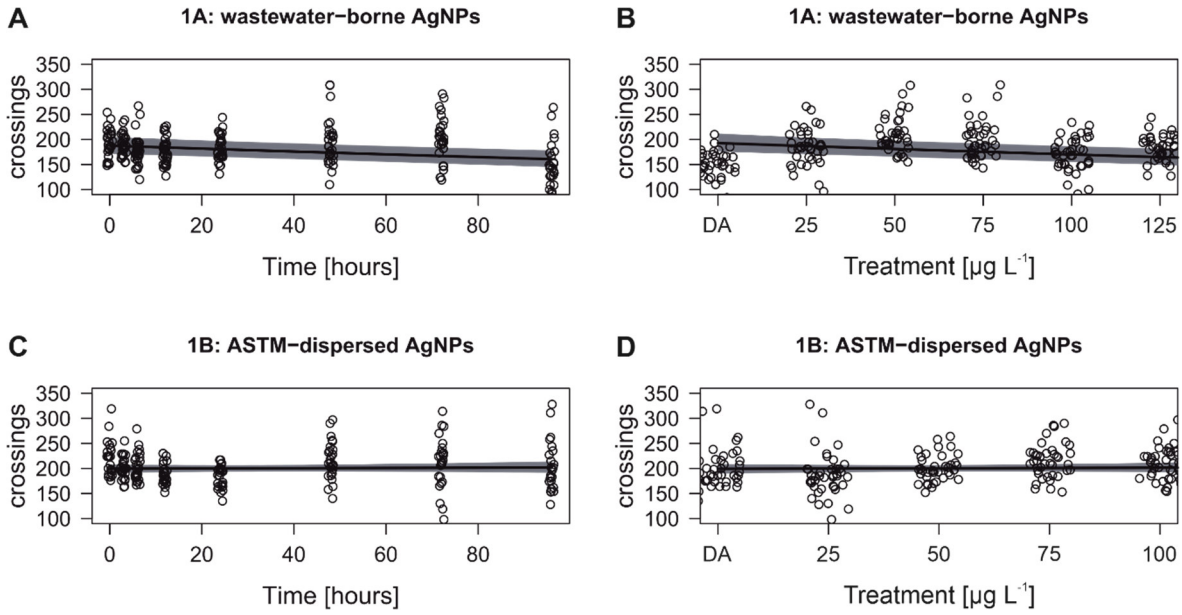


Figure 4-2: Plots of the endpoint “crossings” of *Daphnia magna* exposed to (A + B) wastewater-borne AgNPs and (C + D) ASTM-dispersed AgNPs with respect (A + C) time [hours] and (B + D) treatment [µg/L]. Dots = observed number of “crossings”, solid line = fitted values of GLMM with 95% credible interval (grey area).

The two behavioural-related endpoints “crossings” and “cross backs” were not affected by wastewater-borne AgNPs neither in the observation of the complete test period (0 h – 96 h of exposure), nor in the analysis of specific time periods (e.g. 0 h – 12 h or 0 h – 48 h; GLMM, for statistic details see Table S4-1 and S4-2, Figure 4-2 A + B and Figure 4-3 A + B). However, in some cases (time period of 0 h – 6 h, 0 h - 12 h and 0 h – 96 h) the exposure time had a significant influence, with decreasing number of “crossings” and “cross backs” over time (GLMM, for statistic details see Table S4-1 and S4-2, Figure 4-2 A and Figure 4-3 A). Animals treated with ASTM-dispersed AgNPs, show a significantly higher number of “crossings” with increasing concentrations of AgNPs compared to DA for

the complete test period (0 h – 96 h; GLMM, estimate: 0.036, p = 0.029, Figure 4-2 D) and for all other tested time periods (GLMM, for statistic details see Table S4-1, Figure 4-2 D).

The exposure of ASTM-dispersed AgNPs did not lead to any differences directly after beginning of the experiment (0 h; GLMM, estimate: 0.035, p = 0.273, Figure 4-2 D). The first significant effect compared to DA was found after 3 h, were the animals showed a significantly higher number of "crossings" (GLMM, estimate: 0.043, p = 0.015, Figure 4-2 D). The time effect for the endpoint "crossings" was visible at the exposure periods of 0 h – 3 h, 0 h – 6 h, 0 h – 12 h and 0 h – 24 h (GLMM, for statistic details see Table S4-1, Figure 4-2 C). The endpoint "cross backs" was not affected by ASTM-dispersed AgNPs at exposure time of 0 h – 12 h, 0 h – 24 h, 0 h – 48 h and 0 h – 96 h (GLMM, Table S4-2, Figure 3-9 C + D). However, directly after the exposure (0 h; GLMM, estimate: 0.140, p < 0.001, Figure 3-9 C) and at time periods 0 h – 3 h and 0 h – 6 h, the animals showed a significantly higher number of "cross backs" with increasing concentrations of AgNPs (GLMM, Table S4-2, Figure 3-9 C) in comparison to DA.

No differences were found for the number of "crossings" and "cross backs" in the analysis of EFF and CT of the experiments with wastewater-borne TiO_2NPs and ASTM-dispersed TiO_2NPS (GLMM, for statistic details see Table S4-3 and S4-4, Figure 4-4 and Figure 4-5).

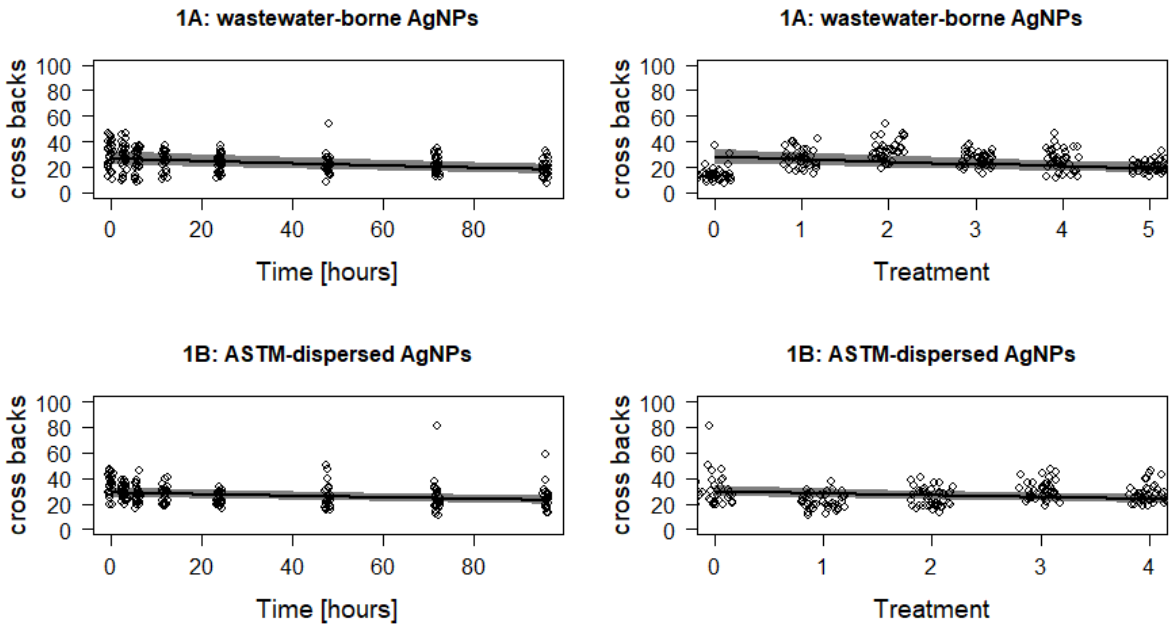


Figure 4-3: Plots of the endpoint "cross backs" of *Daphnia magna* exposed to (A + B) wastewater-borne AgNPs and (C + D) ASTM-dispersed AgNPs with respect (A + C) time [hours] and (B + D) treatment [µg L]. Dots = observed number of "cross backs", solid line = fitted values of GLMM with 95% credible interval (grey area).

In experiment 2A, no time- and treatment-related effects were found for the number of "crossings" and "cross backs" after exposure to wastewater-borne TiO_2NPs (expected time period 0 h – 3 h compared to CT for "crossings"). Also in the experiment with ASTM-dispersed TiO_2NPs no effects were found for the number of "crossings" and "cross backs" directly after the exposure (time point 0 h) in comparison to CT. However, after 3 h of exposure, the animals treated with ASTM-dispersed TiO_2NPs showed a significantly higher number of "cross backs" with increasing concentrations of TiO_2NPs in comparison with CT (GLMM, estimate: 0.085, p = 0.008, Figure 4-5 D) which also applies to all subsequent time periods (GLMM, for statistic details see Table S4-4, Figure 4-5). The same picture was found for the endpoint "crossings" after 6 h of exposure, where all animals had a significantly higher number of "crossings" in comparison to CT (GLMM, for statistic details see Table S4-3, Figure 4-4 D). Time-related effects were found for serval time periods for both, "crossings" and "cross backs" (GLMM, for statistic details see Table S4-3, Figure 4-4 and Figure 4-5) but without a consistent pattern.

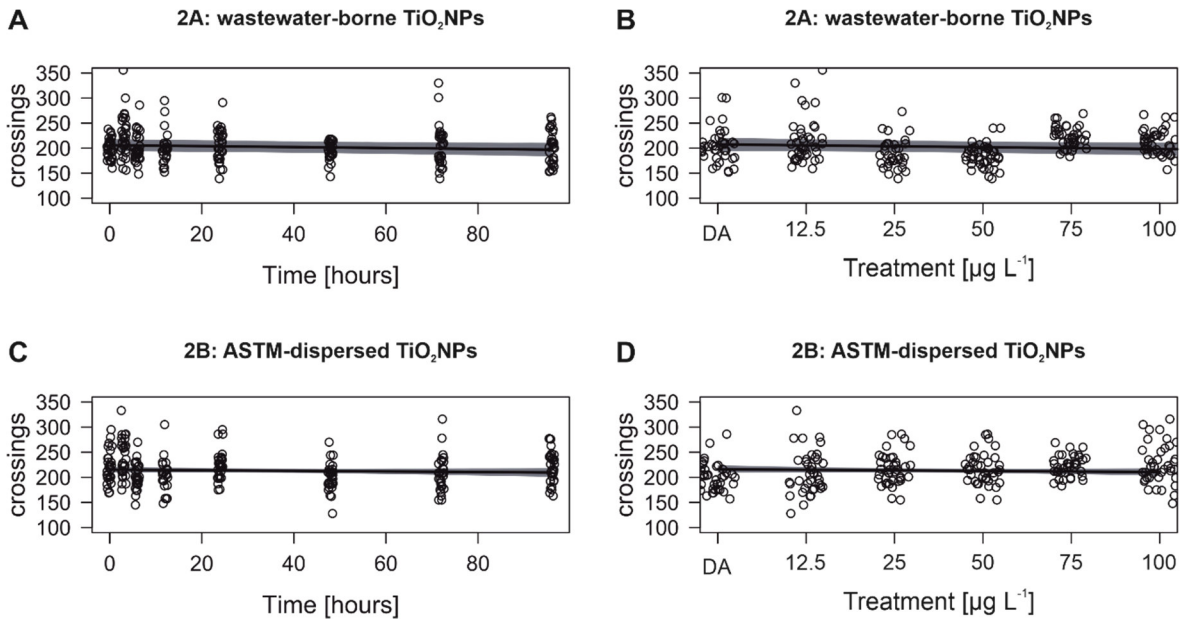


Figure 4-4: Plots of the endpoint "crossings" of *Daphnia magna* exposed to (A + B) wastewater-borne TiO_2NPs and (C + D) ASTM-dispersed TiO_2NPs with respect (A + C) time [hours] and (B + D) treatment [µg/L]. Dots = observed number of "crossings", solid line = fitted values of GLMM with 95% credible interval (grey area).

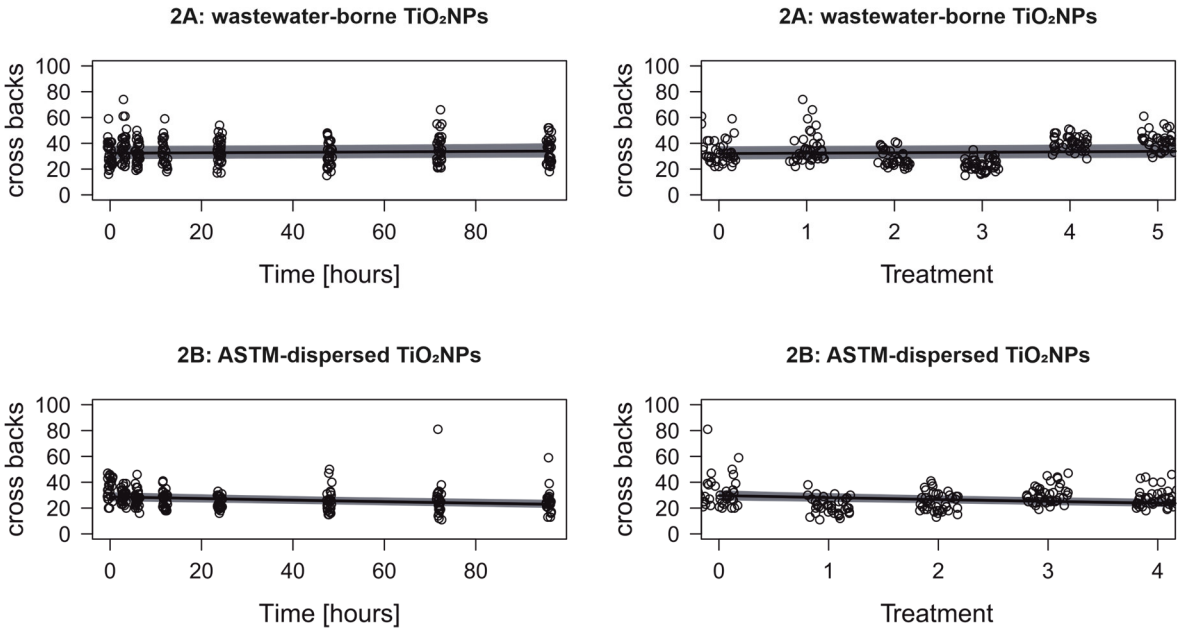


Figure 4-5: Plots of the endpoint "cross backs" of *Daphnia magna* exposed to (A + B) wastewater-borne TiO_2NPs and (C + D) ASTM-dispersed TiO_2NPs with respect (A + C) time [hours] and (B + D) treatment [µg/L]. Dots = observed number of "cross backs", solid line = fitted values of GLMM with 95% credible interval (grey area).

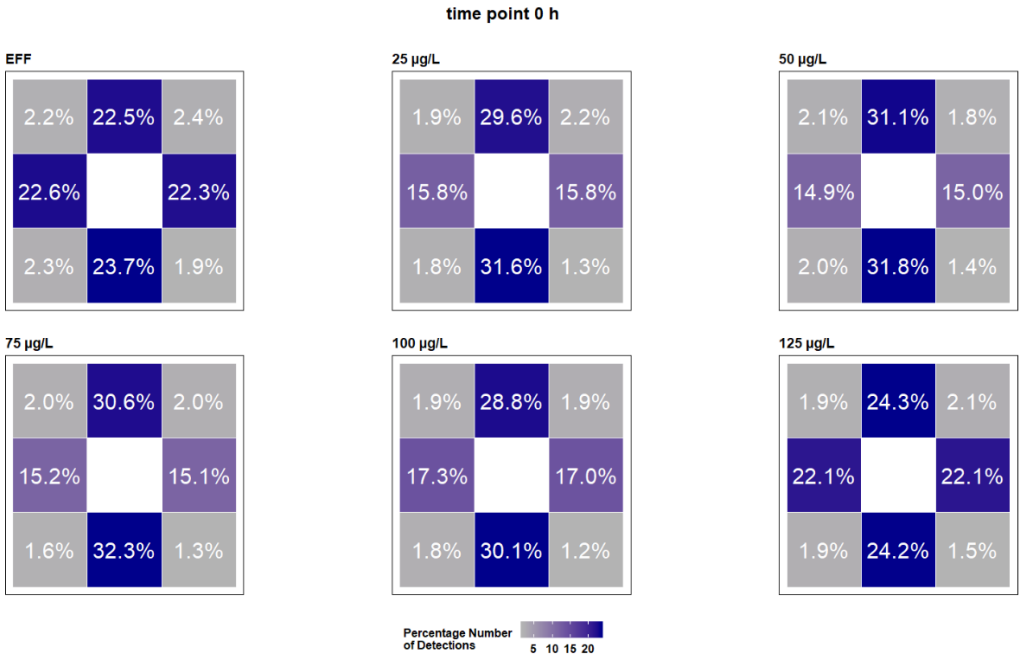


Figure 4-6: Relative percentage change of direction of *Daphnia magna* at time point 0 h and under the exposure with wastewater-borne AgNPs (Exp. 1A).

4.4.2 Swimming direction

At time point 0 h, the animals of EFF did not show any preferred "swimming direction" (χ^2 = 0.033, p = 0.998, Figure 4-6). However, the "swimming direction" was significantly affected by 25 µg/L (χ^2 = 10.089, p = 0.017, Figure 4-6), 50 µg/L (χ^2 = 11.989, p = 0.007, Figure 4-6) and 75 µg/L (χ^2 = 11.217, p = 0.010, Figure 4-6) of wastewater-borne AgNPs. The animals in these treatments swam to the direction "U" and "D" significantly more often than to "L" and "R". The same pattern could be found after 3 h of exposure, *D. magna* treated with 25 µg/L (χ^2 = 16.376, p = 0.001, Figure 4-7), 50 µg/L (χ^2 = 11.731, p = 0.008, Figure 4-7) and 100 µg/L (χ^2 = 13.484, p = 0.003, Figure 4-7) swam significantly more often in the direction "U", and "D" compared und "L" and "R". This movement pattern was weaker after 6 h (Figure S4-1) and not present after 12 h of exposure (Figure S4-2).

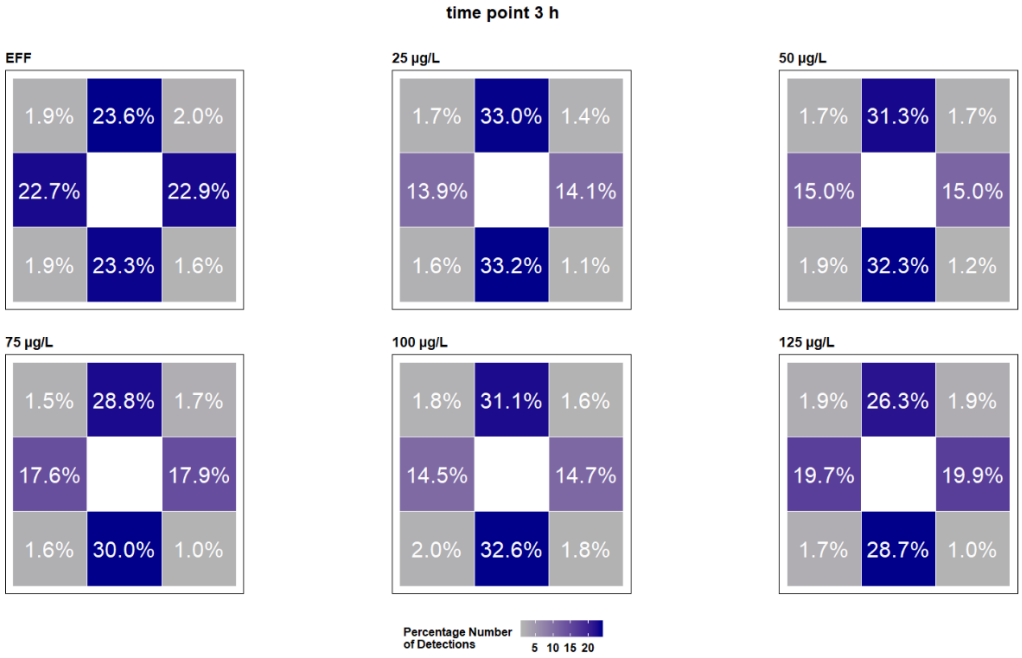


Figure 4-7: Relative percentage change of direction of *Daphnia magna* at time point 3 h and under the exposure with wastewater-borne AgNPs (Exp. 1A).

The exposure to CT (for test statistic see Table S4-5, Figure 4-8 and Figure 4-9; Figure S4- 3 – S4-4) and DA (for test statistic see Table S4-5, Figure 4-8 and Figure 4-9; Figure S4-3 – S4-4) did not show any significant "swimming direction", neither after 0 h of exposure nor after 3 h, 6 h and 12 h of exposure. We found at 0 h, that *Daphnia* of all ASTM-dispersed AgNPs treatments (25 µg/L – 100 µg/L, Figure 4-8) led to a significantly higher percentage (for test statistic see Table S4-5) of the direction "U" and "D" compared to "L" and "R". After 3 h of exposure, the "swimming direction" was significantly affected at

25 µg/L (χ^2 = 10.582, p = 0.014, Figure 4-9) and 50 µg/L (χ^2 = 11, p = 0.011, Figure 4-9) of ASTM-dispersed AgNPs, while the animals used the direction “U” and “D” more often than “L” and “R”. This movement pattern was not available after 6 h (Table S4-5, Figure S4-3) and 12 h (Table S4-5, Figure S4-4) of exposure.

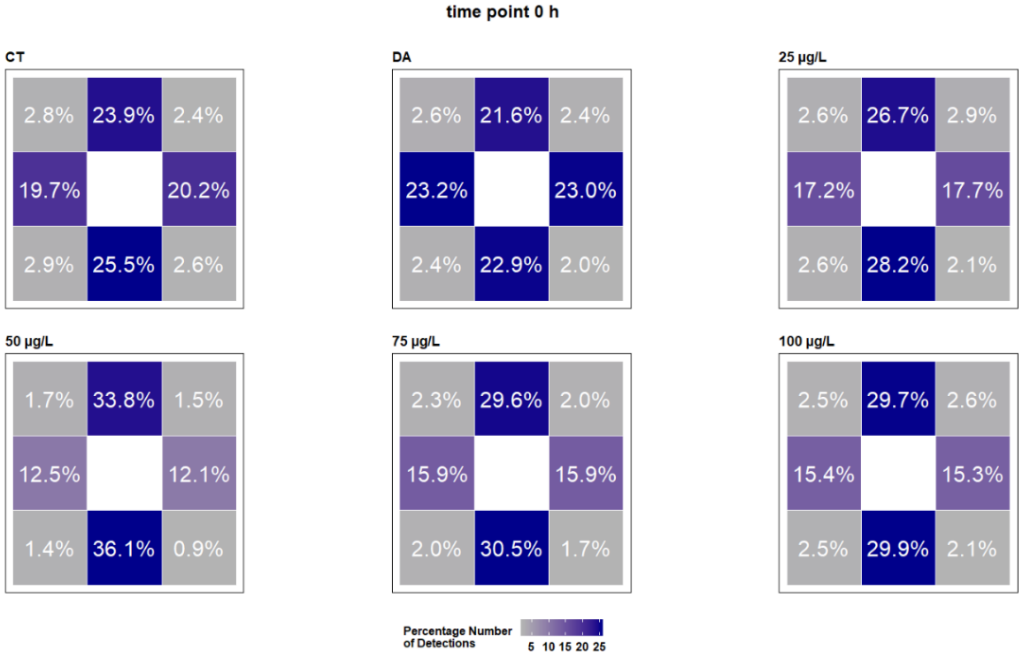


Figure 4-8: Relative percentage change of direction of *Daphnia magna* at time point 0 h and under the exposure with ASTM-dispersed AgNPs (Exp. 1B).

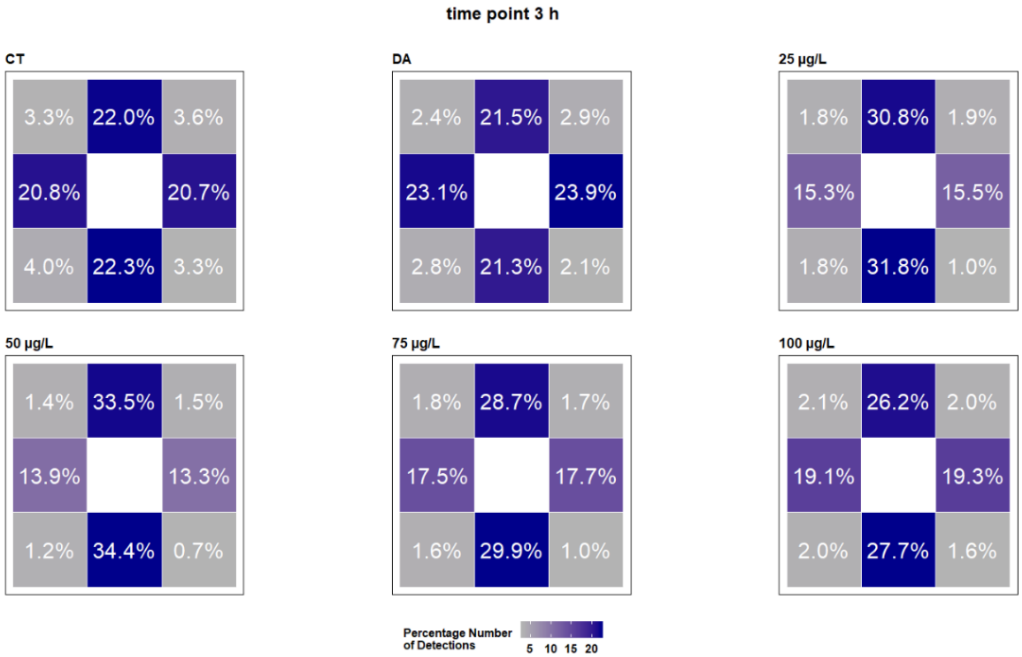


Figure 4-9: Relative percentage change of direction of *Daphnia magna* at time point 3 h and under the exposure with ASTM-dispersed AgNPs (Exp. 1B).

At time point 0 h, 3 h, 6 h and 12 h the “swimming direction” was not affected by wastewater-borne TiO_2NPs and ASTM-dispersed TiO_2NPs (Figure 4-10 – 4-20 and Figure S4-5 – S4-8).

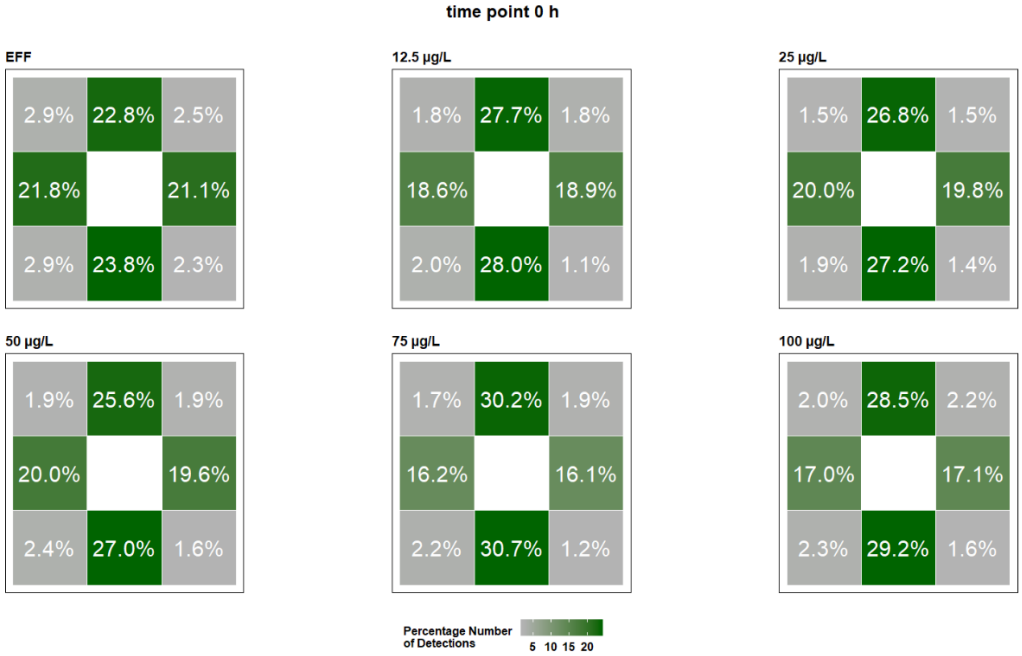


Figure 4-10: Relative percentage change of direction of *Daphnia magna* at time point 0 h and under the exposure with wastewater-borne TiO_2NPs (Exp. 2A).

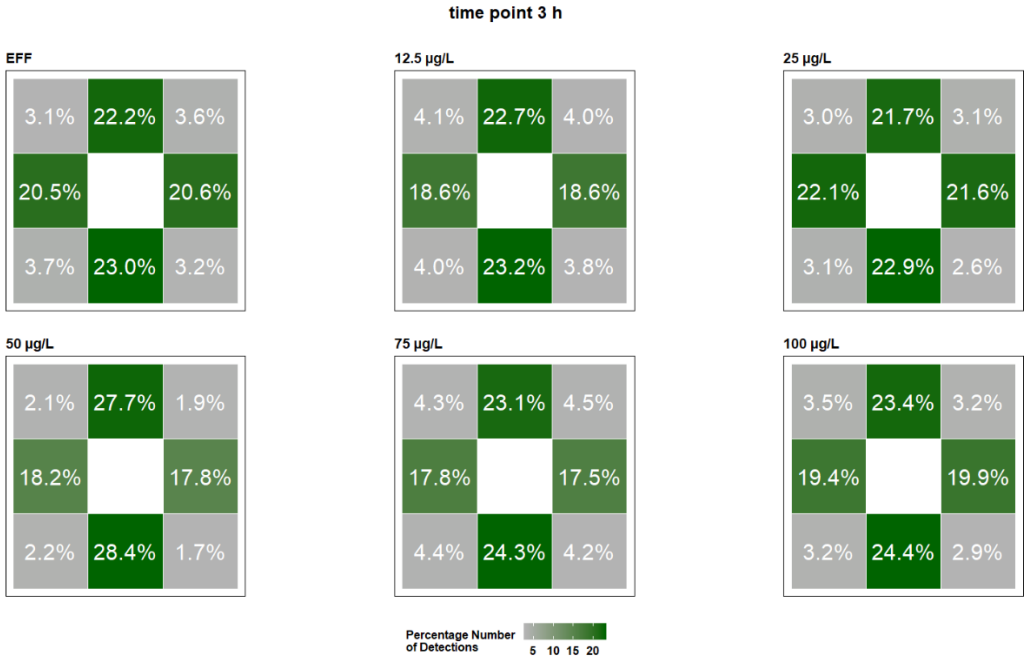


Figure 4-11: Relative percentage change of direction of *Daphnia magna* at time point 3 h and under the exposure with wastewater-borne TiO_2NPs (Exp. 2A).

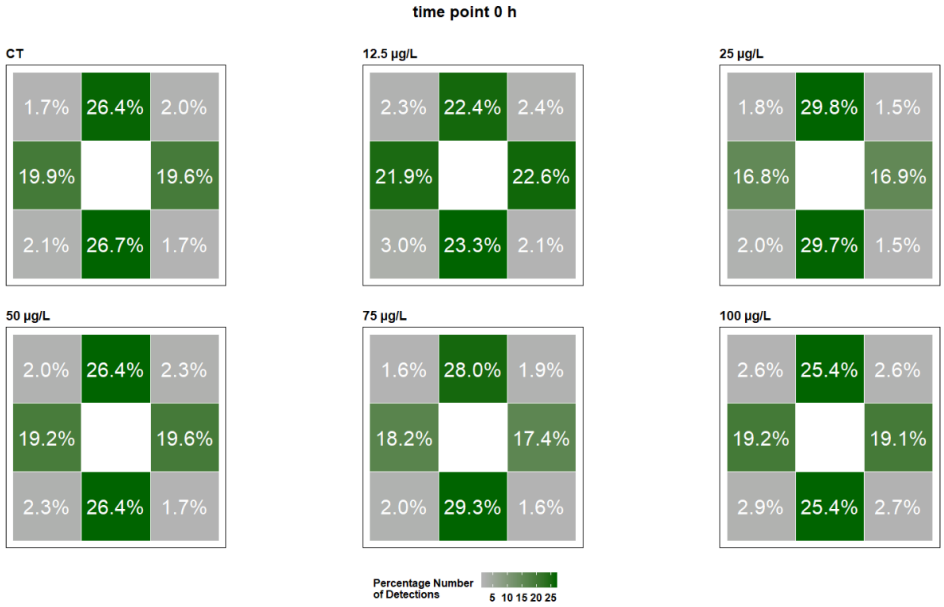


Figure 4-12: Relative percentage change of direction of *Daphnia magna* at time point 0 h and under the exposure with ASTM-dispersed TiO_2NPs (Exp. 2B).

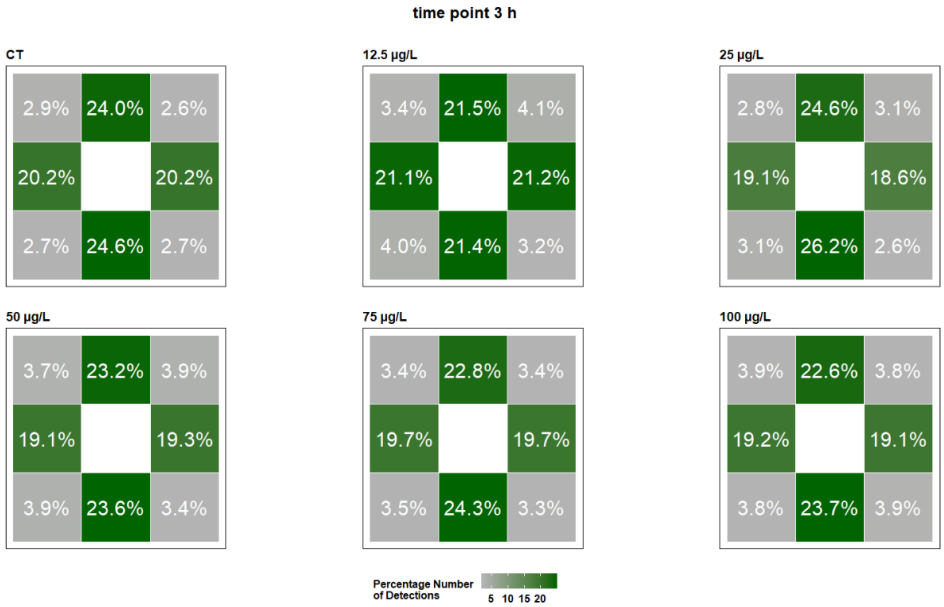


Figure 4-13: Relative percentage change of direction of *Daphnia magna* at time point 3 h and under the exposure with ASTM-dispersed TiO_2NPs (Exp. 2B).

4.5 Discussion

In this study, new behavioural-related endpoints were developed for the detection of nanoparticle contaminations in water systems by using the Grid Count approach. We established the parameters “crossings”, “cross backs” and “swimming direction”. The most sensitive parameter concerning NP exposure was the endpoint “cross backs” since directly

after the exposure (0 h) with ASTM-dispersed AgNPs *Daphnia* showed a significantly higher number of “cross backs” in relation to the control group. Animals treated with ASTM-dispersed TiO_2NPs indicated significant changes of the endpoint “cross backs” after 6 h of exposure, wherefore wastewater-borne AgNPs and wastewater-borne TiO_2NPs had no effect over the whole exposure time. The first indication of a NP-contamination by using the endpoint “crossings” was found after 3 h of exposure with ASTM-dispersed AgNPs and with ASTM-dispersed TiO_2NPs with a significantly higher number of “crossings” of the exposed animals. Wastewater-borne AgNPs and wastewater-borne TiO_2NPs had no effect towards the parameter “crossings”. The newly developed endpoint “swimming direction” showed significant changes of the swimming behaviour for wastewater-borne and ASTM-dispersed AgNPs for the two lowest concentrations directly after exposure (0 h) and for the further analysed timepoints. No differences in the “swimming direction” were found by treating the animals with wastewater-borne and ASTM-dispersed TiO_2NPs.

Commercially available BEWS uses endpoints like swimming height, average distance, swimming speed or vertical distribution and migration (Bownik, 2017; Kokkali and van Delft, 2014). Nevertheless, *Daphnia* show a higher activity under the exposure of natural contaminations and anthropogenic pollutants (Bae and Park, 2014; Jeon et al., 2008). So far, only a few studies detected abnormal swimming activity of *D. magna* with the Grid Count approach as a tool for the identification of toxic compounds, although, analysing activity of aquatic invertebrate serves as a suitable concept in monitoring the water quality (Jeon et al., 2008; Jeong et al., 2014). Jeon et al. (2008) showed that analysing the swimming activity of *D. magna* with the above mentioned approach, leads to a high sensitivity and a fast response time of approximately 7 h to 1 h concerning hyperactivity after exposure with low concentrations of copper (50 µg/L - 400 µg/L). In this study, we analysed the newly developed endpoints “crossings”, “cross backs” and “swimming direction” directly after the exposure (0 h) and after 3 h, 6 h and 12 h of exposure to investigate, how fast animals react in these endpoints towards nanoparticle contaminants. One of the main requirements to serve as a reliable endpoint for a biosensor is the detection of chemical pollutants with a high accuracy and a short response time (Bae and Park, 2014). Firstly we showed, that the analysis of the endpoints “crossings” and “cross backs” confirmed the results of the mobility, swimming height and the allocation time data (described and discussed in Chapter 3), whereas wastewater-borne AgNPs did not show

any toxic effects. On the other side, ASTM-dispersed AgNPs had a significant impact towards the swimming behaviour of *D. magna*. Hence, the new developed endpoints are comparable with standard parameters used in other ecotoxicological behavioural studies (Bownik, 2017). Secondly, the response time of the two mentioned endpoints are fast, since we found significant behavioural changes directly after the exposure (0 h) for the endpoint “cross backs”. A detailed discussion on the effects of ASTM-dispersed and wastewater-borne AgNPs towards *Daphnia´*s behaviour is given in Chapter 3.6. However, when comparing the results of the endpoints “crossings” and “cross backs” of the animals exposed to wastewater-borne TiO_2NPs and ASTM-dispersed TiO_2NPs with the endpoints described in Chapter 3, we saw different response patterns related to their toxicity. We found that both, wastewater-borne and ASTM-dispersed TiO_2NPs, had an effect on the swimming height and the allocation time of the animals after 0 h, but none of the tested particles indicated behavioural-related differences (for the parameters “crossings” and “cross back”) directly after exposure. Nevertheless, ASTM-dispersed TiO_2NPs affected the behaviour of *D. magna* after 3 h, 6 h and 12 h when analysing the number of “crossings” and “cross backs” while wastewater-borne TiO_2NPs had no effect. However, similar toxicity of wastewater-borne and ASTM-dispersed TiO_2NPs is assumed since TiO_2NPs are not transformed while they pass the model STP (*Galhano et al., Under Review*; Georgantzopoulou et al., 2018; *Hartmann et al., 2019*). As discussed in Chapter 3.6, behavioural effects of wastewater-borne TiO_2NPs were lost after 96 h but still available for ASTM-dispersed TiO_2NPs, indicating, that the two particles leading to distinct effects over time. Hence, this is comparable with the results of the two applied endpoints in this Chapter. The toxicity of wastewater-borne TiO_2NPs might be reduced due to the high content of dissolved organic matter (DOM) present in the effluent of a model STP (Bundschuh et al., 2016) or the agglomeration of the particles leading to a reduced bioavailability of the particles to female *Daphnia* (Vale et al., 2016). The response time for the endpoints “crossings” and “cross backs” was delayed in comparison to those analysed in Chapter 3, but the results are similar and therefore served as suitable endpoints for a BEWS. The third parameter “swimming direction” has proven to be unsuitable. The results are inconsistent for both tested NPs and are not comparable with those achieved in Chapter 3. However, the changes in swimming direction also occurred in response to toxic chemicals (Bownik, 2017). Eventually, some adjustments of this endpoint would be useful

by assessing the swimming direction as a change of angle, measured by downward angle and upward angle. This approach was already used by some other studies with fullerenes (C_{60}) (Brausch et al., 2011) or neurotoxic compounds like diazinon, nicotine or imidacloprid (Zein et al., 2014; Zein et al., 2015). Nevertheless, these studies have also found conflicting response patterns.

In general, we showed that the assessment of the toxicity of NPs should be based on multiple endpoints, especially by analysing behavioural changes, since the complexity of the behavioural repertoire depends on the level of neural organisation in the used animals and behavioural events are often species-specific (Dell'Omo, 2002). Nevertheless, with the performed experiments of Chapter 3 and Chapter 4 we indicated that the used organism *D. magna* is a reliable test species for the indication of nanoparticles in water systems since they meet all the requirements as reviewed by Bae and Park (2014). The endpoints analysed in Chapter 3 and the newly developed endpoints in Chapter 4 clearly demonstrated, that under the exposure of low concentrations of NPs the parameters show a high sensitivity and especially the response time of the endpoints “crossings” and “cross backs” were short. The assessment of behavioural changes of *D. magna*, among the other advantages like easy culturing, cost-effective and short generation time, by using the Grid Count approach could act as a powerful tool to identify low concentrations of NPs within water systems and should be included as new behavioural parameters in existing BEWS with *Daphnia*, for instance the DaphTox II system (bbe Moldaenke, Germany).

Besides the test species, the system needs to fulfil specific properties to serve as a reliable BEWS for contamination events with nanoparticles as well. The computer vision system used in Chapter 3 and Chapter 4 has important characteristics like a continuous tracking of the swimming behaviour and performing the statistical analysis of the movements with a high accuracy. To even better support the tracking and data analysis, the system should be further improved based on the following findings: Natural movements of *Daphnia* are more realistically reflected with three-dimensional (3D) swimming trajectories (Bownik, 2017; Noss et al., 2013b). However, only a few studies applied 3D swimming analyses in the field of aquatic ecotoxicology to assess the toxicity of an environmental pollutant (Ekvall et al., 2013; Noss et al., 2013a). So far, there is no free video analysis software on the market that can automatically create 3D trajectories and analyse the swimming behaviour in 3D (Huang et al., 2017). The software Track3D, an add-on module to

EthoVision® XT (Noldus, Netherlands), a video tracking software for behavioural experiments, visualizes the swimming trajectory in a 3D image and calculates movement parameters, like velocity, in 3D space but must be purchased and is therefore not useful for many researchers. Hence, tracking the swimming behaviour of *Daphnia* in 3D would be the best method to identify behavioural-related endpoints with a high sensitivity. The development of a free 3D-tracking-software would be a great benefit for further research. A further valuable tool, which could be integrated in the analysis of the software, is the measurement of the body length, especially if the test duration exceeds periods of more than 24 hours. The swimming velocity and therefore the swimming activity of *D. magna* is influenced by the body size (Baillieul and Blust, 1999) and the detection of differences between treatments is based on differences in body size and not due to chemical stressors (Baillieul and Blust, 1999). Hence, adding this endpoint to the analysis could be a big advantage to take differences in size into consideration during data analysis or using parameters directly which are independent from body size and less prone to physiological characteristics of *Daphnia* (Jeon et al., 2008), as already recommended and used by previous studies (Jeon et al., 2008; Jeong et al., 2014). A further implementation is the setting of a specific alarm line based on significant changes of the swimming behaviour of *D. magna*. In the case of this study, the alarm line has to be specified for critical NPs concentrations. If the contamination exceeds a previous defined threshold, the system should be able to trigger an alarm to serve as a reliable BEWS and to initiate rapid countermeasures to save the environment. Furthermore, the response of organisms is only semi-quantitative (Bae and Park, 2014) and a quantitative information is missing. Because of this, a combination of the computer vision system with an analytical system is advisable. In the case of the detection of significant changes in the swimming behaviour of *Daphnia*, an identification of the chemical pollutant should be carried out. The integrated approach of biological and analytical system achieved a higher sensitivity and a fast determination and identification of the contaminant. So far, the established system (described in detail in Chapter 3) is not able to work as a real time monitoring tool which is essential for a BEWS for a rapid detection of unpredictable changes of the water quality in various water systems (Bae and Park, 2014). Hence, an improvement of the established computer vision system is required in order to act as a useful and reliable BEWS with a high sensitivity of NP contamination and a rapid response time.

4.6 Conclusion

This chapter aimed to developed new behavioural-related endpoints that show a high sensitivity towards nanoparticle contaminations in water systems. The newly developed endpoints can be analysed and evaluated with software developed in the FENOMENO project using existing data and no new experiments are required. In addition, *D. magna* meets all requirements for a sensitive and fast biosensor and the developed method is suitable for use in BEWS as it is cost-effective and real-time analyses are possible. The endpoints “crossings” and “cross backs” have shown the highest reliability against nanoparticle contamination and are therefore suitable for the detection of environmental pollutants in aquatic ecosystems. However, an even higher sensitivity to chemicals can be achieved if further studies are carried out with 3D tracking system, thereby collecting data with natural movements of aquatic organisms, and such systems should be used as BEWS in the future.

4.7 Supporting Information

Table S4-1: Results of the generalized linear mixed effect model of 'time' and 'treatment' on "crossings" of *Daphnia magna*, following the 2-min exposure to different treatments with AgNPs and assessed at different time periods. The coefficient estimates, standard errors (SE), z-values and p-values for fixed effects are shown. The significant differences ($p < 0.05$) within controls, and within NP concentrations and controls are marked in bold. EFF, CT, and DA are the controls (without NPs) for the effluent, ASTM medium, and dispersant agent, respectively.

Experiments and treatments	Fixed effects	Estimate	SE	z value	p
1A: EFF - CT	**CT vs. EFF**				
	(Intercept)	5.177	0.029	177.353	**< 0.001**
	Time	-0.084	0.029	-2.873	**0.004**
	Treatment	-0.233	0.029	-7.941	**< 0.001**
1A: wastewater-borne AgNPs	**EFF vs. 0 h - 96 h**				
	(Intercept)	5.182	0.045	114.015	**< 0.001**
	Time	-0.053	0.012	-4.342	**< 0.001**
	Treatment	0.046	0.045	1.024	0.306
	EFF vs. 0 h - 48 h				
	(Intercept)	5.224	0.034	152.684	**< 0.001**
	Time	0.023	0.021	1.065	0.287
	Treatment	0.029	0.032	0.919	0.358
	EFF vs. 0 h - 24 h				
	(Intercept)	5.168	0.041	126.020	**< 0.001**
	Time	-0.052	0.040	-1.307	0.191
	Treatment	0.025	0.029	0.844	0.399
	EFF vs. 0 h - 12 h				
	(Intercept)	4.991	0.074	66.615	**< 0.001**
	Time	-0.262	0.083	-3.125	**0.001**
	Treatment	0.021	0.031	0.695	0.487
	EFF vs. 0 h - 6 h				
	(Intercept)	4.829	0.158	30.560	**< 0.001**
	Time	-0.442	0.176	-2.515	**0.011**
	Treatment	0.016	0.030	0.552	0.581
	EFF vs. 0 h - 3 h				
	(Intercept)	5.055	0.318	15.860	**< 0.001**
	Time	-0.203	0.343	-0.594	0.553
	Treatment	0.009	0.023	0.427	0.669
	EFF vs. 0 h				
	(Intercept)	5.253	0.036	144.812	**< 0.001**
	Treatment	-0.018	0.036	-0.497	0.619
1B: DA - CT	**CT vs. DA**				
	(Intercept)	5.346	0.0257	207.300	**< 0.001**
	Time	0.024	0.0259	0.925	0.354
	Treatment	-0.064	0.0259	-2.473	**0.013**

Table S4-1: Continued.

1B: ASTM-dispersed AgNPs	**DA vs. 0 h - 96 h**				
	(Intercept)	5.300	0.016	317.665	**< 0.001**
	Time	0.003	0.012	0.313	0.754
	Treatment	0.036	0.016	2.173	**0.029**
	DA vs. 0 h - 48 h				
	(Intercept)	5.301	0.018	285.783	**< 0.001**
	Time	0.006	0.024	0.254	0.799
	Treatment	0.035	0.013	2.530	**0.011**
	DA vs. 0 h - 24 h				
	(Intercept)	5.124	0.033	153.575	**< 0.001**
	Time	-0.227	0.043	-5.204	**< 0.001**
	Treatment	0.034	0.012	2.887	**0.003**
	DA vs. 0 h - 12 h				
	(Intercept)	5.010	0.077	64.826	**< 0.001**
	Time	-0.360	0.091	-3.947	**< 0.001**
	Treatment	0.032	0.018	1.809	0.070
	DA vs. 0 h - 6 h				
	(Intercept)	4.937	0.173	28.523	**< 0.001**
	Time	-0.439	0.194	-2.264	**0.023**
	Treatment	0.053	0.015	3.477	**< 0.001**
	DA vs. 0 h - 3 h				
	(Intercept)	4.394	0.367	11.956	**< 0.001**
	Time	-1.013	0.393	-2.574	**0.010**
	Treatment	0.043	0.017	2.430	**0.015**
	DA vs. 0 h				
	(Intercept)	5.384	0.032	167.525	**< 0.001**
	Treatment	0.035	0.032	1.096	0.273

Table S4-2: Results of the generalized linear mixed effect model of 'time' and 'treatment' on "cross backs" of *Daphnia magna*, following the 2-min exposure to different treatments with AgNPs and assessed at different time periods. The coefficient estimates, standard errors (SE), z-values and p-values for fixed effects are shown. The significant differences ($p < 0.05$) within controls, and within NP concentrations and controls are marked in bold. EFF, CT, and DA are the controls (without NPs) for the effluent, ASTM medium, and dispersant agent, respectively.

Experiments and treatments	Fixed effects	Estimate	SE	z value	p
	CT vs. EFF				
1A: EFF - CT	(Intercept)	3.165	0.043	73.458	**< 0.001**
	Time	0.036	0.042	0.852	0.394
	Treatment	-0.525	0.043	-12.199	**< 0.001**
	EFF vs. 0 h - 96 h				
	(Intercept)	3.156	0.101	31.215	**< 0.001**
	Time	-0.129	0.015	-8.242	**< 0.001**
	Treatment	0.055	0.101	0.550	0.582
	EFF vs. 0 h - 48 h				
	(Intercept)	3.099	0.115	28.046	**< 0.001**
	Time	-0.211	0.035	-6.012	**< 0.001**
	Treatment	0.073	0.109	0.669	0.503
	EFF vs. 0 h - 24 h				
	(Intercept)	2.981	0.121	24.443	**< 0.001**
	Time	-0.366	0.066	-5.506	**< 0.001**
	Treatment	0.079	0.112	0.707	0.48
	EFF vs. 0 h - 12 h				
1A: wastewater-borne AgNPs	(Intercept)	2.819	0.163	17.232	**< 0.001**
	Time	-0.552	0.135	-4.072	**< 0.001**
	Treatment	0.077	0.120	0.639	0.523
	EFF vs. 0 h - 6 h				
	(Intercept)	2.478	0.285	8.667	**< 0.001**
	Time	-0.924	0.290	-3.186	**0.001**
	Treatment	0.065	0.126	0.520	0.602
	EFF vs. 0 h - 3 h				
	(Intercept)	2.957	0.602	4.911	**< 0.001**
	Time	-0.417	0.637	-0.656	0.512
	Treatment	0.028	0.120	0.238	0.812
	EFF vs. 0 h				
	(Intercept)	3.368	0.123	27.308	**< 0.001**
	Treatment	-0.045	0.125	-0.364	0.716
	CT vs. DA				
1B: DA - CT	(Intercept)	3.547	0.040	88.539	**< 0.001**
	Time	0.076	0.040	1.918	0.055
	Treatment	-0.141	0.040	-3.512	**< 0.001**

Table S4-2: Continued.

1B: ASTM-dispersed AgNPs	**DA vs. 0 h - 96 h**				
	(Intercept)	3.280	0.060	54.116	**< 0.001**
	Time	-0.077	0.019	-3.981	**< 0.001**
	Treatment	0.013	0.060	0.219	0.826
	DA vs. 0 h - 48 h				
	(Intercept)	3.226	0.053	60.241	**< 0.001**
	Time	-0.174	0.040	-4.312	**< 0.001**
	Treatment	0.036	0.048	0.743	0.458
	DA vs. 0 h - 24 h				
	(Intercept)	3.009	0.068	43.978	**< 0.001**
	Time	-0.461	0.074	-6.226	**< 0.001**
	Treatment	0.062	0.041	1.504	0.133
	DA vs. 0 h - 12 h				
	(Intercept)	2.772	0.135	20.422	**< 0.001**
	Time	-0.736	0.153	-4.785	**< 0.001**
	Treatment	0.063	0.045	1.372	0.17
	DA vs. 0 h - 6 h				
	(Intercept)	2.241	0.271	8.253	**< 0.001**
	Time	-1.320	0.301	-4.384	**< 0.001**
	Treatment	0.083	0.038	2.188	**0.028**
	DA vs. 0 h - 3 h				
	(Intercept)	1.703	0.548	3.108	**0.001**
	Time	-1.887	0.584	-3.228	**0.001**
	Treatment	0.083	0.034	2.388	**0.016**
	DA vs. 0 h				
	(Intercept)	3.539	0.039	90.442	**< 0.001**
	Treatment	0.140	0.039	3.557	**< 0.001**

Table S4-3: Results of the generalized linear mixed effect model of 'time' and 'treatment' on "crossings" of *Daphnia magna*, following the 2-min exposure to different treatments with TiO_2NPs and assessed at different time periods. The coefficient estimates, standard errors (SE), z-values and p-values for fixed effects are shown. The significant differences ($p < 0.05$) within controls, and within NP concentrations and controls are marked in bold. EFF, CT, and DA are the controls (without NPs) for the effluent, ASTM medium, and dispersant agent, respectively.

Experiments and treatments	Fixed effects	Estimate	SE	z value	p
2A: EFF - CT	**CT vs. EFF**				
	(Intercept)	5.314	0.019	277.518	**< 0.001**
	Time	-0.019	0.019	-0.990	0.322
	Treatment	0.175	0.019	0.911	0.362
2A: wastewater-borne TiO_2NPs	**EFF vs. 0 h - 96 h**				
	(Intercept)	5.312	0.028	184.873	**< 0.001**
	Time	-0.015	0.009	-1.580	0.114
	Treatment	0.009	0.028	0.344	0.731
	EFF vs. 0 h - 48 h				
	(Intercept)	5.289	0.026	198.082	**< 0.001**
	Time	-0.051	0.023	-2.212	**0.027**
	Treatment	0.005	0.023	0.233	0.816
	EFF vs. 0 h - 24 h				
	(Intercept)	5.283	0.045	116.747	**< 0.001**
	Time	-0.058	0.052	-1.123	0.261
	Treatment	0.004	0.024	0.199	0.843
	EFF vs. 0 h - 12 h				
	(Intercept)	5.169	0.105	49.115	**< 0.001**
	Time	-0.190	0.122	-1.547	0.122
	Treatment	0.007	0.022	0.314	0.754
	EFF vs. 0 h - 6 h				
	(Intercept)	5.188	0.218	23.791	**< 0.001**
	Time	-0.169	0.242	-0.697	0.486
	Treatment	0.002	0.026	0.107	0.915
	EFF vs. 0 h - 3 h				
	(Intercept)	6.254	0.454	13.769	**< 0.001**
	Time	0.951	0.483	1.968	**0.049**
	Treatment	-0.004	0.035	-0.136	0.891
	EFF vs. 0 h				
	(Intercept)	5.320	0.036	146.099	**< 0.001**
	Treatment	-0.019	0.036	-0.531	0.596

Table S4-3: Continued.

2B: ASTM-dispersed TiO_2NPs	**CT vs. 0 h - 96 h**				
	(Intercept)	5.362	0.0097	550.372	**< 0.001**
	Time	-0.009	0.0097	-0.936	0.349
	Treatment	0.037	0.0097	3.804	**< 0.001**
	CT vs. 0 h - 48 h				
	(Intercept)	5.331	0.016	329.945	**< 0.001**
	Time	-0.060	0.022	-2.707	**< 0.001**
	Treatment	0.031	0.011	2.840	**< 0.001**
	CT vs. 0 h - 24 h				
	(Intercept)	5.365	0.036	145.955	**< 0.001**
	Time	-0.016	0.048	-0.341	0.733
	Treatment	0.033	0.012	2.726	**0.006**
	CT vs. 0 h - 12 h				
	(Intercept)	5.082	0.092	54.907	**< 0.001**
	Time	-0.344	0.109	-3.150	**0.001**
	Treatment	0.037	0.013	2.716	**0.006**
	CT vs. 0 h - 6 h				
	(Intercept)	4.989	0.189	26.281	**< 0.001**
	Time	-0.446	0.211	-2.106	**0.035**
	Treatment	0.039	0.015	2.584	**0.009**
	CT vs. 0 h - 3 h				
	(Intercept)	6.271	0.390	16.060	**< 0.001**
	Time	0.901	0.416	2.164	**0.003**
	Treatment	0.025	0.018	1.392	0.163
	CT vs. 0 h				
	(Intercept)	5.387	0.024	216.446	**< 0.001**
	Treatment	0.034	0.024	1.383	0.167

Table S4-4: Results of the generalized linear mixed effect model of 'time' and 'treatment' on "cross backs" of *Daphnia magna*, following the 2-min exposure to different treatments with TiO_2NPs and assessed at different time periods. The coefficient estimates, standard errors (SE), z-values and p-values for fixed effects are shown. The significant differences ($p < 0.05$) within controls, and within NP concentrations and controls are marked in bold. EFF, CT, and DA are the controls (without NPs) for the effluent, ASTM medium, and dispersant agent, respectively.

Experiments and treatments	Fixed effects	Estimate	SE	z value	p
	CT vs. EFF				
2A: EFF - CT	(Intercept)	3.471	0.030	113.189	**< 0.001**
	Time	0.016	0.030	0.550	0.582
	Treatment	0.025	0.030	0.832	0.406
	EFF vs. 0 h - 96 h				
	(Intercept)	3.492	0.081	43.102	**< 0.001**
	Time	0.017	0.014	1.202	0.229
	Treatment	0.061	0.081	0.755	0.450
	EFF vs. 0 h - 48 h				
	(Intercept)	3.463	0.082	42.098	**< 0.001**
	Time	-0.026	0.034	-0.744	0.457
	Treatment	0.063	0.080	0.794	0.427
	EFF vs. 0 h - 24 h				
	(Intercept)	3.504	0.096	36.138	**< 0.001**
	Time	0.029	0.074	0.392	0.695
	Treatment	0.057	0.080	0.711	0.477
	EFF vs. 0 h - 12 h				
2A: wastewater-borne TiO_2NPs	(Intercept)	3.431	0.167	20.535	**< 0.001**
	Time	-0.054	0.174	-0.312	0.755
	Treatment	0.069	0.080	0.862	0.389
	EFF vs. 0 h - 6 h				
	(Intercept)	3.586	0.320	11.203	**< 0.001**
	Time	0.115	0.347	0.333	0.739
	Treatment	0.062	0.080	0.777	0.437
	EFF vs. 0 h - 3 h				
	(Intercept)	5.563	0.648	8.575	**< 0.001**
	Time	2.197	0.688	3.193	**0.001**
	Treatment	0.063	0.084	0.751	0.452
	EFF vs. 0 h				
	(Intercept)	3.411	0.087	39.172	**< 0.001**
	Treatment	0.071	0.086	0.831	0.406

Table S4-4: Continued.

2B: ASTM-dispersed TiO_2NPs	**CT vs. 0 h - 96 h**				
	(Intercept)	3.646	0.024	148.275	**< 0.001**
	Time	-0.003	0.013	-0.234	0.815
	Treatment	0.094	0.024	3.827	**< 0.001**
	CT vs. 0 h - 48 h				
	(Intercept)	3.606	0.033	107.590	**< 0.001**
	Time	-0.061	0.034	-1.801	0.071
	Treatment	0.089	0.028	3.134	**0.001**
	CT vs. 0 h - 24 h				
	(Intercept)	3.670	0.058	62.871	**< 0.001**
	Time	0.020	0.071	0.288	0.772
	Treatment	0.090	0.028	3.159	**< 0.001**
	CT vs. 0 h - 12 h				
	(Intercept)	3.3291	0.137	24.231	**< 0.001**
	Time	-0.373	0.159	-2.344	**0.019**
	Treatment	0.1033	0.031	3.289	**0.001**
	CT vs. 0 h - 6 h				
	(Intercept)	3.063	0.280	10.910	**< 0.001**
	Time	-0.663	0.311	-2.130	**0.033**
	Treatment	0.096	0.034	2.797	**0.005**
	CT vs. 0 h - 3 h				
	(Intercept)	4.204	0.622	6.750	**< 0.001**
	Time	0.536	0.664	0.807	0.419
	Treatment	0.085	0.033	2.584	**0.009**
	CT vs. 0 h				
	(Intercept)	3.674	0.048	75.122	**< 0.001**
	Treatment	0.073	0.049	1.498	0.134

Table S4-5: Results of Chi-squared test independently performed for each time point and treatment. Significant p-values are marked bold. χ^2 = test statistic, df = degrees of freedom.

Experiment	time point	Treatment	χ^2	df	p
1A: wastewater-borne AgNPs	0 h	EFF	0.033	3	0.998
		25 µg/L	10.089	3	**0.017**
		50 µg/L	11.989	3	**0.007**
		75 µg/L	11.217	3	**0.010**
		100 µg/L	6.347	3	0.095
		125 µg/L	0.173	3	0.981
	3 h	EFF	0.044	3	0.997
		25 µg/L	16.376	3	**0.001**
		50 µg/L	11.731	3	**0.008**
		75 µg/L	6.347	3	0.095
		100 µg/L	13.484	3	**0.003**
		125 µg/L	2869	3	0.412
	6 h	EFF	3.129	3	0.372
		25 µg/L	8.608	3	**0.034**
		50 µg/L	0.727	3	0.866
		75 µg/L	2.494	3	0.476
		100 µg/L	14.087	3	**0.002**
		125 µg/L	0.560	3	0.905
	12 h	EFF	1.838	3	0.606
		25 µg/L	6.260	3	0.099
		50 µg/L	4.347	3	0.226
		75 µg/L	0.296	3	0.960
		100 µg/L	4.347	3	0.226
		125 µg/L	1.130	3	0.769
2A: wastewater-borne TiO_2NPs	0 h	EFF	0.126	3	0.988
		12.5 µg/L	3.989	3	0.262
		25 µg/L	2.173	3	0.537
		50 µg/L	1.967	3	0.579
		75 µg/L	8.521	3	**0.036**
		100 µg/L	5.835	3	0.119
	3 h	EFF	0.317	3	0.956
		12.5 µg/L	1.024	3	0.795
		25 µg/L	0.046	3	0.997
		50 µg/L	4.488	3	0.213
		75 µg/L	2.111	3	0.549
		100 µg/L	0.976	3	0.806
	6 h	EFF	0.400	3	0.940
		12.5 µg/L	0.284	3	0.963
		25 µg/L	0.126	3	0.988
		50 µg/L	1.967	3	0.579
		75 µg/L	0.976	3	0.806
		100 µg/L	3.000	3	0.391
	12 h	EFF	0.139	3	0.986
		12.5 µg/L	1.083	3	0.781
		25 µg/L	1.162	3	0.761
		50 µg/L	2.173	3	0.537
		75 µg/L	1.481	3	0.686
		100 µg/L	7.044	3	0.070

Table S4-5: Continued.

1B: ASTM-dispersed AgNPs	0 h	CT	1.046	3	0.790
		DA	0.1236	3	0.988
		25 µg/L	8.9091	3	**0.030**
		50 µg/L	21.968	3	**0.001**
		75 µg/L	9.4719	3	**0.023**
		100 µg/L	8.9091	3	**0.030**
	3 h	CT	0.190	3	0.979
		DA	0.181	3	0.980
		25 µg/L	10.582	3	**0.014**
		50 µg/L	11	3	**0.011**
		75 µg/L	5.835	3	0.119
		100 µg/L	2.494	3	0.476
	6 h	CT	0.048	3	0.997
		DA	0.033	3	0.998
		25 µg/L	6.260	3	0.099
		50 µg/L	2.179	3	**0.001**
		75 µg/L	3.197	3	0.362
		100 µg/L	0.0344	3	0.998
	12 h	CT	0.142	3	0.986
		DA	0.614	3	0.893
		25 µg/L	6.890	3	0.075
		50 µg/L	6.170	3	0.103
		75 µg/L	4.868	3	0.181
		100 µg/L	1.382	3	0.709
2B: ASTM-dispersed TiO_2NPs	0 h	CT	2.177	3	0.536
		12.5 µg/L	0.090	3	0.992
		25 µg/L	7.511	3	0.06
		50 µg/L	2.177	3	0.536
		75 µg/L	5.304	3	0.150
		100 µg/L	1.636	3	0.651
	3 h	CT	0.727	3	0.866
		12.5 µg/L	0	3	1
		25 µg/L	2.057	3	0.560
		50 µg/L	0.761	3	0.858
		75 µg/L	0.857	3	0.835
		100 µg/L	0.614	3	0.893
	6 h	CT	1.351	3	0.716
		12.5 µg/L	0.6	3	0.896
		25 µg/L	2.266	3	0.518
		50 µg/L	0.095	3	0.992
		75 µg/L	1.413	3	0.702
		100 µg/L	0.4186	3	0.936
	12 h	CT	2.782	3	0.426
		12.5 µg/L	0.790	3	0.851
		25 µg/L	6.978	3	0.072
		50 µg/L	1.727	3	0.630
		75 µg/L	4.168	3	0.243
		100 µg/L	1.162	3	0.761

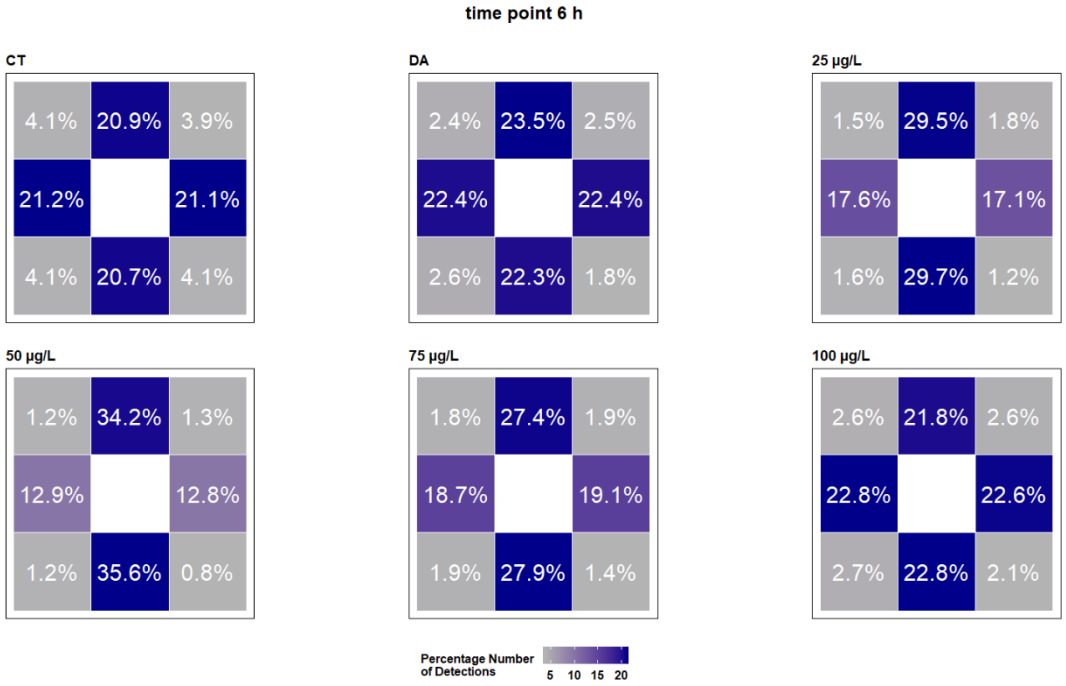


Figure S4-1: Relative percentage change of direction of *Daphnia magna* at time point 6 h and under the exposure with wastewater-borne AgNPs (Exp. 1A).

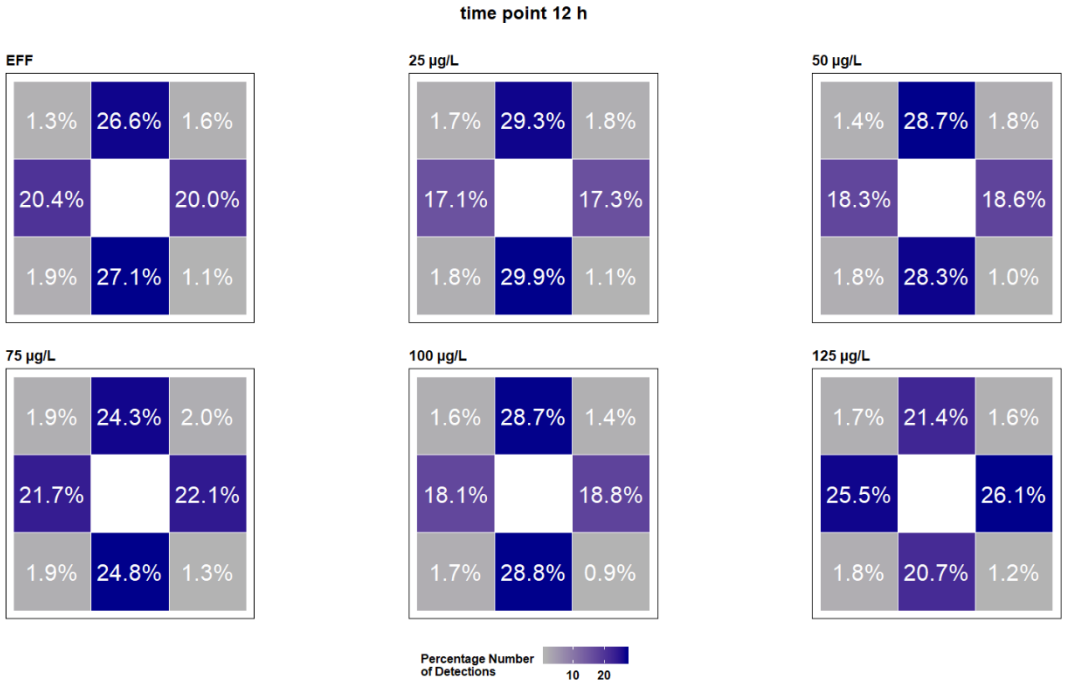


Figure S4-2: Relative percentage change of direction of *Daphnia magna* at time point 12 h and under the exposure with wastewater-borne AgNPs (Exp. 1A).

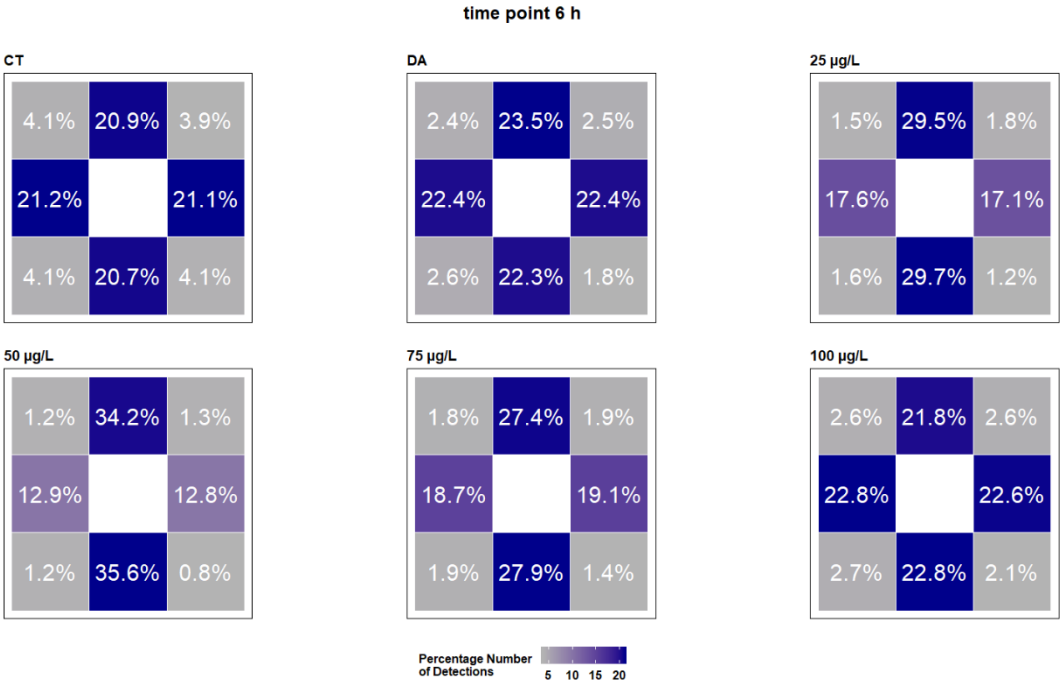


Figure S4-3: Relative percentage change of direction of *Daphnia magna* at time point 6 h and under the exposure with ASTM-dispersed AgNPs (Exp. 1B).

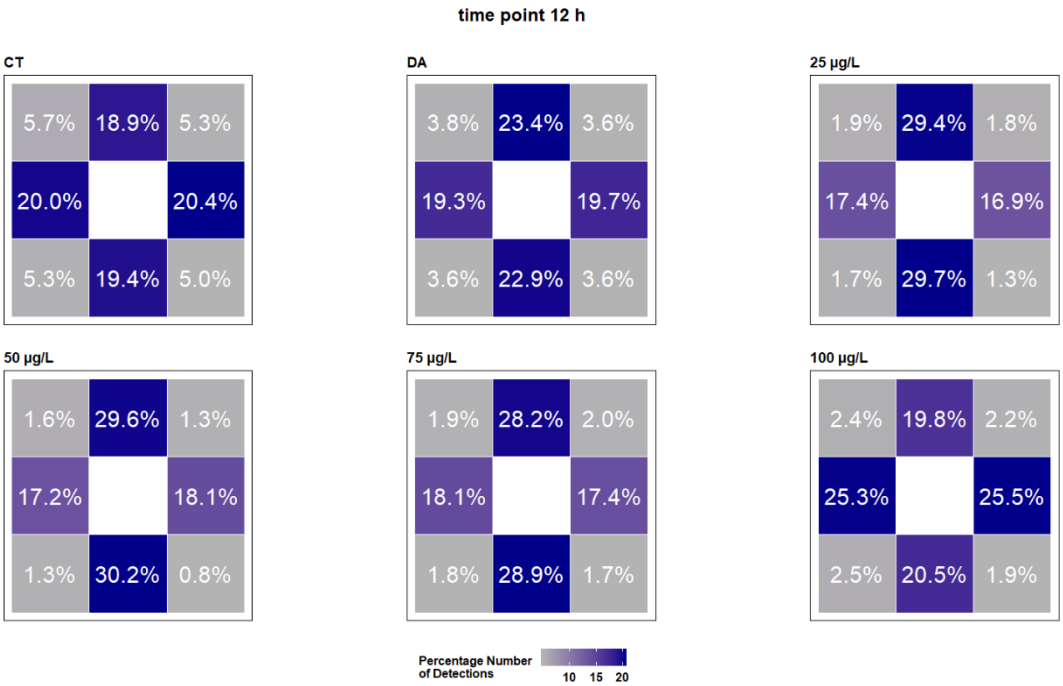


Figure S4-4: Relative percentage change of direction of *Daphnia magna* at time point 12 h and under the exposure with ASTM-dispersed AgNPs (Exp. 1B).

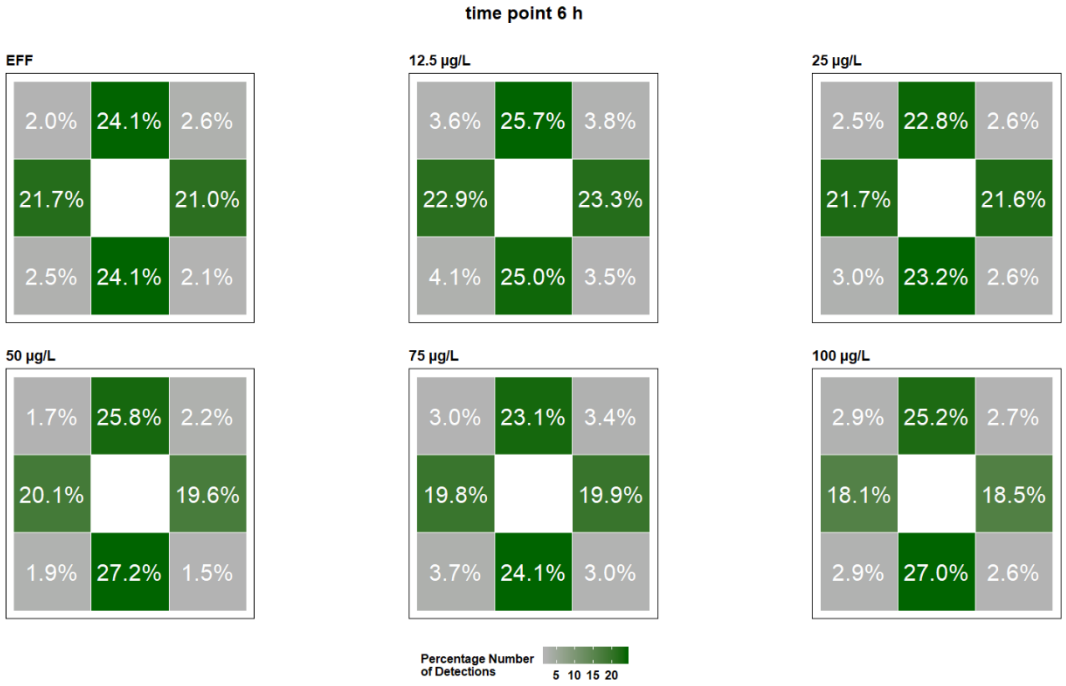


Figure S4-5: Relative percentage change of direction of *Daphnia magna* at time point 6 h and under the exposure with wastewater-borne TiO_2NPs (Exp. 2A).

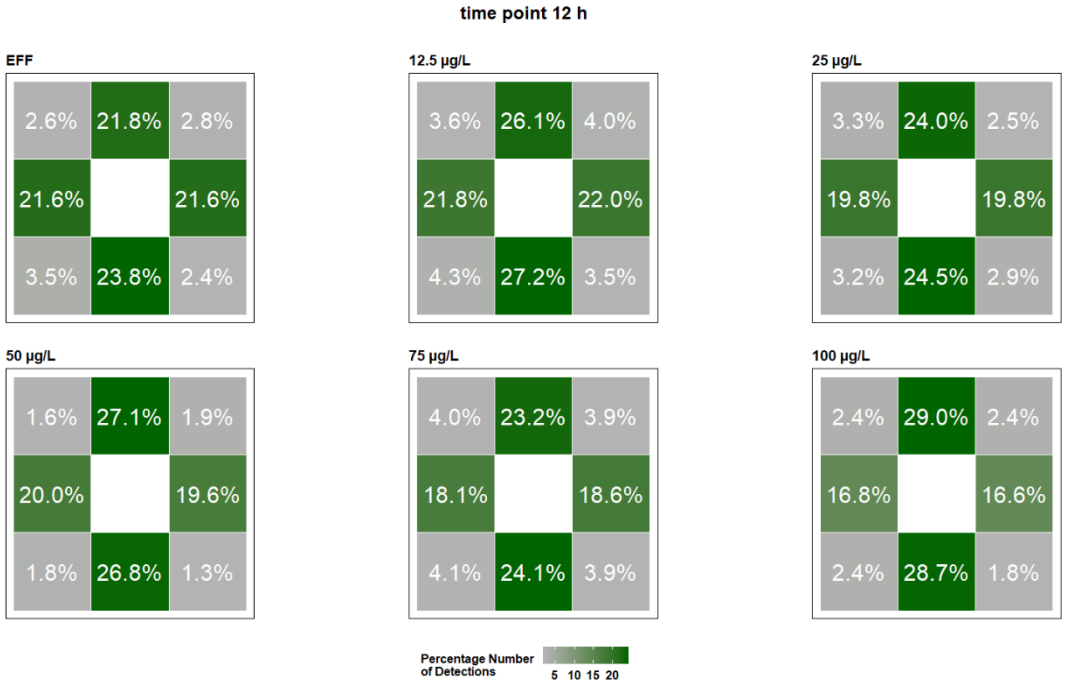


Figure S4-6: Relative percentage change of direction of *Daphnia magna* at time point 12 h and under the exposure with wastewater-borne TiO_2NPs (Exp. 2A).

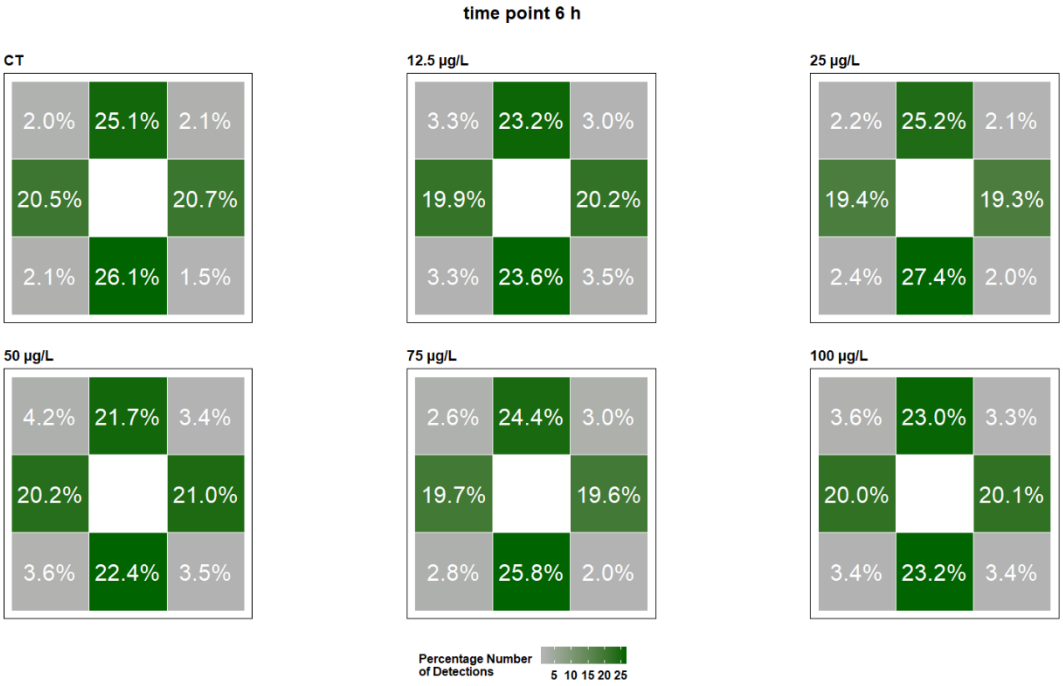


Figure S4-7: Relative percentage change of direction of *Daphnia magna* at time point 6 h and under the exposure with ASTM-dispersed TiO_2NPs (Exp. 2B).

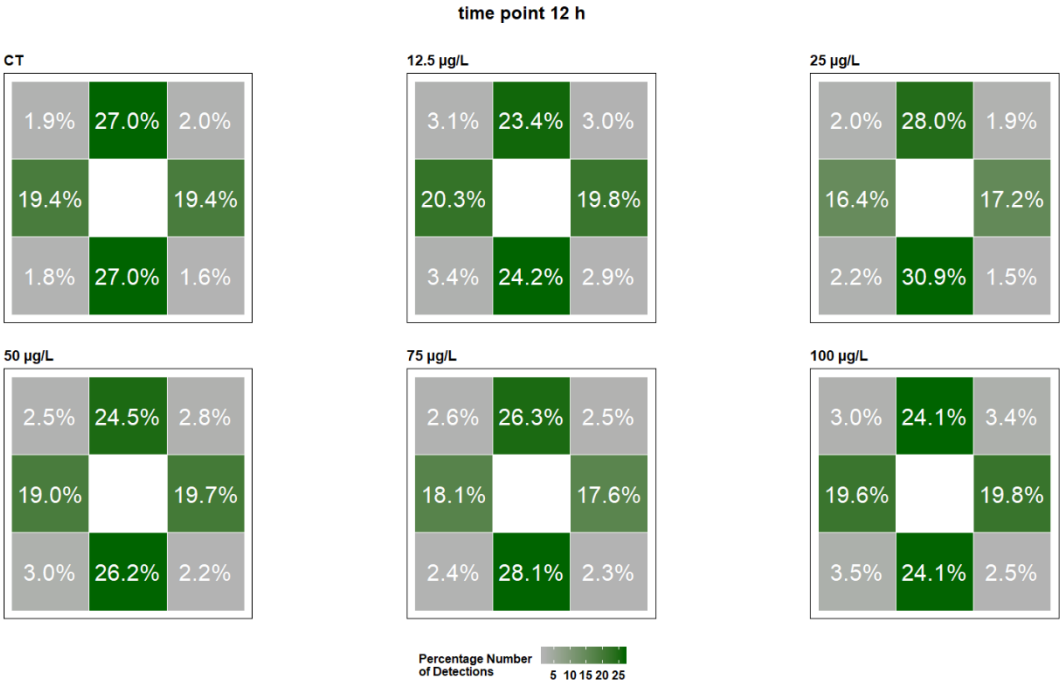
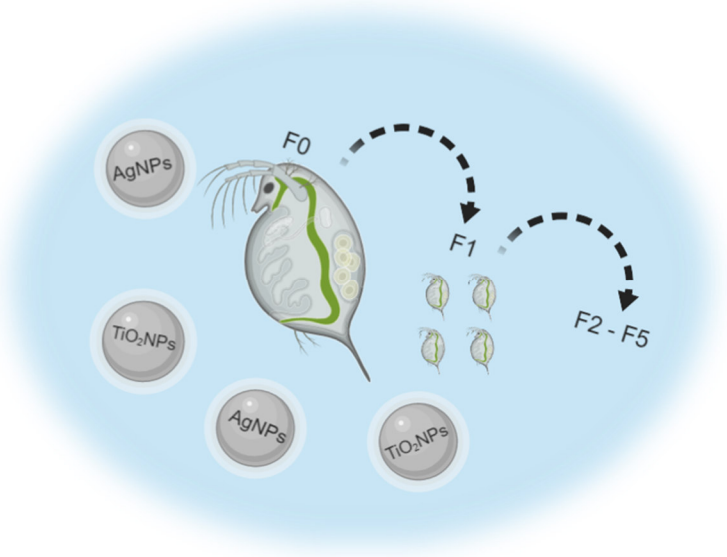


Figure S4-8: Relative percentage change of direction of *Daphnia magna* at time point 12 h and under the exposure with ASTM-dispersed TiO_2NPs (Exp. 2B).

Chapter 5

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*



Rebecca Louch from the University of Manchester helped to collect the data. The project partners Benedikt Steinhoff and Darya Mozhayeva provided the particle characterisation and the analytical data analysed in this chapter.

The study of this chapter has been published in *NanoImpact*:

Hartmann S, Louch R, Zeumer R, Steinhoff B, Mozhayeva D, Engelhard C, Schönherr H, Schlechtriem C, Witte K (2018) „Comparative multi-generation study of long-term effects of pristine and wastewater-borne silver and titanium dioxide nanoparticles on key lifecycle parameters in *Daphnia magna"*. NanoImpact, Vol. 14, 100163, 10.1016/j.impact.2019.100163.

5.1 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 (TiO_2NPs), 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 TiO_2NPs 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 TiO_2 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 TiO_2NPs. 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 Ag_2S 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 TiO_2NPs and wastewater-borne TiO_2NPs. 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 TiO_2NPs were observed. The results confirm that realistic

exposure conditions are required in order to allow for a reliable environmental risk assessment of NPs.

5.2 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 (Fytili 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 (TiO_2NPs) 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 TiO_2NPs range from 0.088 – 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 TiO_2NPs 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., 2015; 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., 2014) estimated a 21 day EC_{50} value of 1.0 µg/L for AgNPs. By contrast, the exposure to $AgNO_3$ leads to a higher toxicity with a 21 day EC_{50} 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 $AgNO_3$) 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 yet (Völker et al., 2013b) 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., 2013a; Zhao and Wang, 2011).

Especially for TiO_2NPs, 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* EC_{50} (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 TiO_2 to the aquatic invertebrate *Daphnia magna* according to OECD TG 211 resulted in an estimated EC_{50} value of 0.46 mg/L for reproduction and an estimated LC_{50} (median lethal concentration) value of 2.62 mg/L (Zhu et al., 2010). The mechanism for toxicity of TiO_2NPs 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 TiO_2NPs 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 Ag_2S, 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). Ag_2S 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 Ag_2S, 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; Kühr 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; Kühr et al., 2018). No information is currently available on the chronic toxicity of TiO_2NPs 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. (2013a) 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 $AgNO_3$ 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 TiO_2NPs of pre-exposed adult *Daphnia* led to a significantly lower 96 h-EC_{50} (median effective concentration after 96 hours 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 TiO_2NPs 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 environmentally conditions.

The aim of this study was, therefore, to investigate the impact of pristine and wastewater-borne AgNPs and TiO_2NPs 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-300K) and TiO_2NPs (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).

5.3 Material and Methods

5.3.1 Study species

Populations of the freshwater cladoceran *Daphnia magna* (clone V; Federal Environment Agency, Berlin, Germany) served as test organism. The culture conditions and the corresponding food source, *Desmodesmus subspicatus,* are described in detail in Chapter 2.1.4.

5.3.2 Preparation of test media and particle characterisation

5.3.2.1 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). Detailed information of the chemical properties of NM300-K are given in Chapter 3.3.1. 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).

5.3.2.2 Titanium dioxide-nanoparticles (NM-105)

The experiments with TiO_2NPs were conducted with the OECD reference nanomaterial (WPMN programme) NM-105. Detailed information of the chemical properties of NM-105 are given in Chapter 3.3.1. Before use, the TiO_2NP 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 (Verleysen et al., 2014). Dilutions were done in PP vials (VWR International, Langenfeld, Germany). The suspension was used immediately.

5.3.2.3 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 AgNP or TiO_2NP 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 (≥ 99.8%, VWR Germany).

5.3.3 Model sewage treatment plant (STP)

Several model sewage treatment plants (STPs) were used to produce effluents in two independent runs with AgNPs or TiO_2NPs according to OECD TG 303a (OECD, 2001) as previously described (Kampe et al., 2018; Kühr 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°09'N 8°16'E, Schmallenberg, 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). Physicochemical 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 5-1) 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_2NP 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_2NPs, 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 5-1. 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 Chapter 5.3.6).

5.3.4 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_2NPs 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. (2013a) 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), 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/*Daphnia*/day). The number of

offspring was counted and removed six times a week. Once a week we measured temperature, 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 S5-1). 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* with a digital camera (Nikon Coolpix L830, Chiyoda, Tokyo, Japan) and analysed body length using the software AxioVision (Carl Zeiss, Jena).

5.3.4.1 **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 TiO_2NPs (i_b) was performed with a control (p-C 2) containing ASTM-medium only and three TiO_2-treatments (p-TiO_2 1-3) with different concentrations of TiO_2NPs. An overview of the preparation of the exposure scenario with pristine NPs are shown in Table 5-1.

5.3.4.2 **Exposure scenario (ii): wastewater-borne NPs**

For the scenario with wastewater-borne AgNPs (ii_a) and with the wastewater-borne TiO_2NPs (ii_b), the collected effluents from the model STPs were shaken for two minutes 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 5-1). 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 5-1. No matrix control was included.

Wastewater-borne TiO_2 (scenario ii_b) was tested by using the control (STP-C 2) and three wastewater-borne TiO_2-treatments (STP-TiO_2 1-3) with different concentrations of TiO_2NPs. Dilution steps for the wastewater-borne TiO_2NP (ii_b) treatments and information on media preparation are presented in Table 5-1.

5.3.5 Collection of media samples for determination of total Ag and Ti concentrations

For the STP effluent with AgNPs and TiO_2NPs, 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.

5.3.6 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 minutes 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

$^{107}Ag^{+}$. 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 $^{107}Ag^{+}$ were ranging from 0.06 to 0.12 µg/L and from 0.19 to 0.38 µg/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. (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) and 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 µg/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 µg/L and from 1.88 to 6.14 µg/L, respectively, depending on the experimental conditions.

The main ICP-MS and ICP-OES instrumental parameters are presented in Table S5-2. 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.

Table 5-1: Exposure scenarios and preparation of test media for the different treatments with respective nominal Ag and Ti concentrations. Concentrations [µg/L] of total Ag and total Ti in the STP effluents (± 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. [µg/L] ± U	Effluent dilution factor	Nominal test media conc. [µg/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
	p-Ag 4	ASTM-medium spiked with NM-300K	n/r	n/r	n/r	10.00
(i_b) pristine TiO_2NPs	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 ± 5.30	1:43	1.25
	STP-Ag 2	AgNP-spiked effluent	2.5	64.45 ± 4.83	1:25	2.5
	STP-Ag 3	AgNP-spiked effluent	3.5	140.70 ± 5.56	1:28	5.00
	STP-Ag 4	AgNP-spiked effluent	6.5	239.03 ± 7.24	1:23	10.00
(ii_b) wastewater-borne TiO_2NPs	STP-C 2	Control effluent from STP	-	-	1:3	-
	STP-TiO_2 1	TiO_2NP-spiked effluent	1	104.34 ± 2.55	1:7	25
	STP-TiO_2 2	TiO_2NP-spiked effluent	2.5	113.67 ± 1.43	1:4	50
	STP-TiO_2 3	TiO_2NP-spiked effluent	5	464.27 ± 6.66	1:8	100

Note: For exposure scenario ii_b the total concentration of the effluent is present as µg/L ± sd; n/r – not required; n/m – not measured; U – expanded uncertainty for measured concentrations

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

5.3.7 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 (± sd), the mean body length (mm ± sd), mortality [%] and the mean time to first brood (days ± 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.

5.4 Results

5.4.1 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 TiO_2NPs formed aggregates of up to

several 100 nm due to the absence of stabilising 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 TiO_2NPs in their pristine state (ASTM-medium) and after passage through a model STP passage. 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 Ag_2S (silver sulphide) which was supported by the atomic Ag/S ratio of ~2 (Figure 5-1B). 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 TiO_2NPs. 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 (Figure 5-2B). 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 TiO_2NPs, it can be assumed that no sulphidation process occurred. This transformation typically requires a reaction with sulphides (S^{2-}) 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.

5.4.2 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 5-1. 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 5-2. In generation F2, the total Ag contents of the treatments with pristine Ag (i_a; ASTM-medium spiked with NM-300K) were 1.98 µg/L (p-Ag 1), 3.35 µg/L (p-Ag 2),

Table 5-2: Total Ag and Ti concentrations (µg/L) of freshly prepared media and aged media samples collected during generation F2 and F4 after 72h of exposure. Given are measured concentrations as mean (± sd) of three individual samples.

Exposure scenario	Treatment	Nominal concentration (µg/L)	Mean measured concentration ± sd (µg/L)	
			Fresh media	Aged media
(i_a) pristine AgNPs	p-C 1	-	3.56 ± 2.61*	1.96 ± 2.11*
	p-M 1	-	1.32 ± 1.13	0.47 ± 3.68
	p-Ag 1	1.25	1.98 ± 0.61	4.86 ± 3.65
	p-Ag 2	2.5	3.35 ± 0.33	3.31 ± 1.67
	p-Ag 3	5.00	6.78 ± 2.58	3.36 ± 3.48
	p-Ag 4	10.00	10.34 ± 1.38	5.83 ± 1.49
(i_b) pristine TiO_2NPs	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 ± 0.75	0.59 ± 0.49
	STP-Ag 1	1.25	0.93 ± 0.28	0.48 ± 0.09
	STP-Ag 2	2.5	2.93 ± 0.11	1.27 ± 0.36
	STP-Ag 3	5.00	6.39 ± 0.47	2.80 ± 1.36
	STP-Ag 4	10.00	10.07 ± 2.96	4.21 ± 0.53
(ii_a) wastewater-borne TiO_2NPs	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

6.78 µg/L (p-Ag 3) or 10.34 µg/L (p-Ag 4, Table 5-2). The effluent spiked with AgNPs (ii_a; STP-Ag 1-4) achieved similar total Ag concentrations after the dilution with ASTM-medium in different treatments (Table 5-2). The total Ag contents for the ASTM-control treatment were 3.56 µg/L (p-C 1), for the control effluent 0.61 µg/L (STP-C 1) and for the matrix control 1.32 µg/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 µg/L (p-TiO_2 1), 13.47 µg/L (p-TiO_2 2) and 68.85 µg/L (p-TiO_2 3) and for the wastewater-borne TiO_2 treatments (ii_b) < 6.14 µg/L (STP-TiO_2 1), 11.59 µg/L (STP-TiO_2 2) and 25.20 µg/L (STP-TiO_2 3), respectively (Table 4-2). The total Ti content of the ASTM-control treatment was estimated with < 0.56 µg/L (p-C 2) and of the control effluent with 14.71 µg/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 5-2). The concentration of p-Ag 1 and STP-TiO_2 1 were higher in comparison to the fresh media content. Additionally, the pooled expanded uncertainty (U) for all measured total Ag and Ti contents are presented in Table S5-3.

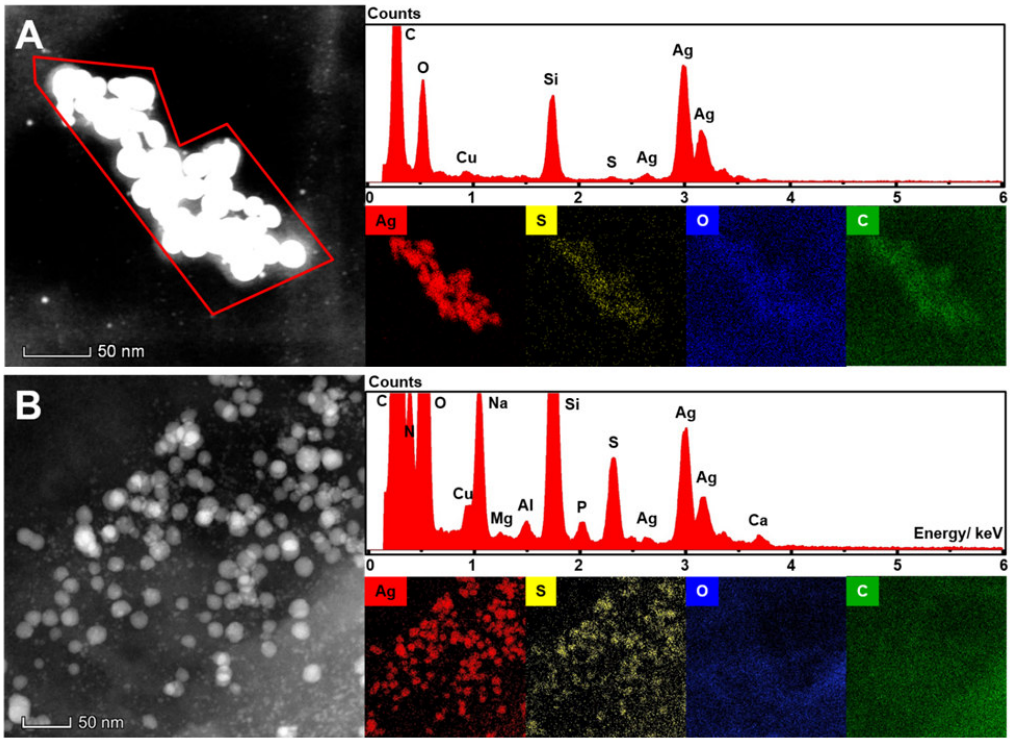


Figure 5-1: STEM images with the corresponding EDX spectra and elemental maps of pristine (A) and wastewater-borne AgNPs (B). The spectrum in A corresponds to the highlighted area in the STEM image, while the spectrum in B corresponds to the entire STEM image.

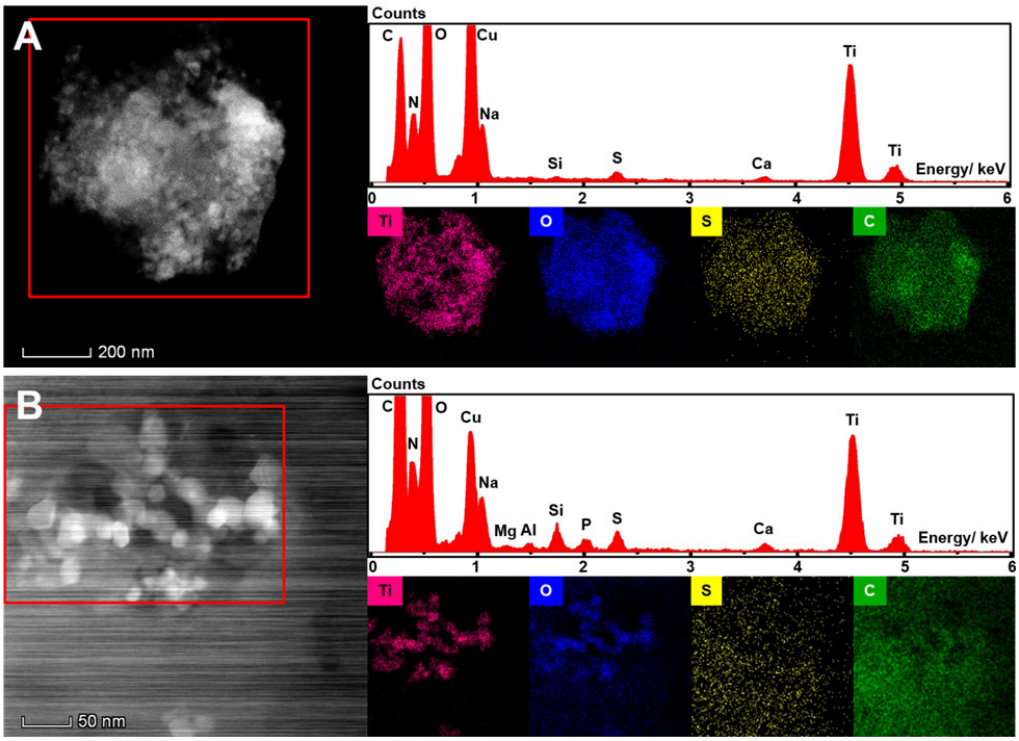


Figure 5-2: STEM images with the respective corresponding EDX spectra and elemental maps of pristine (A) and wastewater-borne TiO_2NPs (B). The spectrum in A and B were captured in the highlighted areas in the STEM images in A and B.

5.4.3 Multi-generation study

5.4.3.1 Reproduction and body length

5.4.3.1.1 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 (Figure 5-3). The cumulative mean number of offspring in generation F0 was affected by all tested concentrations (Kruskal-Wallis test, $\chi^2 = 28.949$, $P \leq 0.001$, Figure 5-3), 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

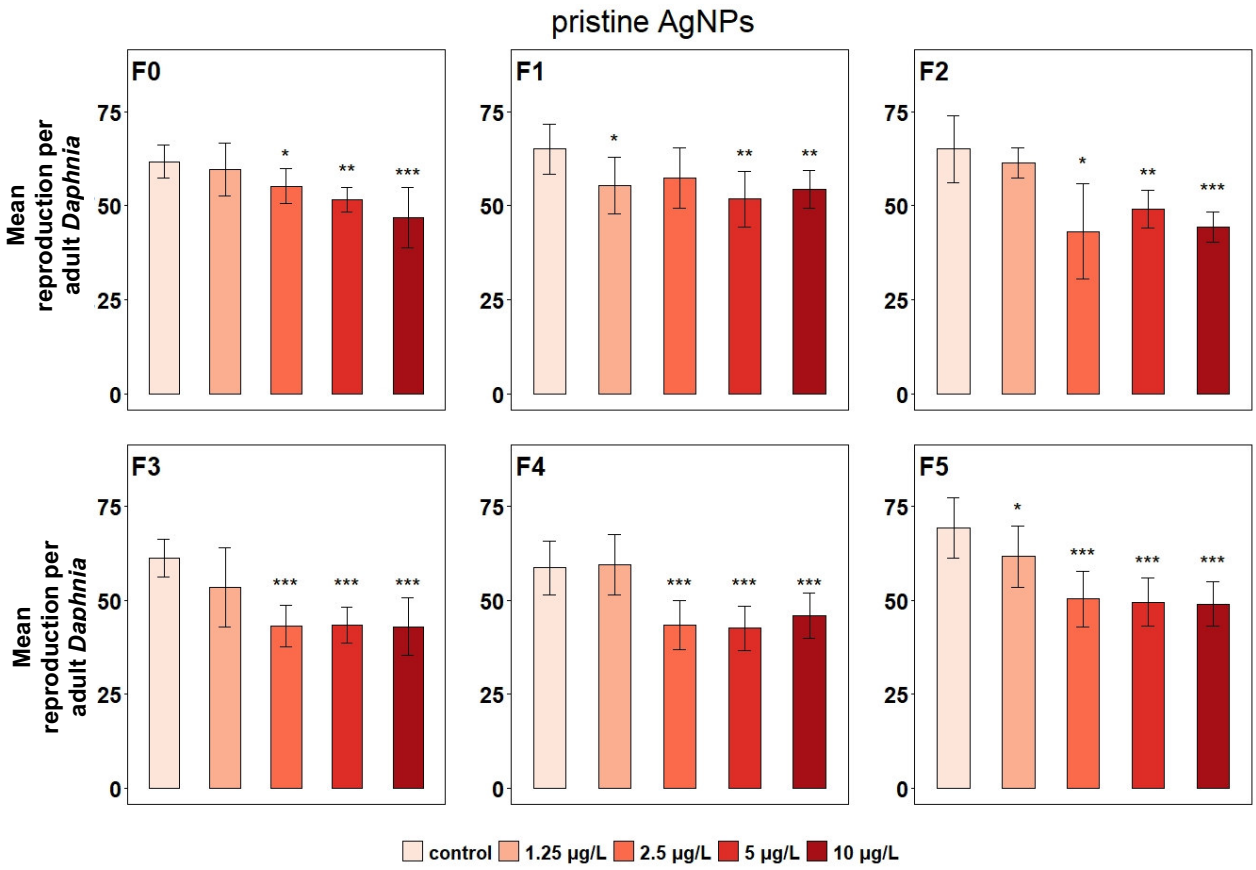


Figure 5-3: Cumulative mean reproduction (as mean number of offspring) per adult *Daphnia* (n = 10; mean ± sd) treated with pristine AgNPs in a multi-generation approach (F0 - F5). Concentrations in treatments are given as nominal concentrations of total Ag in µg/L. Asterisks indicate significant differences compared to the control group: * = P < 0.05, ** = P < 0.01, *** = P < 0.001.

unpaired samples, W = 161; P ≤ 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 ≤ 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 ≤ 0.01, Figure 5-3). 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 ≤ 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 ≤ 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 S5-4.

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 S5-5), 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_2NPs (i_b) had no effect on the reproductive success nor on the adult´s body length in any of the treatments (Figure 5-4, Table S5-4 and S5-5) and over all tested generations (F0 – F5).

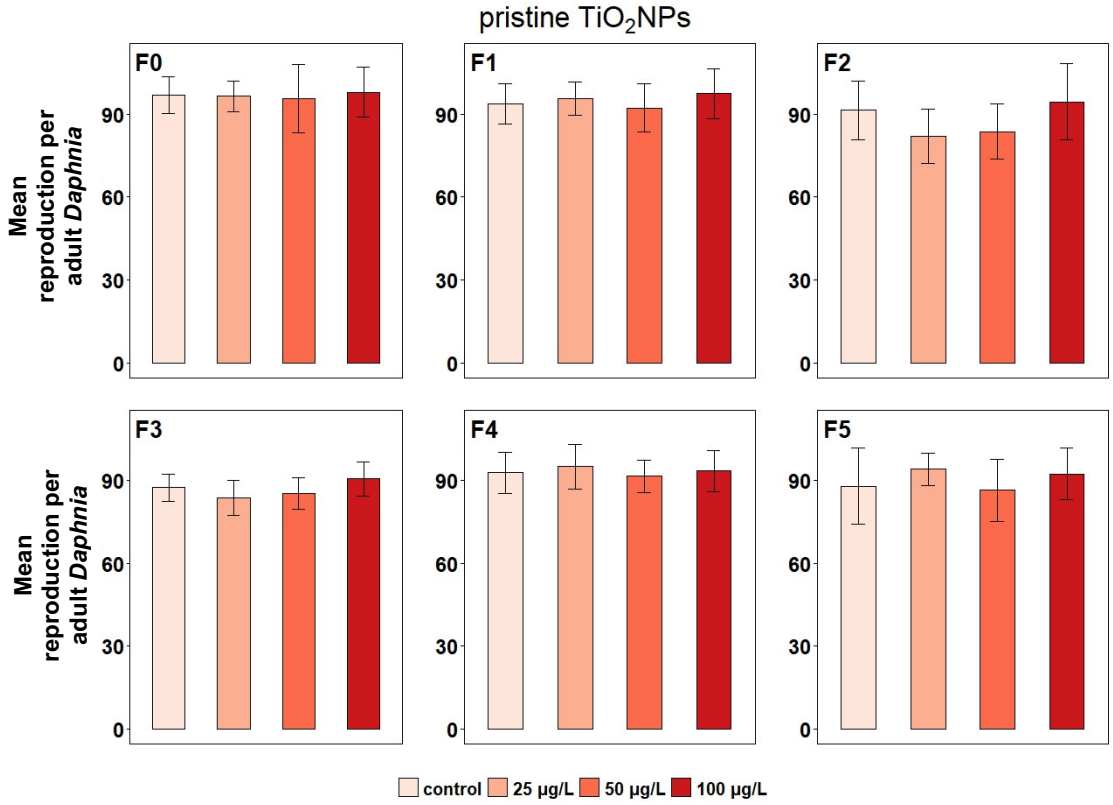


Figure 5-4: Cumulative mean reproduction (as mean number of offspring) per adult *Daphnia* (n = 10; mean ± sd) treated with pristine TiO_2NPs in a multi-generation approach (F0 - F5). Concentrations in treatments are given as nominal concentrations of total Ti in µg/L. No statistically significant differences could be detected.

5.4.3.1.2 Exposure scenario (ii): wastewater-borne NPs

In comparison to pristine AgNPs, the exposure of female *Daphnia* to wastewater-borne AgNPs (ii_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) (Figure 5-5, Table S5-4 and S5-5). Since no matrix control was used for the wastewater-borne AgNP exposure scenario, the control effluent treatment served as the control.

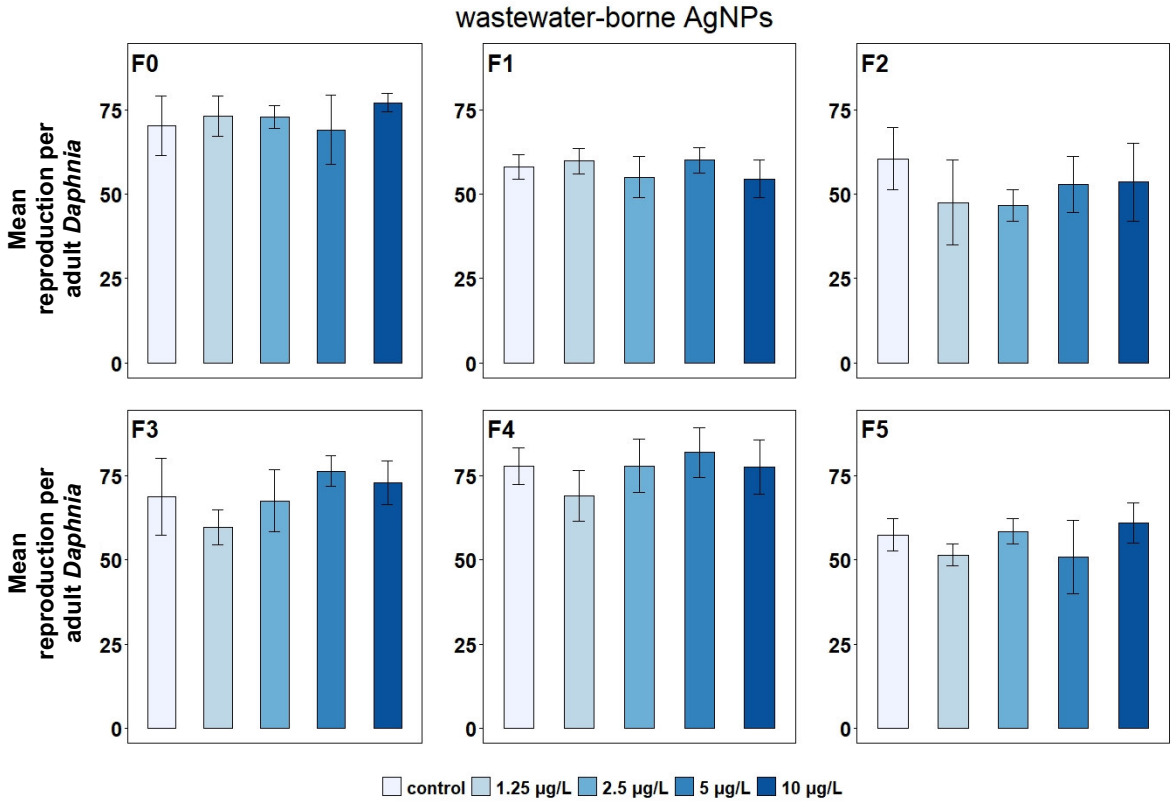


Figure 5-5: Cumulative mean reproduction (as mean number of offspring) per adult *Daphnia* (n = 10; mean ± sd) treated with wastewater-borne AgNPs in a multi-generation approach (F0 - F5). Concentrations in treatments are given as nominal concentrations of total Ag in µg/L. No significant differences could be detected.

In the wastewater-borne exposure scenario with TiO_2NPs (ii_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- TiO_2 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, Figure 5-6, Table S5-4). 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-TiO_2 3 in generation F5 (Table S5-5). 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 S5-5).

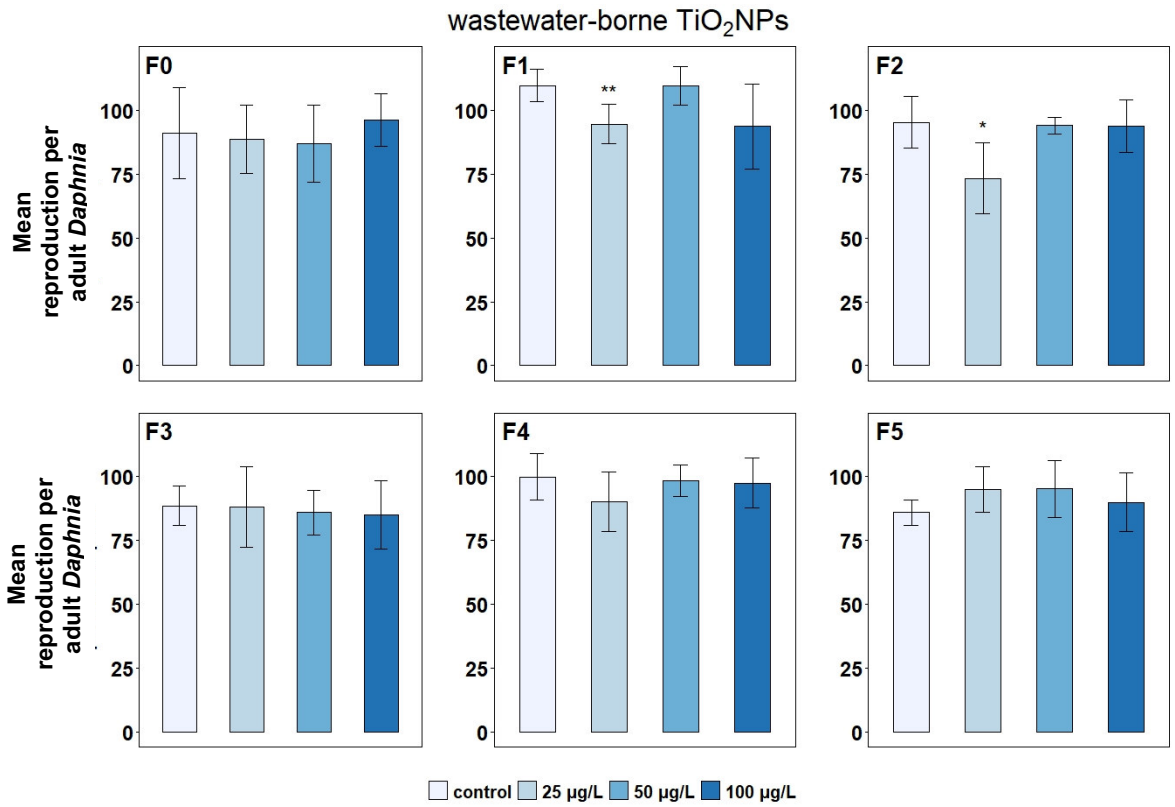


Figure 5-6: Cumulative mean reproduction (as mean number of offspring) per adult *Daphnia* (n = 10; mean ± sd) treated with wastewater-borne TiO_2NPs in a multi-generation approach (F0 - F5). Concentrations in treatments are given as nominal concentrations of total Ti in µg/L. Asterisks indicate significant differences compared to the control group: * = P < 0.05, ** = P < 0.01.

5.4.3.2 **Mortality and mean time to first brood**

Mortality did not differ over all tested generations and between exposure scenarios. However, the mean time to first brood was significantly affected in some of the treatments (F2: p-Ag 3, F3: p- Ag 2, p-TiO_2 1, p-TiO_2 2; STP-Ag 3, STP-Ag 4, F4: p-TiO_2 1, p-TiO_2 3 STP-TiO_2 1, F5: STP-TiO_2 1, STP-TiO_2 2, STP-TiO_2 3), however, no clear relation between NP-exposure and this endpoint could be detected. The results are listed in Table S5-6.

5.5 Discussion

In this study we investigated long-term effects of pristine and wastewater-borne Ag- and TiO_2NPs 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_2NPs demonstrated that neither pristine nor wastewater-borne TiO_2NPs caused any significant effects regarding the reproductive success of *D. magna*. Furthermore, both exposure scenarios with TiO_2NPs 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_2NPs focused on the investigation of the mechanisms and the toxic effects of pristine TiO_2NPs. Concentrations up to 100 times higher compared to this study were applied. To our knowledge, this is the first study on the toxicity of TiO_2NPs 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 TiO_2NPs 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 TiO_2 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 TiO_2NPs in STP effluents are released in pristine form. Since both tested scenarios, exposure to pristine or wastewater-borne TiO_2NPs, respectively, led to similar results, it seems that the toxicity of TiO_2NPs 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 TiO_2NPs do not lead to physiological nor mechanical damage in *Daphnia*. This is in contradiction to the calculated risk quotient (RQ) of TiO_2NPs 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 TiO_2NPs 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 (Struempler, 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 ($110^{m}AgNO_3$) 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 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., 2013a). However, exposure to wastewater-borne AgNPs and TiO_2NPs 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. (Under Review)).*

5.6 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 µg/L) did not affect key lifecycle parameters in *D. magna* over six continuous generations. No transgenerational effects were observed for both exposure scenarios. Furthermore, this study shows that the risk of transformed AgNPs present in the aquatic environment, in the form of Ag_2S, 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 Ag_2S 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 TiO_2NPs (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.

5.7 Supporting Information

Table S5-1: Physico-chemical parameters of the medium used for the multi-generation study in all six generations and different exposure scenarios.

Exposure scenario	Treatment		pH						Oxygen [mg/L]						Temperature [°C]					
			F0	F1	F2	F3	F4	F5	F0	F1	F2	F3	F4	F5	F0	F1	F2	F3	F4	F5
(i_a) pristine AgNPs	p-C 1	1. week	7.88	7.88	7.97	8.97	8.67	8.86	8.49	8.91	7.52	8.37	8.73	8.74	19.4	18.6	19.6	19.4	18.6	20.1
		2. week	7.88	7.96	8.29	8.71	8.87	8.67	7.93	8.33	8.11	8.71	7.94	8.12	20.8	19.9	20.1	20.7	19.7	21.2
		3. week	7.82	7.88	8.56	8.67	8.89	8.84	8.87	8.67	8.45	8.16	8.78	8.42	18.0	19.4	20.5	18.9	20.5	21.4
	p-M 1	1. week	7.87	7.82	7.95	9.02	8.66	8.84	8.60	8.97	7.82	8.08	8.70	8.45	19.4	18.4	19.4	19.0	18.7	20.0
		2. week	7.87	7.94	8.31	8.75	8.89	8.68	8.40	8.41	8.28	8.91	8.26	8.19	20.4	19.6	19.7	20.0	19.8	20.7
		3. week	7.82	7.90	8.59	8.70	8.87	9.29	8.86	8.77	8.41	8.34	8.83	9.14	18.0	19.5	20.1	19.6	20.2	21.1
	p-Ag 1	1. week	7.87	7.84	7.95	9.00	8.69	8.83	8.62	9.02	7.82	8.91	8.71	8.73	19.3	18.3	19.2	18.9	18.7	20.0
		2. week	7.87	7.93	8.31	8.76	8.87	8.68	8.50	8.55	8.28	8.84	8.32	8.23	20.1	19.7	19.7	20.2	19.7	20.8
		3. week	7.76	7.90	8.59	8.71	8.86	8.88	8.64	8.73	8.41	8.47	8.87	8.88	18.0	19.3	20.1	18.6	20.2	20.8
	p-Ag 2	1. week	7.87	7.87	8.95	9.05	8.68	8.83	8.63	9.00	8.02	9.32	8.96	8.67	19.3	18.2	19.2	19.2	18.6	20.0
		2. week	7.87	7.92	8.28	8.80	8.88	8.68	8.54	8.58	8.36	8.83	8.31	8.25	20.0	19.7	20.0	20.2	19.4	20.8
		3. week	7.87	7.88	8.62	8.68	8.86	8.86	8.66	8.80	8.48	8.65	8.78	8.45	18.8	19.4	19.9	18.7	20.3	20.6
	p-Ag 3	1. week	7.89	7.87	7.94	9.02	8.69	8.84	8.69	9.08	8.14	8.46	8.76	8.82	19.4	18.3	19.0	19.3	18.7	20.4
		2. week	7.87	7.92	8.24	8.78	8.87	8.69	8.73	8.59	8.37	9.02	8.28	8.30	20.1	19.7	20.0	19.7	19.3	20.6
		3. week	7.78	7.88	8.87	8.67	8.91	8.79	8.85	8.82	9.00	8.64	8.87	8.51	18.0	19.4	19.7	18.7	20.0	20.6
	p-Ag 4	1. week	7.91	7.89	7.94	9.01	8.68	8.85	8.60	9.07	8.24	9.52	8.73	8.75	19.2	18.2	19.1	19.4	18.8	20.7
		2. week	7.86	7.89	8.24	8.76	8.87	8.68	8.61	8.60	8.38	9.05	8.32	8.29	19.8	19.5	19.6	19.5	19.4	20.6
		3. week	7.90	7.85	8.64	8.67	8.85	8.80	9.21	8.82	8.70	8.73	8.90	8.80	18.1	19.4	19.9	18.8	19.9	20.7
(i_b) pristine TiO_2NPs	p-C 2	1. week	6.76	7.08	7.18	7.45	7.47	7.00	8.79	8.61	8.44	8.41	8.63	8.79	20.9	20.2	20.9	19.8	20.3	20.6
		2. week	7.03	7.18	7.24	7.47	7.62	7.13	8.61	8.66	8.97	8.63	8.91	8.61	20.2	20.9	20.3	20.3	19.8	20.4
		3. week	7.01	7.18	7.12	7.60	7.81	7.12	8.41	8.44	8.89	9.21	8.88	8.79	20.8	20.9	20.4	20.2	20.2	20.3
	p-TiO_2 1	1. week	6.78	6.89	7.17	7.50	7.30	6.90	8.76	8.41	8.43	8.56	8.62	8.83	20.2	20.6	20.8	19.6	19.8	20.5
		2. week	6.89	7.03	7.17	7.30	7.61	7.22	8.41	8.74	9.01	8.62	9.01	8.76	20.6	20.7	20.7	19.8	20.2	20.5
		3. week	7.01	7.17	7.10	7.69	7.51	7.20	8.61	8.43	8.90	9.14	8.76	8.82	20.8	20.8	20.4	20.2	20.4	20.4
	p-TiO_2 2	1. week	6.81	7.02	7.19	7.45	7.19	6.83	8.61	8.61	8.43	8.59	8.61	8.85	21.3	19.9	20.9	19.6	19.8	20.5
		2. week	7.02	6.78	7.06	7.19	7.26	7.42	8.61	8.80	9.02	8.61	8.69	8.76	19.9	20.5	20.3	19.8	20.3	20.4
		3. week	6.95	7.19	6.92	7.51	7.41	7.16	8.61	8.43	8.87	9.16	8.47	8.34	20.8	20.9	20.5	19.9	20.2	20.1
	p-TiO_2 3	1. week	6.87	7.23	7.23	7.44	6.99	6.83	8.87	8.17	8.40	8.58	8.57	8.83	20.8	19.9	21.0	19.7	19.8	20.5
		2. week	7.23	7.03	7.01	6.99	7.21	7.44	8.17	8.77	8.75	8.57	8.56	8.51	19.9	20.5	20.6	19.8	20.2	20.4
		3. week	7.02	7.23	7.29	7.52	7.53	7.49	8.61	8.40	8.81	9.08	8.81	8.72	20.9	21.0	20.4	19.6	20.3	20.3

Table S5-1: Continued.

Exposure scenario	Treatment		pH						Oxygen [mg/L]						Temperature [°C]					
			F0	F1	F2	F3	F4	F5	F0	F1	F2	F3	F4	F5	F0	F1	F2	F3	F4	F5
(ii_a) wastewater-borne AgNPs	STP-C 1	1. week	8.38	7.45	7.59	7.69	8.03	7.96	8.40	8.30	8.79	8.75	7.96	7.76	20.8	21.2	21.0	20.0	20.9	21.1
		2. week	7.84	7.37	8.16	8.07	7.83	7.87	7.73	7.77	8.89	8.87	8.88	8.51	21.5	21.2	19.8	21.5	20.9	21.4
		3. week	7.34	7.76	7.68	7.99	7.99	7.86	8.04	8.04	8.62	7.96	8.35	8.57	21.9	21.3	20.4	20.9	21.9	20.5
	STP-Ag 1	1. week	8.32	7.52	8.07	7.70	8.03	7.99	8.52	8.20	8.43	8.70	8.08	8.58	20.3	21.2	21.3	19.9	20.2	20.7
		2. week	8.10	7.77	8.10	8.07	7.80	7.86	7.68	8.41	8.82	8.43	8.71	8.48	21.3	21.0	19.8	21.3	20.8	21.3
		3. week	7.26	7.58	7.68	7.92	7.98	7.89	8.04	7.81	8.57	8.05	8.30	8.63	20.2	21.1	20.8	20.7	21.6	19.4
	STP-Ag 2	1. week	8.23	7.54	8.21	7.70	8.03	7.98	8.39	8.28	8.26	8.75	8.17	8.52	20.8	21.2	21.3	19.9	21.1	20.6
		2. week	8.17	7.89	8.14	8.07	7.80	7.85	8.21	8.52	8.84	9.16	8.67	8.43	21.2	20.6	19.8	20.8	20.9	21.2
		3. week	7.23	7.68	7.67	7.93	7.96	7.89	7.97	7.86	8.44	8.04	8.28	8.66	20.2	21.0	20.9	20.5	21.7	19.4
	STP-Ag 3	1. week	8.02	7.58	8.23	7.69	8.04	7.98	8.39	8.18	8.57	8.67	8.20	8.48	20.8	21.4	21.4	20.2	21.3	20.8
		2. week	8.13	7.92	8.31	8.07	7.80	7.86	7.76	8.66	8.85	8.91	8.56	8.50	20.8	20.3	19.7	20.5	21.0	21.2
		3. week	7.29	7.73	7.67	7.93	7.96	7.90	8.00	7.88	8.45	8.09	8.29	8.74	21.8	21.0	20.9	21.5	21.8	19.4
	STP-Ag 4	1. week	8.03	7.60	8.21	7.70	8.05	7.97	8.30	8.23	8.40	8.66	8.17	8.47	21.2	21.1	21.4	20.2	21.5	20.8
		2. week	8.18	7.96	8.12	8.06	7.80	7.86	7.78	8.64	8.79	8.66	8.63	8.38	21.8	20.3	19.0	20.9	21.0	21.3
		3. week	7.37	7.77	7.69	7.92	7.95	7.89	7.85	7.90	8.46	8.10	8.25	8.61	21.8	20.9	20.3	21.5	21.8	19.4
(ii_b) wastewater-borne TiO_2NPs	STP-C 2	1. week	7.25	7.12	7.32	7.77	7.16	7.12	8.76	8.16	8.82	8.55	8.48	8.81	20.2	20.2	20.8	20.2	20.7	20.5
		2. week	8.16	7.14	7.35	7.16	7.22	7.27	7.12	8.56	8.95	8.48	8.67	8.71	20.2	21.1	20.6	20.7	20.3	20.5
		3. week	7.12	6.93	7.12	7.91	7.12	7.69	8.01	8.51	8.81	9.28	8.68	8.79	20.9	21.0	20.5	20.4	20.3	20.4
	STP-TiO_2 1	1. week	6.96	7.10	7.16	7.63	7.18	7.61	8.52	8.21	8.84	8.70	8.62	8.79	20.8	19.9	20.4	19.8	20.1	20.4
		2. week	7.10	7.21	7.19	7.18	7.82	7.11	8.21	8.56	9.02	8.62	8.59	8.56	19.9	20.8	20.2	20.1	20.5	20.2
		3. week	7.15	6.98	7.01	7.83	7.69	7.23	8.67	8.47	8.79	9.53	8.71	8.71	20.5	21.1	20.3	20.5	20.3	20.3
	STP-TiO_2 2	1. week	6.72	6.71	7.01	7.47	7.15	7.49	8.09	8.21	8.91	8.70	8.62	8.74	20.1	20.0	20.3	19.6	20.1	20.6
		2. week	6.71	7.22	7.00	7.15	7.32	7.38	8.12	8.61	8.92	8.62	8.76	8.61	20.0	20.9	20.2	20.1	20.4	20.4
		3. week	6.56	6.99	6.89	7.77	7.12	7.61	8.61	8.42	8.41	9.87	8.44	8.13	20.4	21.1	20.6	20.6	20.4	20.3
	STP-TiO_2 3	1. week	6.64	6.76	6.89	7.48	7.20	7.61	8.52	8.34	8.89	8.61	8.61	8.98	20.3	20.1	20.3	19.8	20.2	20.5
		2. week	6.76	7.14	7.00	7.20	7.61	7.46	8.34	8.59	8.96	8.61	8.98	8.79	20.1	20.8	20.3	20.2	20.0	20.3
		3. week	7.01	7.09	7.02	7.69	7.12	7.61	8.61	8.48	8.41	10.01	8.23	8.49	20.6	21.1	20.4	20.5	20.6	20.4

Table S5-2: Main ICP-MS and ICP-OES Instrumental Parameters.

Used for	total Ag measurement: test media exposure scenario (i, ii)	total Ti measurement: test media exposure scenario (i, ii)	total Ti measurement: STP effluent for exposure scenario (ii_b)
	ICP-MS iCAp Qc (Thermo Fisher Scientific, Bremen, Germany)	ICP-OES Arcos, Spectro Analytical Instruments (axial plasma view)	ICP-OES 720, Agilent Technologies (axial plasma view)
Nebulizer	Pneumatic PFA-50 or pneumatic glass nebulizers	Standard cross-flow nebulizer	SeaSpray nebulizer
Spray chamber	Peltier-cooled cyclonic quartz	Standard Scott type	IsoMist Programmable Temperature Spray Chamber
Radio-frequency power	1400 or **1550** W	1200 W	1200 W
Torch injector inner diameter	2.5 mm	2.0 mm	2.4 mm
Cooling flow	14 L/min	13 L/min	15 L/min
Auxiliary flow	0.8 L/min	0.8 L/min	1.5 L/min
Nebulizer flow	1.0 L/min	0.9 L/min	0.75 L/min
Sampling position	4 or **5** mm	n/a	n/a

Note that the values in bold were used with the pneumatic glass nebulizer; n/a – not applicable

Table S5-3: Measurement of total Ag and Ti concentrations of freshly prepared media and aged media samples collected during F2 generation for AgNPs and F4 generation for TiO_2NPs after 72h of exposure. Mean measured concentrations with pooled expanded uncertainty (U, k = 2)

Exposure scenario	Treatment	Mean measured concentration [µg/L ± U]	
		Fresh media	Aged media
(i_a) pristine AgNPs	p-C 1	3.56 ± 0.37*	1.96 ± 0.22*
	p-M 1	1.32 ± 0.15	0.47 ± 0.11
	p-Ag 1	1.98 ± 0.22	4.86 ± 0.58
	p-Ag 2	3.35 ± 0.30	3.31 ± 0.30
	p-Ag 3	6.78 ± 0.65	3.36 ± 0.36
	p-Ag 4	10.34 ± 0.74	5.83 ± 0.50
(i_b) pristine TiO_2NPs	p-C 2	< 0.56	n/m
	p-TiO_2 1	7.59 ± 1.15	n/m
	p-TiO_2 2	13.47 ± 2.85	< 1.84
	p-TiO_2 3	68.85 ± 6.55	< 6.14
(ii_a) wastewater-borne AgNPs	STP-C 1	0.61 ± 0.12	0.59 ± 0.10
	STP-Ag 1	0.93 ± 0.14	0.48 ± 0.13
	STP-Ag 2	2.93 ± 0.28	1.27 ± 0.20
	STP-Ag 3	6.39 ± 0.48	2.80 ± 0.31
	STP-Ag 4	10.07 ± 0.66	4.21 ± 0.27
(ii_a) wastewater-borne TiO_2NPs	STP-C 2	14.71 ± 2.66	< 6.14
	STP-TiO_2 1	< 6.14	11.34 ± 4.86
	STP-TiO_2 2	11.59 ± 2.58	8.61 ± 2.99
	STP-TiO_2 3	25.20 ± 2.80	18.07 ± 5.23

Note that combined U is calculated for all total silver concentrations out of three replicates (n = 3), except for the value "*" marked, where n = 2. Only one replicate was measured for total Ti content; n/m – not measured

Table S5-4: Statistical bidirectional comparisons in mean number of offspring between all exposure scenarios in all generations and the control, respectively. All p-values are two-tailed.

Exposure scenario	Treatment	F0	F1	F2	F3	F4	F5
(i_a) pristine AgNPs	p-C 1						
	p-Ag 1	W = 99 P = 1[c]	W = 139.5 P = 0.01[c]	W = 55.5 P = 0.28[c]	W = 149 P = 0.01[c]	P = 0.99[a]	P = 0.04[a]
	p-Ag 2	W = 151.5 P = 0.01[c]	W = 91.5 P = 0.08[c]	W = 93 P = 0.004[c]	W = 199 P < 0.001[c]	P < 0.001[a]	P < 0.001[a]
	p-Ag 3	W = 161 P < 0.001[c]	W = 117.5 P < 0.001[c]	W = 95.5 P =0.002[c]	W = 200 P < 0.001[c]	P < 0.001[a]	P < 0.001[a]
	p-Ag 4	W = 143.5 P < 0.001[c]	W = 144.5 P < 0.001[c]	W = 140 P < 0.001[c]	W = 198.5 P < 0.001[c]	P < 0.001[a]	P < 0.001[a]
(i_b) pristine TiO_2NPs	p-C 2						
	p-TiO_2 1	X^2 = 0.388 P = 0.94[b]	P = 0.89[a]	X^2 = 5.852 P = 0.119[b]	P = 0.34[a]	P = 0.83[a]	P = 0.46[a]
	p-TiO_2 2	X^2 = 0.388 P = 0.94[b]	P = 0.95[a]	X^2 = 5.852 P = 0.119[b]	P = 0.75[a]	P = 0.85[a]	P = 0.97[a]
	p-TiO_2 3	X^2 = 0.388 P = 0.94[b]	P = 0.57[a]	X^2 = 5.852 P = 0.119[b]	P = 0.48[a]	P = 0.99[a]	P = 0.68[a]
(ii_a) wastewater-borne AgNPs	STP-C 1						
	STP-Ag 1	X^2 = 7.203 P = 0.12[b]	P = 0.89[a]	X^2 = 7.512 P = 0.11[b]	W = 61.5 P = 0.24[c]	W = 65.5 P = 0.11[c]	W =75.5 P = 0.5[c]
	STP-Ag 2	X^2 = 7.203 P = 0.12[b]	P = 0.53[a]	X^2 = 7.512 P = 0.11[b]	W = 44.5 P = 1[c]	W = 36.5 P = 1[c]	W = 40.5 P = 1[c]
	STP-Ag 3	X^2 = 7.203 P = 0.12[b]	P = 0.78[a]	X^2 = 7.512 P = 0.11[b]	W = 20.5 P = 0.355[c]	W = 32 P = 1[c]	W = 42 P = 1[c]
	STP-Ag 4	X^2 = 7.203 P = 0.12[b]	P = 0.46[a]	X^2 = 7.512 P = 0.11[b]	W = 30.5 P = 0.42[c]	W = 42.5 P = 1[c]	W = 25 P = 0.73[c]
(ii_b) wastewater-borne TiO_2NPs	STP-C 2						
	STP-TiO_2 1	P = 0.96[a]	W = 95.5 P = 0.001[c]	W = 73.5 P = 0.01[c]	X^2 = 1.601 P = 0.65[b]	X^2 = 5.702 P = 0.12[b]	P = 0.09[a]
	STP-TiO_2 2	P = 0.86[a]	W = 51 P = 1[c]	W = 47 P = 1[c]	X^2 = 1.601 P = 0.65[b]	X^2 = 5.702 P = 0.12[b]	P = 0.08[a]
	STP-TiO_2 3	P = 0.76[a]	W = 80 P = 0.72[c]	W = 41.5 P = 1[c]	X^2 = 1.601 P = 0.65[b]	X^2 = 5.702 P = 0.12[b]	P = 0.66[a]

[a]: one-way ANOVA and Dunett´s test

[b]: Kruskal-Wallis test or [c]: Wilcoxon runk sum test for unpaired samples

Table S5-5: Mean adult body length (mm± sd) for all exposure scenarios and generations (F0 – F5). ** = P < 0.01 *** = P < 0.001

Exposure scenario	Treatment	Generation					
		F0	F1	F2	F3	F4	F5
(i_a) pristine AgNPs	p-C 1	4.13 ± 0.10	4.12 ± 0.10	4.05 ± 0.24	4.08 ± 0.17	4.10 ± 0.38	4.22 ± 0.10
	p-Ag 1	4.24 ± 0.30	4.05 ± 0.30	3.82 ± 0.48	3.99 ± 0.17	4.39 ± 0.38	4.54 ± 0.30
	p-Ag 2	3.97 ± 0.38	4.11 ± 0.38	4.10 ± 0.16	4.28 ± 0.28	3.98 ± 0.20	4.44 ± 0.38
	p-Ag 3	4.09 ± 0.31	4.18 ± 0.31	4.15 ± 0.27	4.12 ± 0.28	3.81 ± 0.29	4.39 ± 0.31
	p-Ag 4	4.01 ± 0.15	4.26 ± 0.15	4.07 ± 0.21	4.09 ± 0.20	4.00 ± 0.28	4.75 ± 0.15 ***
(i_b) pristine TiO_2NPs	p-C 2	4.19 ± 0.34	3.94 ± 0.43	3.99 ± 0.18	3.85 ± 0.19	3.99 ± 0.24	3.94 ± 0.14
	p-TiO_2 1	4.01 ± 0.31	3.65 ± 0.30	3.83 ± 0.21	3.97 ± 0.19	4.19 ± 0.09	3.98 ± 0.24
	p-TiO_2 2	4.01 ± 0.44	4.08 ± 0.13	3.96 ± 0.15	3.96 ± 0.05	4.08 ± 0.13	4.05 ± 0.13
	p-TiO_2 3	4.26 ± 0.26	4.06 ± 0.15	3.83 ± 0.26	3.96 ± 0.13	4.00 ± 0.20	3.95 ± 0.15
(ii_a) wastewater-borne AgNPs	STP-C 1	3.96 ± 0.23	4.07 ± 0.28	4.13 ± 0.13	4.02 ± 0.27	4.36 ± 0.29	4.10 ± 0.25
	STP-Ag 1	4.05 ± 0.33	4.17 ± 0.51	3.96 ± 0.28	3.98 ± 0.17	4.06 ± 0.18	3.95 ± 0.17
	STP-Ag 2	4.13 ± 0.33	3.84 ± 0.29	3.79 ± 0.64	4.16 ± 0.26	4.25 ± 0.28	4.08 ± 0.20
	STP-Ag 3	4.25 ± 0.22	4.11 ± 0.26	4.03 ± 0.1	4.24 ± 0.23	4.35 ± 0.23	3.94 ± 0.19
	STP-Ag 4	4.26 ± 0.23	3.73 ± 0.22	4.16 ± 0.24	3.98 ± 0.30	4.31 ± 0.18	3.94 ± 0.16
(ii_b) wastewater-borne TiO_2NPs	STP-C 2	4.44 ± 0.40	4.23 ± 0.50	3.97 ± 0.17	3.87 ± 0.25	4.14 ± 0.10	4.02 ± 0.16
	STP-TiO_2 1	4.32 ± 0.50	4.25 ± 0.55	3.94 ± 0.36	4.01 ± 0.18	4.03 ± 0.16	4.12 ± 0.01
	STP-TiO_2 2	4.24 ± 0.29	4.15 ± 0.11	4.16 ± 0.14	3.99 ± 0.24	4.19 ± 0.10	4.09 ± 0.19
	STP-TiO_2 3	4.03 ± 0.24	4.01 ± 0.25	3.90 ± 0.24	4.04 ± 0.15	4.11 ± 0.21	4.28 ± 0.17 **

Table S5-6: Key life cycle parameters: number of offspring (mean ± sd), mean time to first day of reproduction (days ± sd) and mortality [%] for both exposure scenarios and all treatments. *P < 0.05, **P < 0.01, ***P < 0.001.

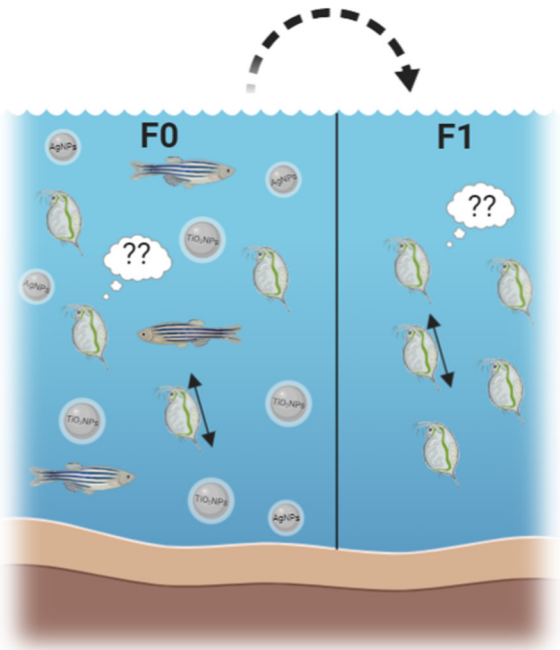
Exposure scenario	Treatment	Mean (± sd) number of offspring						Mean (days± sd) time to first brood						Mortality [%]					
		F0	F1	F2	F3	F4	F5	F0	F1	F2	F3	F4	F5	F0	F1	F2	F3	F4	F5
(i_a) pristine AgNPs	p-C 1	61.7 ± 4.4	65.0 ± 6.6	65.0 ± 8.9	61.2 ± 4.9	58.6 ± 7.1	69.1 ± 7.9	10.1 ± 0.3	11.2 ± 1.1	10.6 ± 0.6	10.8 ± 0.5	10.8 ± 1.0	10.5 ± 0.6	0	5	30	0	0	10
	p-Ag 1	59.6 ± 7.2	55.3 ± 7.6 *	61.3 ± 4.1	53.4 ± 10.5	59.4 ± 8.1	61.6 ± 8.2*	10.0 ± 0.0	11.5 ± 2.5	11.1 ± 0.9	11.0 ±0.6	10.3 ± 0.9	11.1 ± 0.7	0	10	50	0	0	0
	p-Ag 2	55.2 ± 4.6 *	57.3 ± 7.9*	43.1 ± 12.6 *	43.1 ± 5.4 ***	43.4 ± 6.5 ***	50.3 ± 7.3***	10.0 ± 0.0	10.4 ± 1.7	11.2 ± 0.4	11.6 ±0.5 *	10.4 ± 1.0	10.7 ± 0.9	0	30	30	0	0	0
	p-Ag 3	51.5 ± 3.2**	51.7 ± 7.4 **	49.0 ± 5.0 **	43.4 ± 4.8 ***	42.6 ±5.9 ***	49.4 ± 6.4***	10.3 ± 0.6	11.8 ± 1.2	11.5 ±0.8 *	11.2 ± 1.1	10.4 ± 0.6	10.3 ± 0.8	10	30	20	0	0	10
	p-Ag 4	46.8 ± 8.1 **	54.3 ± 5.1 **	44.2 ± 3.9 ***	43.0 ± 7.6 ***	46.0 ±6.0 ***	49.0 ± 5.9***	10.2 ± 1.0	11.4 ± 0.5	10.6 ± 0.5	10.9 ± 0.5	10.6 ±1.4	10.3 ± 0.7	10	0	10	0	30	10
(i_b) pristine TiO_2NPs	p-C 2	96.8 ± 6.6	93.7 ± 7.2	91.3 ± 10.6	87.4 ± 4.8	92.8 ± 7.4	88.0 ± 13.8	7.7 ± 0.4	8.4 ± 0.5	9.0 ± 0.4	9.0 ± 0.0	8.9 ± 0.5	10.4 ± 0.5	10	0	20	0	0	0
	p-TiO_2 1	96.0 ± 5.4	92.3 ± 6.0	83.6 ± 9.9	85.2 ± 6.3	91.6 ± 8.0	86.5 ± 5.7	8.0 ± 0.5	8.4 ± 0.6	9.3 ± 0.8	8.4 ± 0.5*	8.0 ± 0.0**	9.9 ± 0.5	10	0	20	10	0	0
	p-TiO_2 2	96.2 ± 12.4	95.7 ± 8.7	82.0 ± 9.9	83.6 ± 5.7	95.0 ± 5.8	94.2 ± 11.2	8.2 ± 0.6	8.1 ± 0.3	9.0 ± 0.8	8.0 ± 0.0** *	9.0 ± 0.0	10.3 ± 0.4	0	0	30	0	0	10
	p-TiO_2 3	98.0 ± 9.0	97.5 ± 9.0	94.5 ± 13.8	90.6 ± 6.1	93.5 ± 7.4	92.4 ± 9.4	8.0 ± 0.0	8.1 ± 0.3	8.8 ± 0.6	8.8 ± 0.6	8.0 ± 0.0**	10.2 ± 0.4	0	0	20	10	0	0

Table S5-6: Continued.

Exposure scenario	Treatment	Mean (± sd) number of offspring						Mean (days ± sd) time to first brood						Mortality [%]					
		F0	F1	F2	F3	F4	F5	F0	F1	F2	F3	F4	F5	F0	F1	F2	F3	F4	F5
(ii_a) wastewater-borne AgNPs	STP-C 1	70.3 ± 8.8	58.0 ± 3.6	60.4 ± 9.2	68.7 ± 11.2	77.7 ± 5.4	57.4 ± 4.7	7.4 ± 0.7	8.3 ± 0.5	9.1 ± 1.0	10.7 ± 2.3	8.0 ± 1.0	8.2 ± 0.9	10	30	40	10	10	10
	STP-Ag 1	73.1 ± 5.9	59.7 ± 3.6	47.5 ± 12.6	59.7 ± 5.1	69.0 ± 7.5	51.5 ± 3.3	7.3 ± 0.5	8.0 ± 0.0	9.6 ± 2.1	9.7 ± 0.4	8.6 ± 0.8	8.1 ± 0.3	10	30	20	0	0	0
	STP-Ag 2	72.9 ± 3.4	55.0 ± 6.0	46.5 ± 4.6	67.5 ± 9.1	77.8 ± 7.8	58.5 ± 3.6	7.3 ± 0.4	8.5 ± 0.5	9.1 ± 0.3	9.4 ± 0.6	8.2 ± 0.6	8.0 ± 0.6	0	20	40	10	10	0
	STP-Ag 3	69.0 ± 10.1	60.0 ± 3.8	52.8 ± 8.4	76.4 ± 4.6	81.9 ± 7.3	50.8 ± 10.9	7.4 ± 0.5	8.2 ± 0.4	9.7 ± 1.9	9.1 ±0.3*	7.6 ± 0.5	8.3 ± 1.7	0	20	40	0	0	30
	STP-Ag 4	77.0 ± 2.7	54.5 ± 5.5	53.5 ±11.4	72.9 ± 6.5	77.5 ± 8.1	60.8 ± 6.0	7.3 ± 0.4	8.4 ± 0.5	8.7 ± 0.6	9.1 ±0.3*	7.6 ± 0.9	7.2 ± 0.4	0	20	40	0	10	10
(ii_b) wastewater-borne TiO_2NPs	STP-C 2	91.0 ± 17.9	109.7 ± 6.4	95.2 ± 10.0	88.6 ± 7.7	99.9 ± 8.9	85.9 ± 5.0	8.6 ± 0.9	8.2 ± 0.4	8.6 ± 0.5	8.8 ± 0.4	9.0 ± 0.0	8.3 ± 0.0	0	0	10	0	0	0
	STP-TiO_2 1	88.6 ± 13.3	94.6 ± 7.7*	73.3 ± 13.9*	88.0 ± 15.7	90.1 ± 11.6	95.1 ± 8.8	8.3 ± 0.5	8.0 ± 0.0	8.6 ± 0.5	8.3 ± 0.6	8.1 ± 0.3**	8.9 ± 0.3*	0	0	10	0	0	0
	STP-TiO_2 2	87.0 ± 15.0	109.6 ± 7.5	94.0 ± 3.2	85.9 ± 8.6	98.4 ± 6.1	95.3 ± 11.1	8.1 ± 0.4	8.7 ± 0.9	9.6 ± 0.6	9.2 ± 0.6	9.1 ± 0.5	9.2 ± 0.5**	0	0	10	0	0	0
	STP-TiO_2 3	96.1 ± 10.2	93.7 ± 16.8	93. 7 ± 10.4	85.0 ± 13.3	97.5 ± 9.8	89.9 ± 11.5	8.0 ± 0.4	8.7 ± 1.2	8.7 ± 0.4	8.6 ± 0.5	8.7 ± 0.4	9.1 ± 0.4*	0	0	10	0	0	0

Chapter 6

Do silver and titanium dioxide nanoparticles influence the anti-predator defence in *Daphnia magna* and in their next generation?



Anna Beasley from the University of Manchester helped to collect the data analysed in this chapter and Darya Mozhayeva provided the analytical data.

Parts of this chapter have been submitted to Scientific Reports:

Hartmann S, Beasley A, Witte K (submitted). Defective defence: silver nanoparticles inhibit anti-predator defence in offspring of *Daphnia magna*. Scientific Reports

6.1 Abstract

One major environmental problem of our time is the discharge of chemicals into the aquatic environment. Due to their antimicrobial properties and high photocatalytic activity silver nanoparticles (AgNPs) and titanium dioxide nanoparticles (TiO_2NPs), respectively, are the most commonly used and studied nanoparticles. However, as a result of their small size, NPs are not completely filtered out at wastewater treatment plants and therefore continuously introduced to the aquatic environment. Although negative effects of AgNPs and TiO_2NPs on aquatic organisms have been extensively studied, there is still a gap in knowledge on how this chemical stressor interacts with natural cues on the maternal and subsequent generation of aquatic organisms. We tested whether AgNPs affected the kairomone-induced adaptive predator defence mechanism in adult *D. magna* and their offspring. Although adult *D. magna* developed typical anti-predator defence mechanisms when exposed to kairomones and AgNPs, their offspring could not develop such adaptive defensive traits. The lack of this defence mechanism in offspring could have dramatic negative consequences for the entire complex food web in the aquatic ecosystem. The induction of defensive traits in adult *D. magna* and their offspring was not affected by TiO_2NPs. For a realistic risk assessment, it is extremely important to test combinations of chemical stressors because aquatic organisms are exposed to several natural and artificial chemical stressors at the same time.

6.2 Introduction

Since the end of the 18th century, the industrial revolution has led to enormous technical, health and economic improvements for human welfare. However, technological progress is interfering with global cycles that could lead to negative changes in the environment (Fent, 2013; Parry et al., 2007; Walker et al., 2016). One major environmental problem of our time is the discharge of chemicals into the environment (Fent, 2013; Parry et al., 2007). In recent decades, pollution of the aquatic environment has risen to new levels (Borcherding, 2006) due to the release of an increasing variety and amount of chemicals, such as organic and radioactive substances, heavy metals and nanomaterials into aquatic ecosystems. The most commonly used nanomaterials are silver nanoparticles (AgNPs) due to their antimicrobial properties and titanium dioxide nanoparticles (TiO_2NPs) based on their high photocatalytic activity and UV-light absorbing. Many medical products, such

as wound dressings, bandages and sanitation devices use AgNPs (Benn and Westerhoff, 2008). But also common household objects, food containers, and sports clothing contain AgNPs, and even washing machines are impregnated with AgNPs to reduce bacterial growth and odour (Benn and Westerhoff, 2008). In addition, TiO_2NPs are applied in environmental remediation, consumer products, coatings and UV-protective clothing (CPI, 2019; Menard et al., 2011; Robichaud et al., 2009; Spengler, 2018). Based on their small size (less than 100 nm in size in two dimensions), NPs are not completely filtered out at waste-water treatment plants (Gaiser et al., 2011), and a significant amount of NPs are still continuously released into freshwater ecosystems (Gottschalk and Nowack, 2011). Maurer-Jones et al. (2013) estimated that the predicted environmental concentrations (PECs) for AgNPs and TiO_2NPs in surface water range from 0.088 to 10.000 ng/L and 0.021 to 10 µg/L, respectively.

Besides the numerous studies on the negative effects of high concentrations of AgNPs on aquatic organisms such as *Daphnia* (Bowman et al., 2012; Newton et al., 2013; Ribeiro et al., 2014; Seitz et al., 2015; Völker et al., 2013b), it has been shown that AgNPs affect aquatic organisms even at low, environmentally relevant concentrations. Hartmann et al. (*2019)* showed that low concentrations of AgNPs (1.25 – 10 µg/L) lead to a significant reduction in reproductive success in *Daphnia magna*, a key species within the complex aquatic food web and a standard model species for ecotoxicological studies (Kim et al., 2015; OECD, 2004; OECD, 2012). Chronic exposure of *Daphnia similis* to PVP-coated AgNPs (0.02 and 1 µg/L) inhibits reproduction due to down regulation of key fatty acids which are required for egg production, larval development and environmental sex determination (Wang et al., 2018). Zhao and Wang (2011) report a significant reduction in body length in adult *D. magna* exposed to AgNPs (carbonate-coated) at a concentration of less than 5 µg/L. On the contrary, negative effects of TiO_2NPs towards aquatic organism were only detected at high concentrations (Zhu et al., 2010, Hou et al., 2018) and environmentally relevant concentrations of TiO_2NPs did not affect important life cycle parameters of *D. magna* as shown by Hartmann et al. (2019).

Although many effects of AgNPs and TiO_2NPs on aquatic organisms are well studied, there is still a gap of knowledge about how NPs interact with natural chemical stressors in water systems and how this interaction affects aquatic organisms. Kairomones are chemical stimuli emitted by the predator which indicates the presence of a predator to the

prey. It might be possible that kairomones from a predator are reduced or neutralized by NPs, or NPs might result in a lack of a predator induced response by prey as low concentrations of citrate-AgNPs (2 µg Ag/L) defect the sensory system of *Daphnia* (Pokhrel and Dubey, 2012). To the best of our knowledge, effects of AgNPs and TiO_2NPs on a kairomone-mediated anti-predator defence in *Daphnia spp*. and their offspring has never been investigated. Whether NPs affect the kairomone-induced anti-predator defence in *Daphnia spp.* or not is very important to know because in aquatic systems *Daphnia* are exposed to both chemical stressors simultaneously. Furthermore, investigating the effect of the combined stressors is a much more realistic scenario and will lead to a better risk assessment of AgNPs and TiO_2NPs in the environment. *Daphnia,* is an excellent model species to investigate the development of defensive traits in response to the presence of predators indicated by kairomones (Trotter et al., 2019) and to the presence of NPs. It has been shown several times that in the presence of a predator species, many species of *Daphnia* change life history, behavioural and morphological traits (Laforsch and Tollrian, 2004b; Rabus and Laforsch, 2011). The kairomone-mediated response in *Daphnia* includes growth of defensive morphology, e.g. growth of a helmet (Laforsch and Tollrian, 2004a), neckteeth (Hunter and Pyle, 2004; Tollrian, 1993), a crown of thorns (Petrusek et al., 2009), an elongated tail spine and an increase in overall body size (Rabus and Laforsch, 2011). Typical predators for *Daphnia* are the phantom midge larvae *Chaborus*, the heteroperan *Notonecta* sp. or small fishes (Barbosa et al., 2014; Tollrian, 1995; Weiss et al., 2012). In the presence of fish predators, *Daphnia* react with an earlier sexual maturity, an increased fecundity and the production of resting eggs (Ślusarczyk et al., 2013; Stibor and Lüning, 1994; Weiss et al., 2012). The presence of predators even leads to new defensive traits in the next generation. These protected neonates have a better chance of survival from the moment they are born (Hesse et al., 2012). Offspring of adult *Daphnia* exposed to predatory kairomones develop a longer tail spine relative to their total body length, than offspring of adult *Daphnia* that were not exposed to kairomones (Barbosa et al., 2014).

Thus, the aim of this study was to test whether adult *Daphnia* exposed to kairomones released from a fish predator and exposed to different environmentally relevant concentrations of AgNPs (NM-300K) and TiO_2NPs (NM-105) are able to develop defensive morphological traits, and/or whether the exposure of the maternal generation to

kairomones and to different concentrations of AgNPs or TiO_2NPs would lead to adaptations in the offspring or not. We tested different low concentrations of AgNPs and TiO_2NPs to cover a spectrum of possible environmentally relevant contaminations and to exclude single concentration effects. If AgNPs or TiO_2NPs inhibit a predator induced defence in adults and/or offspring, this would have dramatic impacts on *Daphnia* populations and therefore on the entire complex food web in the aquatic environment with *Daphnia* as a key species in that food web.

6.3 Material and Methods

6.3.1 Study species

For the experiments, we used the laboratory-cultured *Daphnia magna* (clone V) originally provided by the Federal Environment Agency (Berlin, Germany). The culture conditions of *D. magna* and the corresponding food source, *Desmodesmus subspicatus,* are described in detail in Chapter 2.1.4.

6.3.2 Test substances

6.3.2.1 Silver nanoparticles (NM-300K)

In this study, we used NM-300K particles (polyvinylpyrrolidone- (PVP-) coated) from the OECD Working Party on Manufactured Nanomaterials (WPMN) Sponsorship (Klein et al., 2011) as AgNPs. Detailed information of the chemical properties of NM-300K are given in Chapter 3.3.1. A working stock with a nominal concentration of 50 mg/L was prepared in ASTM medium to set the test concentrations. As a matrix control, the AgNP-free stabilization agent NM-300K DIS was used. A dispersant stock solution was prepared accordingly. In this solvent with AgNP-free stabilization agent NM-300K DIS we diluted kairomones for Treatment I_b.

6.3.2.2 Titanium dioxide nanoparticles (NM-105)

The OECD reference nanomaterial (WPMN programme) NM-105 were used to perform the experiments with TiO_2NPs. Detailed information of the chemical properties of NM-105 are given in Chapter 3.3.1. The NM-105 powder was dispersed in ASTM medium to generate a working stock dispersion with a nominal concentration of 1 g/L. According to the method described in Verleysen et al., 2014, 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. All dilutions were done in PP vials (VWR International, Langenfeld, Germany). To prevent agglomeration of the TiO_2NPs, the suspension was prepared freshly prior each water exchange and was used immediately.

6.3.3 Preparation of kairomone stock media

Kairomone stock medium (predator medium, PM) was prepared in accordance with Barbosa et al. (2014). In total, we used eight randomly selected adult wild-type zebrafish, *Danio rerio*, from West Aquarium GmbH (Bad Lauterberg, Germany) with a body length of about 40 mm and kept them in one 8 L glass tank filled with ASTM medium (without additional salts and vitamins) for 24 h in an temperature controlled room (26 ± 1 °C) under a light-dark cycle of 14:10 hours. Fish were fed with 160 *D. magna* of varying sizes and ages one day before collecting the predator medium (PM). No extra fish flake food was given. After 24 h, when all *D. magna* were consumed by *D. rerio*, adult fish were returned to their home tank (80 x 40 x 35 cm^3) and debris was allowed to settle down for 10 minutes before the medium, containing fish kairomones (predator medium) was directly used in the experiment. The predator medium (PM) was taken out from the glass tank with a 1 L glass beaker without any additional filtering. The freshly prepared PM was made every day under the same conditions as described above to ensure a high concentration of fish kairomones from *D. rerio* for the experiment. In their home tank, *D. rerio* were cultured in 112 L glass tanks (80 x 40 x 35 cm^3) in groups of 100 animals with a sex ratio of 50:50 under a light-dark cycle of 14:10 hours and a water temperature of 26 ± 1°C, a pH-value of 7-7.5 and a conductivity of 450 µS/cm. Water exchange (40 %) took place two times a week. Water in the tank was aerated and filtered continuously. In their home tank, animals were fed daily in the morning with dry flake food (JBL GmbH & Co. KG, Germany), and additionally three times a week in the afternoon with brine shrimp, *Artemia salina*.

6.3.4 Experimental procedure and treatments

In this study, we followed the guidelines of the *Daphnia magna* reproduction test (OECD, 2012). In all experiments, a single *D. magna* was placed in a glass beaker (100 mL, Rotilabo, Carl Roth GmbH + Co. KG, Karlsruhe), filled with 50 ml of test medium. Each *D. magna* was less than 24 h old at the start of the experiment. In each treatment group, *D. magna* (n = 12) were exposed for 21 days. Medium renewal took place daily to ensure a high kairomone concentration throughout the complete test period. The O_2 (mg/L), pH and

temperature (°C) of old and fresh medium for one test beaker of each treatment group were measured once a week with a WTW Multi 3430 (WTW GmbH, Weilheim, Germany). *D. magna* were fed daily with green algae *Desmodesmus subspicatus* with 0.2 mg C/*Daphnid*/day algae suspension. We determined 'time to first brood', 'reproduction' (as the number of offspring), 'adult body length (aBL)' (as distance from naupliar eye to the base of the dorsal spine) and 'adult spine length (aSL)', and calculated 'relative spine length of adults (aRSL)' after each moult and after 21 days at the end of the experiment. We checked the beaker for offspring daily. We removed offspring of each brood from the beaker as soon as possible and measured 'offspring body length (oBL)', 'offspring spine length (oSL)', and 'relative spine length of offspring (oRSL)' as morphological traits. We took pictures of body length and spine length with a digital camera (Nikon Coolpix L830, Chiyoda, Tokyo, Japan) and analysed pictures using the software AxioVision (Carl Zeiss, Jena).

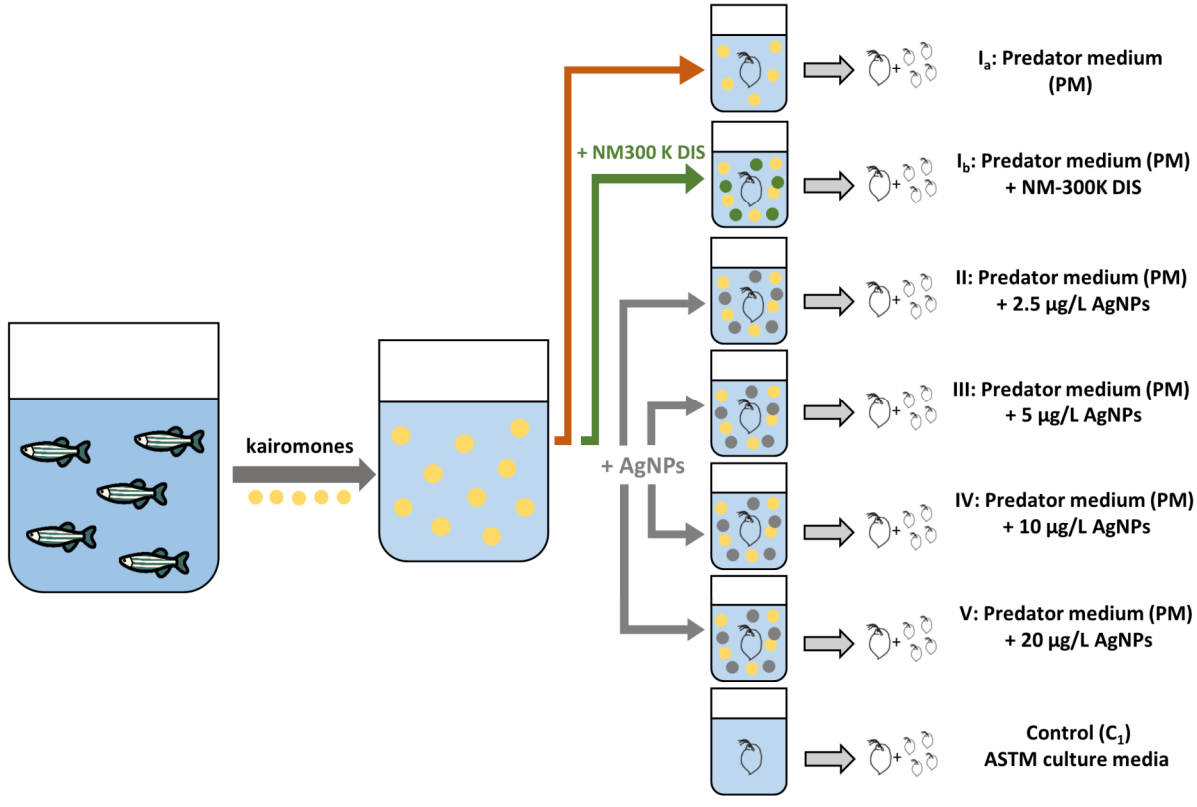


Figure 6-1: Illustration of the experimental set-up. Treatments were as follows: *Daphnia magna* exposed to predator medium only (PM, Treatment I_a), exposed to predator medium and NM-300K Dis (Treatment I_b), exposed to kairomones and different concentrations of AgNPs (Treatment II - V), and *D. magna* exposed to ASTM culture medium which served as a control (C_1). Yellow dots indicate kairomones released from zebrafish (*Danio rerio*). Within each Treatment, we analysed adult *D. magna* and their released offspring.

With AgNPs, we performed the following controls and treatments (Figure 6-1):

I_a. PM: Predator medium (PM) containing solely kairomones of *D. rerio* as a positive control for a kairomone induced response

I_b. PM + NM-300K DIS: Predator medium (PM) enriched with NM-300K DIS as a matrix control to exclude possible effects of the stabilization agent

II. PM + 2.5 µg Ag/L: Predator medium (PM) enriched with 2.5 µg/L of AgNPs

III. PM + 5 µg Ag/L: Predator medium (PM) enriched with 5 µg/L of AgNPs

IV. PM + 10 µg Ag/L: Predator medium (PM) enriched with 10 µg/L of AgNPs

V. PM + 20 µg Ag/L: Predator medium (PM) enriched with 20 µg/L of AgNPs

C_1. Control: ASTM culture media as a reference

Because we have shown that NM-300K DIS alone did not affect any morphological or life history traits in *D. magna* (*Hartmann et al., 2019*), we did not perform this additional control here.

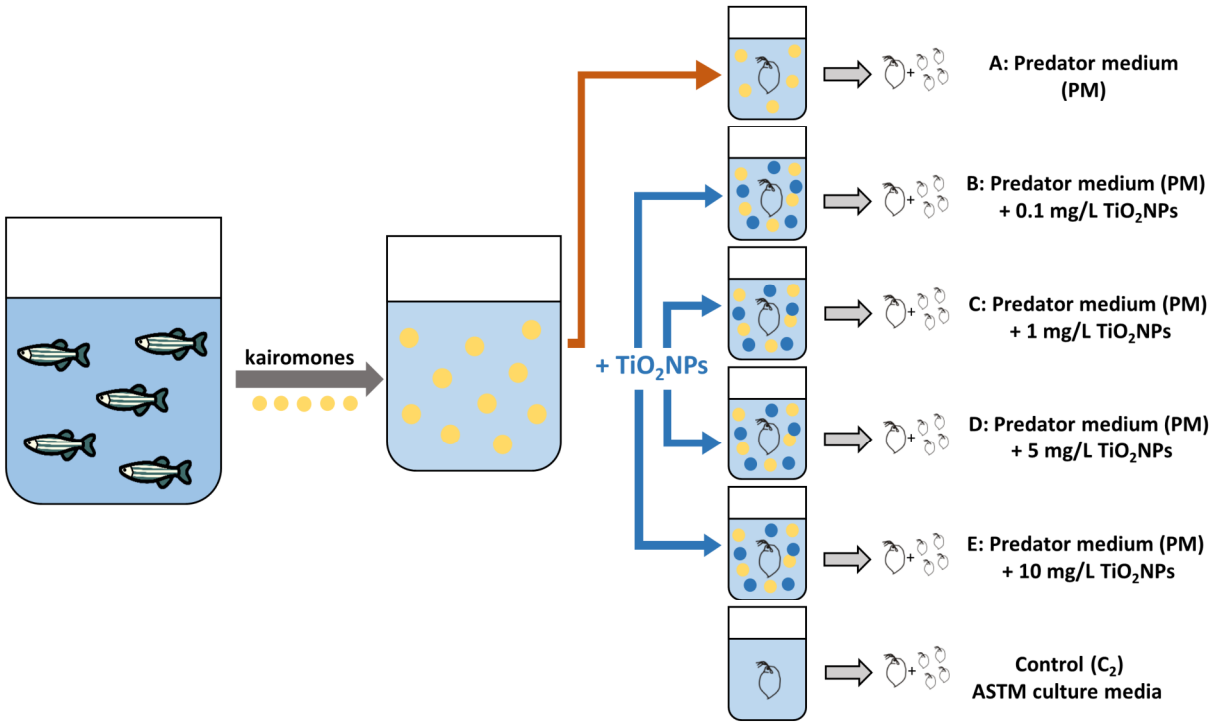


Figure 6-2: Illustration of the experimental set-up. Treatments were as follows: *Daphnia magna* exposed to predator medium only (PM, Treatment A), exposed to kairomones and different concentrations of TiO_2NPs (Treatment B - E), and *D. magna* exposed to ASTM culture medium which served as a control (C_2). Yellow dots indicate kairomones released from zebrafish (*Danio rerio*). Within each Treatment, we analysed adult *D. magna* and their released offspring.

With TiO_2NPs, we performed the following controls and treatments (Figure 6-2):

A. PM: Predator medium (PM) containing solely kairomones of *D. rerio* as a positive control for kairomone induced response

B. PM + 0.1 mg/L TiO_2NPs: Predator medium (PM) enriched with 0.1 mg/L of TiO_2NPs

C. PM + 1 mg/L TiO_2NPs: Predator medium (PM) enriched with 1 mg/L of TiO_2NPs

D. PM + 5 mg/L TiO_2NPs: Predator medium (PM) enriched with 5 mg/L of TiO_2NPs

E. PM + 10 mg/L TiO_2NPs: Predator medium (PM) enriched with 10 mg/L of TiO_2NPs

C_2. Control: ASTM culture media as a reference

6.3.5 Determination of total Ag and total Ti in media samples

A single set (N = 1) of fresh and aged test media samples were collected during the 21-day test period to determine total Ag and Ti concentrations. The fresh media sample was taken on day 15 of the reproduction study and the aged media sample 24 h later (day 16), represent the longest period without water exchange. The aqueous samples were stored at 4°C prior the analysis. Total Ag content was determined with ICP-MS (inductively coupled plasma mass spectrometry); and total Ti content with ICP-OES (inductively coupled plasma optical emission spectrometry), the detailed experimental procedures and experimental parameters are presented in Chapter 5.3.6 and in Table S6-1, respectively.

6.3.6 Statistical analysis

The statistical analysis was performed using the statistical program R version 3.2.4 (R Core Team, 2016). For all parameters, we first compared parameters between *D. magna* from the control (ASTM medium, C_1 + C_2) and from Treatment I_a + A (PM) to test whether *Danio rerio* was a useful predator for testing anti-predator defence mechanism in *D. magna*. Secondly, we analysed the differences between Treatment I_a (PM) and Treatments II – V (PM + different concentration of AgNPs), including Treatment I_b (PM + NM-300K DIS) to analyse the influence of PM in combination with AgNPs and to exclude possible effects of the dispersant agent on test animals and the differences between Treatment A (PM) and Treatments B – E (PM + different concentration of TiO_2NPs). For each treatment we calculated the life-history parameters reproduction (cumulative mean number of offspring) ± standard deviation (sd), time to first brood (days ± sd), adult body length (aBL; mm ± sd), offspring body length (oBL; mm ± sd), adult spine length (aSL; mm

± sd), offspring spine length (oSL; mm ± sd), relative adult spine length (aRSL; % ± sd), relative offspring spine length (oRSL; % ± sd), and checked the data for normal distribution (Shapiro-Wilk test) and for homogeneity of variances (Bartlett´s test). If both requirements met, we performed a one-way analysis of variances (ANOVA), followed by a Dunnett´s post hoc-test for multiple comparisons to test for statistical differences within the treatments. Was one requirement not fulfilled, the nonparametric alternative, the Kruskal-Wallis test and afterwards the Dunn's Test of multiple comparisons using rank sums (Dinno, 2015) was used. Because relative spine length of adults (aRSL) and relative spine length in offspring (oRSL) are bounded (Barbosa et al., 2014), the data were analysed as dependent variables by using a '*glmer*' (Generalized Linear Mixed Effect Model) of the package *lme4* (Bates et al., 2014). As fixed factor, we added treatment as the categorical variable to each model. Relative spine length of adults (aRSL) and relative spine length in offspring (oRSL) were modelled using a Gamma error distribution and a Log link function (Barbosa et al., 2014). We included the number of moults and identity of test animals as nested random effects to the model. Model assumptions were checked visually. The p-values were adjusted with Bonferroni correction. The data of the fifth moult of the experiment with TiO_2NPs are missing and were not included within the analysis. Significant p-values were marked with asterisks (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$). All p-values are two tailed.

6.4 Results

6.4.1 Total Ag and Ti concentrations in test media samples

To verify the calculated nominal concentration the total Ag and Ti content of the test media was analysed. The measured total Ag concentration in the fresh and aged media for PM (Treatment I) and Control (Treatment C_1) were below < 0.098 µg/L. The total Ag concentration of the fresh media samples with PM + AgNPs were 2.21 µg/L (Treatment II), 4.38 µg/L (Treatment III), 9.30 µg/L (Treatment IV) and 18.65 µg/L (Treatment V; Table 6-1). The content of total Ti for the PM exposure (Treatment A) reached a concentration of 4.22 µg/L while the control treatment (C_2) was below LOD while. The analysis of the total Ti content of treatments enriched with PM + TiO_2NPs determined concentrations of 36.45 µg/L for Treatment B, 247.62 µg/L for Treatment C, 2148.65 µg/L for Treatment D and 5138.84 µg/L for Treatment E (Table 6-1). The analysis of the aged medium showed for nearly all tested treatments a decrease in the total Ag and Ti content (Table 6-1).

Table 6-1: Concentration of total Ag and Ti [µg/L ± U] of the respective treatments measured with ICP-MS and ICP-OES, respectively, of freshly prepared media and aged media samples after 24h of exposure.

Treatment	Nominal concentrations (µg/L)	Mean measured concentration (µg/L) ± U	
		Fresh media	Aged media
I_a	-	< 0.098	< 0.098
I_b	-	< 0.099	< 0.099
II	2.5	2.21 ± 0.26	1.96 ± 0.22
III	5	4.38 ± 0.24	3.06 ± 0.25
IV	10	9.30 ± 0.59	8.16 ± 0.54
V	20	18.65 ± 0.90	15.50 ± 0.68
C_1	-	< 0.098	< 0.099
A	-	4.22 ± 1.34	4.02 ± 1.51
B	100	36.45 ± 2.40	11.39 ± 1.74
C	1000	247.62 ± 8.49	160.69 ± 9.49
D	5000	2148.65 ± 74.60	85.41 ± 5.00
E	10000	5138.84 ± 294.13	162.60 ± 6.21
C_2	-	< LOD	< LOD

Note: For AgNPs: N = 1, n = 10 and for TiO_2NPs: N = 1, n = 3; N = biological replicates; n = internal replicates

6.4.2 Silver nanoparticles

We found that offspring of adult female *D. magna* which had been exposed to kairomones released from zebrafish, *Danio rerio,* and simultaneously exposed to AgNPs of different environmentally relevant concentrations [2.5, 5, 10 and 20 µg/L] (Treatments II-V, Figure 6-1), did not develop a typical defence mechanism as compared to offspring of adult *Daphnia* which had been exposed to kairomones only (Treatment I_{a+b}) (Tables 6-2 + 6-3). Instead, offspring of adult females which had been exposed to kairomones and different concentrations of AgNPs had a smaller relative spine length as compared to the other offspring (Tables 6-2 + 6-3).

Adult *Daphnia magna* exposed to the AgNP-free predator medium (PM; Treatment I_{a+b}, Figure 6-1), served as a positive control and developed typical defence mechanisms (Figure 6-3 A + B; Table 6-2). Adult *Daphnia* exposed to kairomones (PM) and to different concentrations of AgNPs (Treatment II-V; for more details see Material and Methods section, Figure 6-1) simultaneously developed defensive traits as well (Figure 6-3 C + D, Table 6-2). In the control (C_1) which served as a general reference, adult *D. magna* were exposed to the culture medium (ASTM) containing neither kairomones nor AgNPs (Control

Table 6-2: Mean body length (mm ± sd), mean spine length (mm ± sd) and relative spine length (% ± sd) of adult *Daphnia magna* (n = 12) at the end of the experiment (Day 21) and their offspring (n > 1000) for all AgNPs and all TiO_2NPs treatments and controls. # indicated significant differences between control and predator medium (PM). * showed significant differences between respective treatment and predator medium (PM). # P < 0.05; ### P < 0.001; * P < 0.05; ** P < 0.01; *** P < 0.001.

	Treatment	Offspring			Adult		
		mean body length (mm ± sd)	mean spine length (mm ± sd)	mean relative spine length (% ± sd)	mean body length (mm ± sd)	mean spine length (mm ± sd)	mean relative spine length (% ± sd)
AgNPs	Predator medium (PM)	0.91 ± 0.07 #	0.51 ± 0.04 #	36.01 ± 1.69 ###	4.17 ± 0.12 #	0.82 ± 0.13 #	22.47 ± 5.82 ###
	PM + 2.5 µg/L AgNPs	0.90 ± 0.06	0.51 ± 0.03	35.88 ± 1.69 *	4.26 ± 0.07	0.88 ± 0.07	22.24 ± 5.19
	PM + 5 µg/L AgNPs	0.92 ± 0.06 *	0.52 ± 0.04*	35.72 ± 1.61 ***	4.45 ± 0.29 *	0.90 ± 0.10	22.32 ± 5.40
	PM + 10 µg/L AgNPs	0.94 ± 0.08 *	0.51 ± 0.04	35.83 ± 1.81 **	4.09 ± 0.07	0.82 ± 0.13	22.39 ± 5.20
	PM + 20 µg/L AgNPs	0.92 ± 0.08	0.50 ± 0.03 *	35.86 ± 1.98 *	4.29 ± 0.07	0.93 ± 0.07	22.49 ± 5.05
	Control C_1	0.89 ± 0.07	0.49 ± 0.04	35.73 ± 1.90	3.83 ± 0.31	0.66 ± 0.10	20.19 ± 6.73
TiO_2NPs	Predator medium (PM)	0.93 ± 0.08 #	0.49 ± 0.07	35.59 ± 3.38 ###	4.37 ± 0.14#	1.00 ± 0.20#	22.33 ± 7.16
	PM + 0.1 mg/L TiO_2NPs	0.94 ± 0.08	0.52 ± 0.06	35.58 ± 3.38	4.44 ± 0.11	1.05 ± 0.14	23.51 ± 5.22
	PM + 1 mg/L TiO_2NPs	0.91 ± 0.07	0.50 ± 0.06	35.38 ± 2.92	4.35 ± 0.16	0.99 ± 0.16	23.01 ± 4.95
	PM + 5 mg/L TiO_2NPs	0.92 ± 0.08	0.50 ± 0.06	35.41 ± 3.06	4.35 ± 0.15	0.97 ± 0.19	23.39 ± 4.98
	PM +10 mg/L TiO_2NPs	0.91 ± 0.07	0.50 ± 0.06	35.44 ± 3.27	4.43 ± 0.16	0.98 ± 0.04	23.23 ± 4.75
	Control C_2	0.89 ± 0.08	0.48 ± 0.06	33.75 ± 2.93	4.53 ± 0.13	0.80 ± 0.16	21.75 ± 6.06

(C_1), Figure 6-1) and developed no defensive traits (Figure 6-3 A + B, Table 1). Because AgNPs were dissolved and stabilized with NM-300K DIS, we exposed adult *D. magna* to NM-300K DIS and PM to test for any effects from the solvent (Treatment I_b, Figure 6-1).

Female *D. magna* exposed to kairomones only (PM, Treatment I_a, Figure 6-1) reproduced significantly earlier (Kruskal-Wallis-test and Dunn´s test, $\chi^2 = 6.131$, $P < 0.01$, Figure 6-3 A), produced a significantly greater number of offspring (one-way ANOVA and Dunnett´s test, $P < 0.01$, Figure 6-3 B), developed a significantly larger body length (aBL) (Kruskal-Wallis-test and Dunn´s test, $\chi^2 = 7.491$, $P < 0.01$, Table 6-2), a significantly larger spine length (aSL) (Kruskal-Wallis-test and Dunn´s test, $\chi^2 = 6.687$, $P < 0.01$, Table 6-2) and a significantly larger relative spine length (aRSL) (GLMM, Estimate = 0.115, $p < 0.001$, Tables 6-2 + 6-3) at the end of the experiment (Day 21) in comparison to *Daphnia* females in the control (C_1) with ASTM culture medium only. Similarly, the offspring of female *Daphnia* in Treatment I, exposed to kairomones only (PM), developed a significantly larger body length (oBL) (Kruskal-Wallis-test and Dunn´s test, $\chi^2 = 51.1924$, $P < 0.01$, Table 6-2), a significantly longer spine length (oSL) (Kruskal-Wallis-test and Dunn´s test, $\chi^2 = 122.1717$, $P < 0.01$, Table 6-2) and a significantly larger relative spine length (oRSL) (GLMM, Estimate: 0.009, $p < 0.001$, Table 6-2 + 6-3) compared to offspring from adult *Daphnia* in the control (C_1). These changes in morphology and in life-history parameters are well described as kairomone-mediated anti-predator defence mechanisms in response to fish predators. Hence, the induction of defensive traits in *D. magna* was successful in adult females and their offspring, when AgNPs were absent.

Because we found no differences in any of the measured parameters between female *Daphnia* in Treatment I_a (PM, Figure 6-1) and those in Treatment I_b (PM + NM-300K DIS, Figure 6-1) we combined these data for further comparison mentioned below as Treatment I (data not shown).

The most pronounced effects were observed in the offspring of adult *Daphnia* exposed to kairomones in combination with different environmentally relevant concentrations of AgNPs in Treatments II-V. These offspring even developed a significantly smaller relative spine length (oRSL) when adult *D. magna* were exposed to PM and 2.5 µg Ag/L (GLMM, Estimate: - 0.004, $p = 0.015$, Table 6-2 + 6-3), PM and 5 µg Ag/L (GLMM, Estimate: - 0.007, $p < 0.001$, Table 6-2 + 6-3), PM and 10 µg Ag/L (GLMM, Estimate: - 0.005 $p = 0.06$, Table 6-3 + 6-4), and PM and 20 µg Ag/L (GLMM, Estimate: - 0.007, $p < 0.05$, Table 6-2

+ 6-3) compared to offspring born by females exposed to kairomones only (Treatment I, PM). No clear dose response pattern was found for the body length (oBL) and the spine length (oSL) of offspring from adult *D. magna* exposed to PM and AgNPs in comparison to offspring from Treatment I (PM only) (Table 6-2).

Female *Daphnia* simultaneously exposed to kairomones and different concentrations of AgNPs did not differ from females exposed to kairomones only (Treatment I) in the time to first brood, with one exception. Females exposed to PM and 10 µg Ag/L reproduced significantly later (mean of 9.66 days) than *Daphnia* exposed to PM only (mean of 8.30 days) (Kruskal-Wallis test with Dunn´s test, $\chi^2 = 33.241$, $P < 0.01$, Figure 6-3 C). The number of offspring did not differ between female *Daphnia* exposed to kairomones only and those animals simultaneously exposed to kairomones and different concentrations of AgNPs (Kruskal-Wallis test with Dunn´s test, $\chi^2 = 15.928$, $P > 0.05$, Figure 6-3 D). No concentration dependent pattern was found for adult *Daphnia* in Treatment I compared to Treatments II-V regarding body length (aBL) and spine length (aSL) after each moult (Table S6-2).

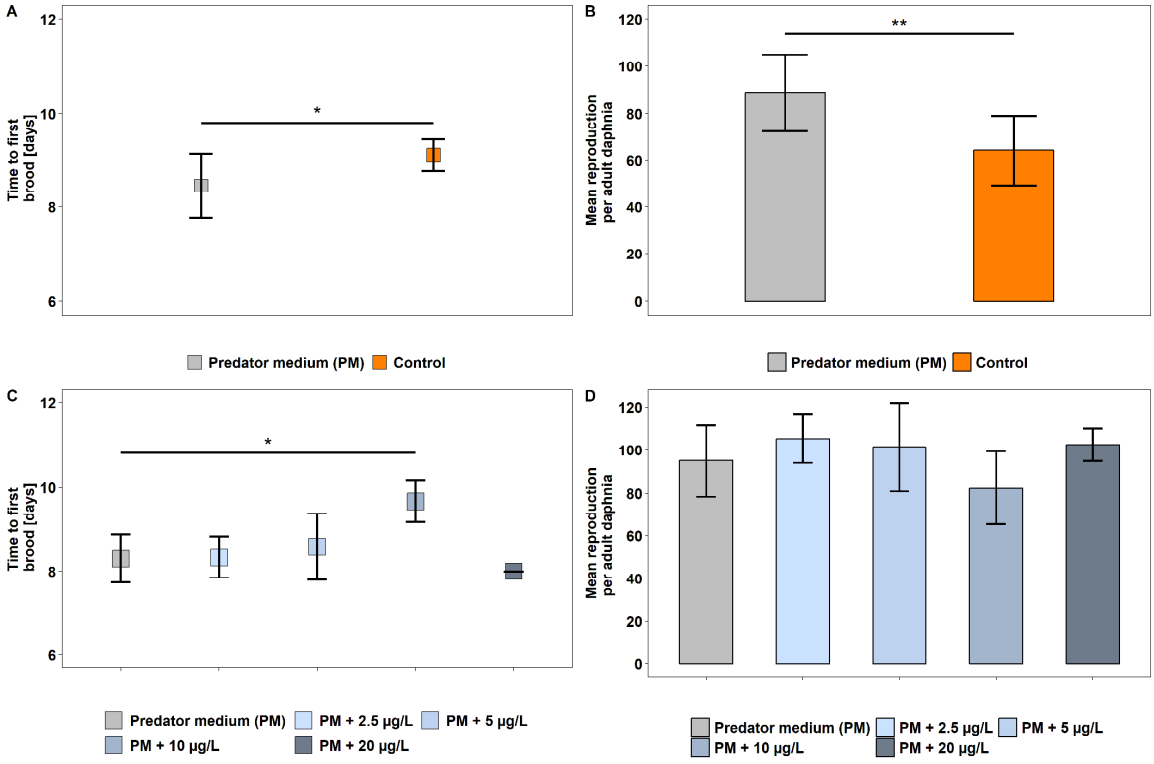


Figure 6-3: Time to first brood [days] ± sd (n = 12 in each treatment) and cumulative mean reproduction ± sd over 21 days of *Daphnia magna* exposed to predator medium (PM) or to ASTM (Control C_1) (A + B), and *Daphnia* exposed to predator medium (PM) only or to PM + different concentrations of AgNPs (C + D). * $P < 0.05$, ** $P < 0.01$.

6.4.3 Titanium dioxide nanoparticles

On day 21, adult *D. magna* treated with TiO_2NPs-free PM (Treatment A, Figure 6-2) released significantly more offspring (one-way ANOVA and Dunnett´s test, $P < 0.01$, Figure 6-4 B), had a significantly smaller body length (aBL) (Kruskal-Wallis-test and Dunn´s test, $\chi^2 = 3.88$, $P < 0.05$, Table 6-3) and a significantly larger spine length (aSL) (Kruskal-Wallis-test and Dunn´s test, $\chi^2 = 5.08$, $P < 0.01$, Table 6-3) compared to animals exposed to control medium (C_2). At moult 6, 7 and 8 the body length (aBL) was significantly smaller (Kruskal-Wallis-test and Dunn´s test, $P < 0.05$, Table S6-3) and the spine length (aSL) was significantly larger (Kruskal-Wallis-test and Dunn´s test, $P < 0.05$, Table S6-3) of adult *D. magna* exposed to PM only (Treatment A, Figure 6-2) compared to those of control medium (C_2). The day of the first brood (Kruskal-Wallis-test, $\chi^2 = 0.193$, $P= 0.660$, Figure 6-4 A) and the relative spine length (aRSL) (GLMM, Estimate = - 0.026, $p = 0.592$, Table 6-3) of animals treated with PM only was not affected compared to animals of the control group (C_2).

The body length (oBL) was significantly larger and spine length (oSL) of offspring of female *D. magna* in Treatment A exposed to kairomones only (PM) did not differ compared to the offspring of Treatment C_2 (Kruskal-Wallis-test, $P > 0.05$, Table 6-2). The relative spine length (oRSL) of offspring of adult *D.magna* exposed to PM only (Treatment A) was significantly larger (GLMM, Estimate: - 0.052, $p < 0.001$, Table 6-3) compared to those of Treatment C_2. The exposure with PM + 10 mg/L TiO_2NPs (Treatment E) lead to a significantly fewer number of offspring (one-way ANOVA and Dunnett´s test, $P < 0.01$, Figure 6-4 D) compared to PM only (Treatment A). No differences were found between PM and the other tested treatments with PM + TiO_2NPs (Treatment B - D). No delayed or prematurely first brood were found for any of the treatments (Kruskal-Wallis, $\chi^2 = 4.228$, $P = 0.376$, Figure 6-4 C). The life history traits body length (aBL), spine length (aSL) (Table 6-2), relative spine length (aRSL) of adult *D. magna* (GLMM, for test statistic details see Table 6-2 + 6-3) exposed to PM + TiO_2NPs (Treatment B - E) was not affected compared to animals exposed to PM only (Treatment A). Similar to this, the body length (oBL), spine length (oSL) (Kruskal-Wallis-test, $P > 0.05$, Table 6-2) and relative spine length (oRSL) (GLMM, for test statistic details see Table 6-2 + 6-3) of the offspring of adult *D. magna* exposed to PM + TiO_2NPs (Treatment B - E) did not differ to the offspring released by adult *Daphnia* treated with PM (Treatment A) only.

Table 6-3: GLMM estimates for the effects on relative spine length of adult and juvenile *D. magna*. Significantly differences (** P < 0.01, *** P < 0.001) within treatments compared to predator medium are marked in bold. PM = predator medium, *t* = test statistics.

Fixed effects	Estimate	Standard error	*t*	P
		AgNPs		
Offspring - Treatment I - V				
(Intercept)	3.585	0.001	2402.713	**< 0.001**
PM + 2.5 µg/L AgNPs	-0.004	0.001	-2.429	**0.015**
PM + 5 µg/L AgNPs	-0.007	0.001	-3.933	**< 0.001**
PM + 10 µg/L AgNPs	-0.005	0.001	-2.705	**0.006**
PM + 20 µg/L AgNPs	-0.007	0.001	-2.202	**0.02**
Offspring – Treatment C_1 and I				
(Intercept)	3.574	0.002	1351.248	**< 0.001**
Predator medium (PM)	0.009	0.002	3.465	**< 0.001**
Adults – Treatment I - V				
(Intercept)	3.017	0.082	36.601	**< 0.001**
PM + 2.5 µg/L AgNPs	0.000	0.012	0.011	0.991
PM + 5 µg/L AgNPs	0.000	0.012	0.068	0.946
PM + 10 µg/L AgNPs	-0.002	0.012	-0.166	0.868
PM + 20 µg/L AgNPs	0.010	0.012	0.870	0.384
Adults – Treatment C_1 and I				
(Intercept)	2.929	0.129	22.602	**< 0.001**
Predator medium (PM)	0.115	0.019	5.803	**< 0.001**
		TiO_2NPs		
Offspring – Treatments A - E				
(Intercept)	3.571	0.004	862.963	**< 0.001**
PM + 0.1 mg/L TiO_2NPs	- 0.0001	0.005	- 0.022	0.982
PM + 1 mg/L TiO_2NPs	- 0.0058	0.005	- 1.105	0.269
PM + 5 mg/L TiO_2NPs	- 0.0052	0.005	- 0.938	0.348
PM +10 mg/L TiO_2NPs	- 0.0043	0.005	- 0.846	0.398
Offspring – Treatments A and C_2				
(Intercept)	3.520	0.005	617.018	**< 0.001**
Predator medium (PM)	0.052	0.006	7.708	**< 0.001**
Adults – Treatments A - E				
(Intercept)	3.105	0.027	113.052	**< 0.001**
PM + 0.1 mg/L TiO_2NPs	0.051	0.037	1.396	0.163
PM + 1 mg/L TiO_2NPs	0.030	0.037	0.819	0.413
PM + 5 mg/L TiO_2NPs	0.046	0.036	1.270	0.204
PM +10 mg/L TiO_2NPs	0.039	0.036	1.083	0.279
Adults – Treatments A and C_2				
(Intercept)	3.105	0.0349	88.851	**< 0.001**
Predator medium (PM)	0.026	0.0489	- 0.592	0.592

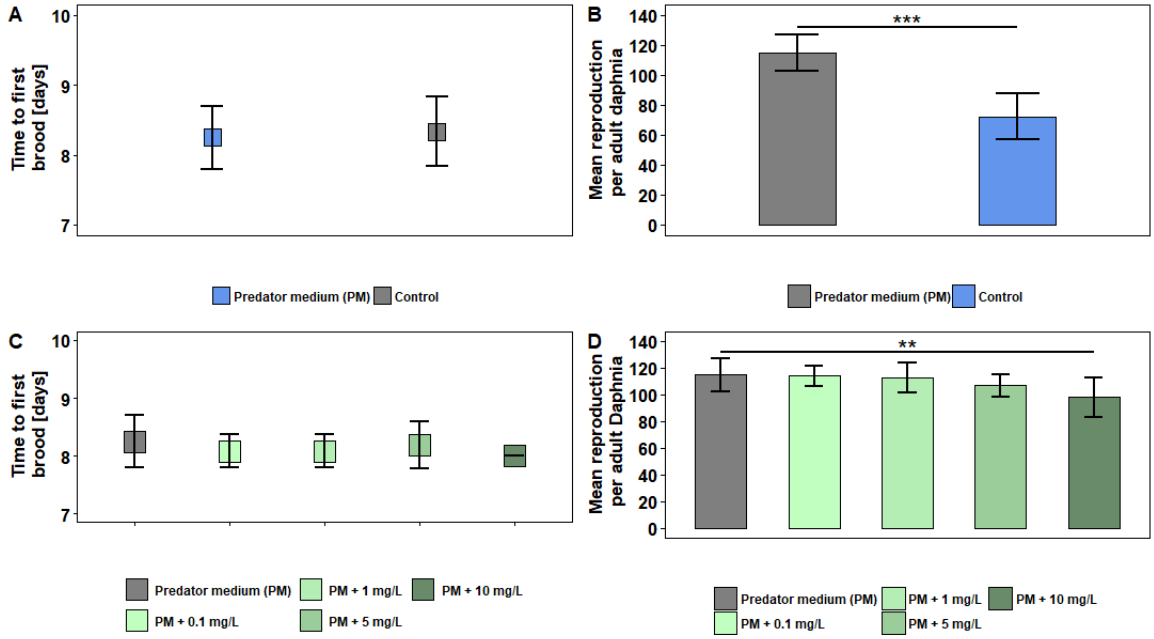


Figure 6-4: Time to first brood [days] ± sd (n = 12 in each treatment) and cumulative mean reproduction ± sd over 21 days of *Daphnia magna* exposed to predator medium (PM) or to ASTM (Control) (A + B), and *Daphnia* exposed to predator medium (PM) only or to PM + different concentrations of TiO_2NPs (C + D). * P < 0.05, ** P < 0.01.

6.5 Discussion

In this study, we detected a defective kairomone-mediated anti-predator defence mechanism to zebrafish *D. rerio* in *Daphnia* offspring in the presence of environmentally relevant low concentrations of AgNPs but not for TiO_2NPs. Although adult *D. magna* exposed to kairomones and different concentrations of AgNPs developed typical defensive traits, their offspring did not exhibit such traits. They developed a significantly smaller relative spine length which probably makes them even more vulnerable to predators. This is the first study showing that environmentally relevant low concentrations of AgNPs can have a dramatic negative impact on offspring, although they were never directly in contact with these AgNPs (protected by the brood pouch of adult *Daphnia*). TiO_2NPs did not affect the anti-predator defence mechanism neither of adult *D. magna* nor of their released offspring. Our results indicate that adult *D. magna* are not able to produce offspring with an adaptive defence mechanism against fish predators when exposed to PM and AgNPs. The lack of this effective and adaptive defence mechanism will have a dramatic negative impact on *Daphnia* populations and therefore potentially on the entire food web in the aquatic environment.

In our study, adult *D. magna* treated with kairomones only exhibited a typical kairomone-mediated anti-predator defence mechanism as expected. In the Treatment I and Treatment A with kairomones (PM) only the reproductive success of female *D. magna* was significantly higher, they reproduce significantly earlier, and their spine length was significantly larger in comparison to *Daphnia* of the control with ASTM-medium only (C_1 and C_2). Thus, our results are in accordance with studies of Barbosa et al. (2014) and Ślusarczyk et al. (2013) who showed that the exposure to kairomones from fish predators leads to a significantly increase in number of offspring, in body size and an earlier first reproduction of adult *D. magna*. In the presence of fish predation, *Daphnia* invest most of their resources into reproduction than into somatic growth (Stibor and Lüning, 1994), leading to an early sexual maturity with more but smaller neonates (Weiss et al., 2012). This predator defence mechanism is adaptive because *D. magna* that sexually mature earlier at smaller body size are less conspicuous to fish predators since fish predators selecting larger prey due to visual hunting (Weber and Declerck, 1997).

So far, it is known that environmental pollutants can affect the kairomone-mediated anti-predator defence mechanism in adult *Daphnia*. Trotter et al. (2019) found that microplastics inhibit the induction of defensive traits in adult *D. longicephala*, when they were exposed to kairomones of *Notonecta glauca* and plastic waste. Further, Pokhrel and Dubey (2012) showed that adult *Daphnia* treated with low concentrations of citrate-AgNPs and predator medium of the dragonfly *Anax junius*, were not able to detect the presence of the predator in a vertical migration test. The authors assumed that the exposure to AgNPs impairs the sensory system of *Daphnia*, or that the chemoreceptors might be compromised. The chemoreceptors for perception of kairomones are located on the first antennae of *Daphnia* (Weiss et al., 2015). The chemosensilla of the first antennae is developed already in the juvenile stages of a *Daphnia*'s life cycle, allowing them to detect predators via the chemical signals released into the aquatic environment (Laforsch and Tollrian, 2004a; Weiss et al., 2015).

In our study, *Daphnia* females exposed to kairomones and different low concentrations of AgNPs developed similar defensive traits as *Daphnia* exposed to kairomones only. This is interesting because our previous long-term multi-generation study on *D. magna* exposed to similar low concentrations of AgNPs showed that *D. magna* reproduced less offspring over six successive generations in comparison to *D. magna* not exposed to AgNPs

(*Hartmann et al., 2019*). Thus, the presence of AgNPs leads to a reduction in the reproductive success. In the present study, however, the presence of kairomones only led to an increase in the number of offspring, which is the opposite effect. The fact that adult *D. magna* exposed to both kairomones and AgNPs reproduced a similarly high number of offspring as *D. magna* exposed to kairomones only, might indicate that the effect of kairomones prevails the effect of AgNPs. We could observed the same for the exposure with TiO_2NPs, reproductive success was only significantly reduced by the exposure with 10 mg/L but not by lower concentrations, although the toxicity of TiO_2NPs at similar concentrations to the offspring of female *Daphnia* was investigated by several studies (Jacobasch et al., 2014; Seitz et al., 2013; Zhu et al., 2010). Hence, it might be that the presence of kairomones from *D. rerio* inhibits the chronic toxicity of TiO_2NPs in the concentration range of 0.1 mg/L – 5 mg/L mainly due to the high content of dissolved organic matter (DOM) within the test medium (Bundschuh et al., 2016), or the formation of reactive oxygen species (ROS) was too low to exceeded the organism antioxidant response capacity leading to no oxidative stress-mediated toxicity (Vale et al., 2016). However, most interestingly, we found, that the exposure with TiO_2NPs did not affect any kairomone-mediated defensive traits, different compared to the results with AgNPs.

The fact that *Daphnia* females exposed to kairomones and different low concentrations of AgNPs developed similar defensive traits as *Daphnia* exposed to kairomones only, gives the first impression that AgNPs in combination with kairomones had no negative impact on the reproductive success of adult *Daphnia*. However, we detected a lack of the adaptive kairomone-mediated anti-predator defence mechanism in the offspring of females exposed to both chemical stressors. Even worse, these offspring had a smaller relative spine length than offspring of Treatment I (PM). But why did these offspring not develop typical kairomone-mediated defence mechanisms? A study by Hales et al. (Hales et al., 2017) found that different gene expression programs are involved in kairomone-mediated anti-predator defence mechanisms in the maternal generation and offspring of *Daphnia ambigua*, when exposed to kairomones of redbreast sunfish (*Lepomis auritus*). They provide evidence that the gene expression program within a generation (from moult to moult) and the gene expression program involved in transgenerational responses (from female to offspring) are distinct programs and regulated independently (Hales et al., 2017). Thus, such differences in these two types of gene expression programs might explain, why

adult females responded to kairomones in the presence of AgNPs but their offspring did not. Further studies are required to identify the mechanisms behind this impairment and the role of NPs in gene expression programs in *Daphnia* and other aquatic organisms. Further it seems like, that the impairment of NPs in gene expression programs are substance-specific because the offspring of adult female *Daphnia* exposed to high concentrations of TiO_2NPs show kairomone mediated anti-predator defence mechanism. Thus, further studies are required to identify the mechanism behind this impairment and the role of NPs in gene expression programs.

6.6 Conclusion

This study firstly shows, that even environmentally relevant, low concentrations of AgNPs in aquatic environments have a clear negative impact on the adaptive kairomone-mediated anti-predator defence mechanism in *D. magna*. Although adult *Daphnia* developed typical anti-predator defence mechanisms when exposed to kairomones and AgNPs, their offspring could not develop such adaptive defensive traits. This lack of defence mechanism in offspring of *Daphnia* could therefore have dramatic impacts and consequences on *Daphnia* population structure in the presence of predation risk, and thus on the entire complex food web. However, TiO_2NPs did not affect defensive traits in *D. magna* neither in adults nor in their released offspring. Hence, this study provides strong evidence, that anthropogenic pollutants released into the aquatic environment interfere with evolutionary adaptation strategies in cladoceran. Our study is the first one investigating the effect of two chemical stressors on an evolved predator defence strategy in *Daphnia* with dramatic effects in the offspring. This shows that it is extremely important to test a combination of chemical stressors simultaneously instead of testing them individually. This is a more realistic exposure scenario for an aquatic organism which is typically exposed to several natural and artificial chemical stressors at the same time. Additionally, this set up enables us to detect possible interacting effects. Additionally, we should not only focus on one generation in risk assessment studies but include at least the following generation as well. Further research is needed to understand how AgNPs affect the kairomone-mediated anti-predator defence mechanism in *Daphnia* species.

6.7 Supporting Information

Table S6-1: Instrumental Parameters of ICP-MS and ICP-OES to determine total Ag and total Ti in aqueous test samples.

Used for	total Ag measurement	total Ti measurement
	ICP-MS iCAp Qc (Thermo Fisher Scientific, Bremen, Germany)	ICP-OES Arcos, Spectro Analytical Instruments (axial plasma view)
Nebulizer	C400d (Savillex, Eden Prairie, MN, USA)	Standard cross-flow nebulizer
Spray chamber	Peltier-cooled cyclonic quartz	Standard Scott type
Radio-frequency power	1550 W	1200 W
Torch injector inner diameter	2.5 mm	2.0 mm
Cooling flow	14 L/min	13 L/min
Auxiliary flow	0.8 L/min	0.8 L/min
Nebulizer flow	1.0 L/min	0.9 L/min
Sampling position	5 mm	n/a

Table S6-2: Mean body length (mm ± sd) and mean spine length (mean ± sd) of adult *D. magna* exposed to predator medium (PM) and PM + different concentrations of AgNPs and ASTM medium (Control) after each moult. Asterisks indicated significant differences compared to predator medium. * P < 0.005. n = 12.

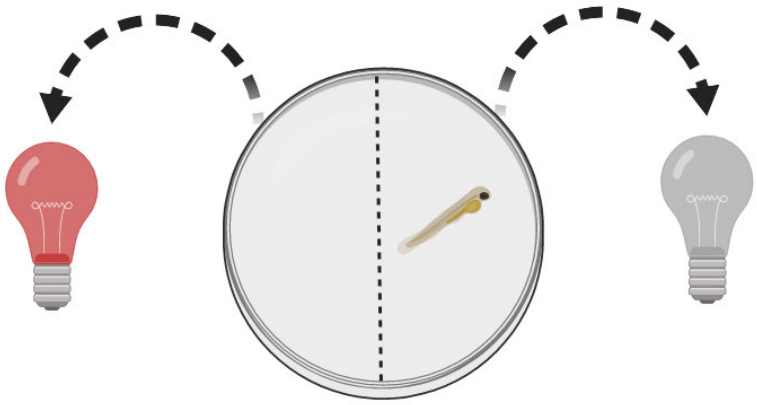
Moult	mean body length (mm ± sd)						mean spine length (mm ± sd)					
	Treatment						Treatment					
	Predator medium (PM)	PM + 2.5 µg/L AgNPs	PM + 5 µg/L AgNPs	PM + 10 µg/L AgNPs	PM + 20 µg/L AgNPs	Control C_1	Predator medium (PM)	PM + 2.5 µg/L AgNPs	PM + 5 µg/L AgNPs	PM + 10 µg/L AgNPs	PM + 20 µg/L AgNPs	Control C_1
0	0.95 ± 0.09	1.04 ± 0.05	1.07 ± 0.11	0.98 ± 0.03	1.05 ± 0.13	0.97 ± 0.14	0.50 ± 0.02	0.53 ± 0.02	0.52 ± 0.04	0.46 ± 0.04	0.50 ± 0.04	0.50 ± 0.04
1	1.22 ± 0.19	1.35 ± 0.19	1.41 ± 0.22	1.23 ± 0.21	1.32 ± 0.35	1.33 ± 0.13	0.49 ± 0.08	0.52 ± 0.04	0.52 ± 0.04	0.47 ± 0.02	0.49 ± 0.09	0.44 ± 0.11
2	1.54 ± 0.31	1.69 ± 0.09	1.82 ± 0.27 *	1.48 ± 0.06	1.73 ± 0.22	1.68 ± 0.18	0.54 ± 0.09	0.55 ± 0.07	0.60 ± 0.06	0.51 ± 0.02	0.57 ± 0.03	0.47 ± 0.15
3	1.92 ± 0.40	2.15 ± 0.24	2.38 ± 0.54 *	1.80 ± 0.30	2.25 ± 0.32	2.15 ± 0.38	0.58 ± 0.10	0.63 ± 0.08	0.67 ± 0.10	0.55 ± 0.05	0.65 ± 0.03	0.52 ± 0.13
4	2.51 ± 0.46	2.73 ± 0.22	2.97 ± 0.43	2.26 ± 0.33	2.85 ± 0.26	2.67 ± 0.33	0.64 ± 0.16	0.71 ± 0.09	0.77 ± 0.10	0.63 ± 0.08	0.70 ± 0.06	0.57 ± 0.12
5	2.95 ± 0.43	3.21 ± 0.21	3.42 ± 0.39 *	2.76 ± 0.38	3.32 ± 0.20	3.17 ± 0.28	0.70 ± 0.15	0.79 ± 0.07	0.84 ± 0.08	0.72 ± 0.06	0.81 ± 0.06	0.64 ± 0.13
6	3.46 ± 0.32	3.63 ± 0.13	3.72 ± 0.31	3.26 ± 0.20	3.67 ± 0.14	3.44 ± 0.25	0.78 ± 0.14	0.84 ± 0.06	0.88 ± 0.08	0.78 ± 0.05	0.87 ± 0.04	0.68 ± 0.13
7	3.65 ± 0.23	3.81 ± 0.15	3.98 ± 0.30 *	3.63 ± 0.21	3.85 ± 0.23	3.62 ± 0.27	0.81 ± 0.15	0.85 ± 0.08	0.89 ± 0.09	0.83 ± 0.05	0.88 ± 0.06	0.70 ± 0.13
8	3.92 ± 0.23	4.09 ± 0.12	4.13 ± 0.20	3.83 ± 0.23	4.04 ± 0.24	3.71 ± 0.30	0.81 ± 0.16	0.88 ± 0.06	0.88 ± 0.11	0.78 ± 0.11	0.90 ± 0.09	0.69 ± 0.12

Table S6-3: Mean body length (mm ± sd) and spine length (mean ± sd) of adult *D. magna* exposed to predator medium (PM) and PM + different concentrations of TiO_2NPs and ASTM medium (Control) after each moult. The data of the fifth moult are missing. Asterisks indicated significant differences compared to predator medium. * P < 0.05. n = 12.

Moult	mean body length (mm ± sd)						mean spine length (mm ± sd)					
	Treatment						Treatment					
	Predator medium (PM)	PM + 0.1 mg/L TiO_2NPs	PM + 1 mg/L TiO_2NPs	PM + 5 mg/L TiO_2NPs	PM + 10 mg/L TiO_2NPs	Control C_2	Predator medium (PM)	PM + 0.1 mg/L TiO_2NPs	PM + 1 mg/L TiO_2NPs	PM + 5 mg/L TiO_2NPs	PM + 10 mg/L TiO_2NPs	Control C_2
0	1.08 ± 0.03	1.10 ± 0.02	1.11 ± 0.09	1.09 ± 0.02	1.08 ± 0.02	1.08 ± 0.03	0.54 ± 0.01#	0.53 ± 0.04	0.51 ± 0.05	0.51 ± 0.02	0.52 ± 0.02	0.52 ± 0.02
1	1.38 ± 0.07	1.41 ± 0.04	1.38 ± 0.04	1.38 ± 0.04	1.40 ± 0.05	1.40 ± 0.17	0.57 ± 0.02	0.59 ± 0.02	0.55 ± 0.04	0.57 ± 0.03	0.56 ± 0.03	0.55 ± 0.03
2	1.80 ± 0.09	1.83 ± 0.06	1.80 ± 0.07	1.75 ± 0.13	1.86 ± 0.19	1.78 ± 0.15	0.56 ± 0.19	0.62 ± 0.09	0.60 ± 0.05	0.62 ± 0.05	0.63 ± 0.04	0.61 ± 0.03
3	2.24 ± 0.16	2.35 ± 0.07	2.29 ± 0.11	2.33 ± 0.10	2.34 ± 0.06	2.30 ± 0.17	0.66 ± 0.18	0.70 ± 0.10	0.68 ± 0.06	0.72 ± 0.03	0.74 ± 0.03	0.67 ± 0.04
4	2.93 ± 0.10	2.96 ± 0.08	2.94 ± 0.12	2.95 ± 0.11	3.00 ± 0.09	2.99 ± 0.27	0.76 ± 0.22	0.80 ± 0.13	0.76 ± 0.12	0.80 ± 0.06	0.83 ± 0.05	0.74 ± 0.08
6	3.31 ± 0.11#	3.39 ± 0.10	3.34 ± 0.14	3.39 ± 0.09	3.41 ± 0.10	3.51 ± 0.12	0.84 ± 0.23#	0.90 ± 0.09	0.86 ± 0.14	0.89 ± 0.09	0.89 ± 0.06	0.77 ± 0.11
7	3.72 ± 0.12#	3.74 ± 0.08	3.67 ± 0.17	3.75 ± 0.11	3.70 ± 0.10	3.93 ± 0.11	0.86 ± 0.11#	0.93 ± 0.14	0.90 ± 0.08	0.91 ± 0.13	0.90 ± 0.04	0.81 ± 0.11
8	3.96 ± 0.15#	4.01 ± 0.11	3.98 ± 0.12	4.00 ± 0.11	4.02 ± 0.12	4.20 ± 0.13	0.83 ± 0.32#	0.95 ± 0.12	0.93 ± 0.14	0.92 ± 0.13	0.92 ± 0.06	0.81 ± 0.09

Chapter 7

The sensitivity of zebrafish larvae to near-infrared light



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7.1 The visual sense of fish

For the interaction with their environment, fish have a series of sensory systems, which provide them with information about the surrounding area and physical characteristics of the water (Wootton, 2012). The categories of fish sensory systems include chemoreception (taste, olfaction), mechanoreception (hearing, lateral line), electroreception, magnetic reception and vision (Helfman et al., 2009; Wootton, 2012). The morphology of the eyes of fish are similar to humans and other vertebrates indicating well-developed eyes (Helfman et al., 2009; Wootton, 2012). The main differences are that the lens of a fish eye is more spherical compared to those of terrestrial vertebrates (Helfman et al., 2009).

Visual information passes through a transparent cornea before it enters the eye by the pupil (Helfman et al., 2009). After passing through the lens, the visual information encounters the retina, the photosensory system of the eye. The retina of fish contains two general types of sensory cells, rods for vision in dim light and cones for vision in bright light and colour vision (Helfman et al., 2009; Wootton, 2012). The structure of the retina (number of rods and cones) correlates with the optical properties of the habitat where the fish species lives (Wootton, 2012). For instance, deep-sea fish have a retina, which is dominated by rod cells, sensitive to low light intensity. Deep coastal water fish have, in addition to rods, two cone types with a maximum sensitivity to blue and green light. Fresh and shallow water fish have three types of cone cells, with a light maximum to red light whereby, coral reef fish have cones types which are sensitive to ultraviolet (UV) light (Helfman et al., 2009; Wootton, 2012). However, it is also known, that some fish could detect polarized light, maybe to enhance the underwater vision for a better detection for prey, predators or mates (Helfman et al., 2009).

7.2 Abstract

Zebrafish larvae (*Danio rerio*) are among the most used model species to test biological effects of different substances in biomedical research, neuroscience and ecotoxicology. Most tests are based on changes in swimming activity of zebrafish larvae by using commercially available high-throughput screening systems. These systems record and analyse behaviour patterns using visible (VIS) and near-infrared (NIR) light sources, to simulate day (VIS) and night (NIR) phases, which allow continuous recording of the

behaviour using a NIR sensitive camera. So far, however, the sensitivity of zebrafish larvae to NIR has never been tested experimentally, although being a critical piece of information for interpreting their behaviour under experimental conditions. Here, we investigated the swimming activity of 96 hpf (hours post fertilization) and 120 hpf zebrafish larvae under light sources of NIR at 860 nm and at 960 nm wavelength and under VIS light. A thermal source was simultaneously presented opposite to one of the light sources as control. We found that zebrafish larvae of both larval stages showed a clear negative phototactic response towards 860 nm NIR light and to VIS light, but not to 960 nm NIR light. Our results demonstrated that zebrafish larvae are able to perceive NIR at 860 nm, which is almost identical to the most commonly used light source in commercial screening systems (NIR at 850 nm) to create a dark environment. These tests, however, are not performed in the dark from the zebrafish´s point of view. We recommend testing sensitivity of the used test organism before assuming no interaction with the applied light source of commonly used biosensor test systems. Previous studies on biological effects of substances to zebrafish larvae should be interpreted with caution.

7.3 Introduction

Fish in general are sensitive model species and have been frequently used in automated biological monitoring systems as biosensors (Gruber et al., 1994). The analysis of behavioural parameters such as swimming activity, respiration, shoaling behaviour or rheotactic behaviour are of particular interest (Gruber et al., 1994; Legradi et al., 2015; Miller and Gerlai, 2007). Measuring sublethal effects as behavioural endpoints leads to a higher significance and, compared to mortality, a 10 - 100 times higher sensitivity can be achieved (Gerhardt, 2007; Robinson, 2009).

During the last few decades the zebrafish (*Danio rerio*), a small tropical freshwater fish, became one of the most used model species in the field of biomedical research, neuroscience and ecotoxicology (Ahmad et al., 2012; Burgess and Granato, 2007; de Esch et al., 2012a; Ingebretson and Masino, 2013; Padilla et al., 2011; Powers et al., 2011). Breeding and cultivating this fish species in the lab is cost-effective and embryo development is very fast (Kimmel et al., 1995). Additionally, the complete genome of the zebrafish is known (Howe et al., 2013) and has broad homologies to other vertebrate species (Ali et al., 2012; Guo, 2004; Guo, 2009), and genes involved in behaviour are

highly conserved between humans and zebrafish (Ahmet et al., 2012). Thus, monitoring of zebrafish swimming activity serves as a sensitive and powerful tool for identifying toxic compounds in several fields of research (Gruber et al., 1994, Legradi et al. 2015). These behavioural measurements are highly economical and appropriate for e.g. ecotoxicological research (Bae and Park, 2014). They are mostly based on swimming activity of adult zebrafish and zebrafish larvae, which are monitored using video tracking systems and corresponding software to detect and quantify changes in locomotion pattern. So far, commercially available high-throughput tracking systems, such as the DanioVision from Noldus (Wageningen, Netherlands) or the Zebrabox from Viewpoint (Lyon, France) allow tracking of locomotion parameters of zebrafish larvae in multi-well plates. Many studies use light/dark transition tests to investigate possible effects of several substances (e.g. ethanol, cadmium, microplastic, neurotoxic drugs) on swimming activity of zebrafish larvae. The dark sections are lit with NIR light, so that the behaviour can still be recorded with an IR sensitive camera (Ali et al., 2012; Burgess and Granato, 2007; Chen et al., 2017; Ellis et al., 2012; Emran et al., 2008; Irons et al., 2010; Legradi et al., 2015; MacPhail et al., 2009; Nüßer et al., 2016). The Zebrabox system e.g. uses an infrared light illumination at a wavelength of 850 nm to record the swimming activity under “dark” conditions (Liu et al., 2015). So far, it has been assumed that zebrafish larvae cannot perceive NIR light and do not respond to it (Brockerhoff et al., 1997; Dowling, 2002; Legradi et al., 2015). This assumption was based, first, on the anatomy of the vision system in adult zebrafish. Adult zebrafish have four different types of photoreceptors with specific visual sensitivity in the red spectrum (~570 nm), in the green spectrum (~480 nm), in the blue spectrum (~415 nm) and in the ultraviolet spectrum (~362 nm) (Brockerhoff et al., 1997; Fleisch and Neuhauss, 2006; Robinson et al., 1993). Secondly, in an optokinetic experiment, zebrafish larvae showed no eye movement in response to rotating stripes that were illuminated with 750 nm NIR light (Brockerhoff et al., 1995). It is known, however, that the visual system can change during ontogeny in fish (Colwill and Creton, 2011b; de Esch et al., 2012b). Nevertheless, it is precarious to extrapolate from the visual sensitivity in adult fish to the visual sensitivity in fish larvae within a species.

Recently, only a few researchers focused on near-infrared (NIR) sensitivity in fish, although the absorption spectra of light in natural aquatic ecosystems covers wavelengths higher than 700 nm (Kirk, 1994; Schwoerbel and Brendelberger, 2010). The sensitivity

towards NIR illumination was reported in the common carp *Cyprinus carpio* and the Nile tilapia *Oreochromis niloticus* (Matsumoto and Kawamura, 2005). Both species showed a visual sensitivity towards the near infrared light at 865 nm, and a perception of even longer wavelengths (936 nm) was indicated in the common carp (Matsumoto and Kawamura, 2005). Due to the experimental set-up, the authors concluded that the NIR light was detected by the eyes and not by the pineal organ in both species, the common carp and the Nile tilapia (Matsumoto and Kawamura, 2005). In the cichlid *Pelvicachromis taeniatus* it could be shown that a direct illumination of the prey organism *Gammarus pulex* with NIR wavelengths between 780 and 920 nm led to stronger foraging responses compared to non-illuminated *G. pulex* (Meuthen et al., 2012) which indicates prey-detection through NIR sensitivity. Shcherbakov et al. (2013) analysed the NIR detection under different light conditions as a parameter for spectral sensitivity in different fish species: the Mozambique tilapia (*Oreochromis mossambicus*) and the Nile tilapia (*O. nilticus*) showed a high sensitivity to wavelengths above 930 nm, and they found an upper threshold for the green swordtail (*Xiphophorus helleri*) at 825 - 845 nm. Furthermore, the authors investigated the response of adult zebrafish to NIR light and determined a threshold at 845 - 910 nm, as clear evidence for the perception of NIR by adult zebrafish (Shcherbakov et al., 2013). The authors explained the sensitivity to NIR as an evolutionary adaptation to environmental conditions and suggested long wavelength sensitive cones as a potential candidate for NIR perception in fish (Shcherbakov et al., 2013).

Here, we investigated the behavioural reaction of zebrafish larvae at two larval stages, (96 hours post fertilization (hpf) and 120 hpf), to different wavelengths of NIR light (860 nm and 960 nm), to test for a phototactic response according to Jékley (2009). As Shcherbakov et al. (2013) found that adult zebrafish are able to sense NIR light up to 910 nm, we hypothesized that zebrafish larvae might perceive a similar NIR light spectrum. We, therefore, conducted three experiments under specific light characteristics: NIR light with a spectral peak at 860 nm and 960 nm, and blue-white light as a visible light source (VIS, 440 - 700 nm). Our results showed for the first time that zebrafish larvae at both larval stages showed a clear negative phototactic response towards NIR light at 860 nm as well as towards VIS light, but not to NIR light at 960 nm wavelength. Our results are, therefore, highly relevant to all experiments using zebrafish larvae in standard testing procedures under NIR light conditions because most experimental devices use 850 nm

light sources to simulate a dark environment. These tests, however, are not carried out in the dark from the zebrafish´s point of view.

7.4 Materials and Methods

7.4.1 Ethics statement

All experiments were non-invasive behavioural tests. The performed experiments were in line with the German Animal Welfare Act (Deutsches Tierschutzgesetz) and approved by the internal animal protection commissioner Dr. Urs Gießelmann, University of Siegen, Germany, and the national Veterinary Authority (Kreisveterinaeramt Siegen-Wittgenstein, Germany).

7.4.2 Study species

The culture conditions of the adult *D. rerio* are described in Chapter 2, section 2.2.2. Fertilized and healthy eggs were kept in 60 mL crystallisation dishes (60 x 35 mm²) for development until larvae were 96 hpf and 120 hpf and were cultured under the same conditions (water temperature: 26 ± 1°C; light-dark cycle 14:10) as adult zebrafish. Fish eggs were checked daily and dead and abnormal embryos were removed. Only normally developed and hatched larvae were used for testing.

7.4.3 Phototactic experiments and video tracking

Phototactic experiments followed Shcherbakov et al. (2012) with some modifications. All experiments were performed in a room with a constant temperature of 26 ± 1 °C. We used a custom-built, light-isolated chamber coated with black PVC plates (49 x 90 x 45 cm^3), to record movements of the zebrafish larvae in a petri dish (diameter: 35 mm), which served as the test vessel (Figure 7-1 A), under specific light conditions for 5 min. Movement was recorded with an IR sensitive camera (Manta G-235C, Allied Vision, Stadtroda, Germany) with a frame rate of 35/s, fixed 35 cm above the test vessel. The camera was connected to a PC to control, start and manage the experimental settings. We performed three experiments with different light sources to test the sensitivity of the zebrafish larvae at an age of 96 hpf or of 120 hpf towards two different wavelengths of NIR light and VIS light. In each experiment a light source was provided simultaneously with a thermal source positioned opposite to the light source in 10.5 cm distance from that vessel (Figure 7-1 A + B) to control for a thermal gradient within the test vessel. The thermal source was an

860 nm light source equipped with a 950-IR high-pass filter (IR-filter 950 nm, Delamax, Germany) that cut off all shorter wavelengths and additionally equipped with a UV-IR-cut filter (HD2130, Ningbo Haida Photo supplies Co., Ltd., Ningbo China) to cut off wavelengths between UV and NIR, so that light was entirely blocked (Figure S7-1). To test for temperature differences, we measured the temperature of each light source 30 times after each 5 min test period with an IR thermometer at the position of the test vessel in 10.5 cm distance (IRT-350 IR thermometer, Base Tech, Hirschau, Germany).

To test for a thermal gradient within the test vessel we measured the water temperature within the test vessel at five different positions (left and right side, centre, top and bottom) with a digital thermocouple (digital probe thermometer POCKET-DIGITEMP, TFA Dostmann GmbH & Co. KG, Wertheim-Reicholzheim, Germany) 5 min after onset of light, thus under same conditions as in trials. Furthermore, we measured the light intensity as

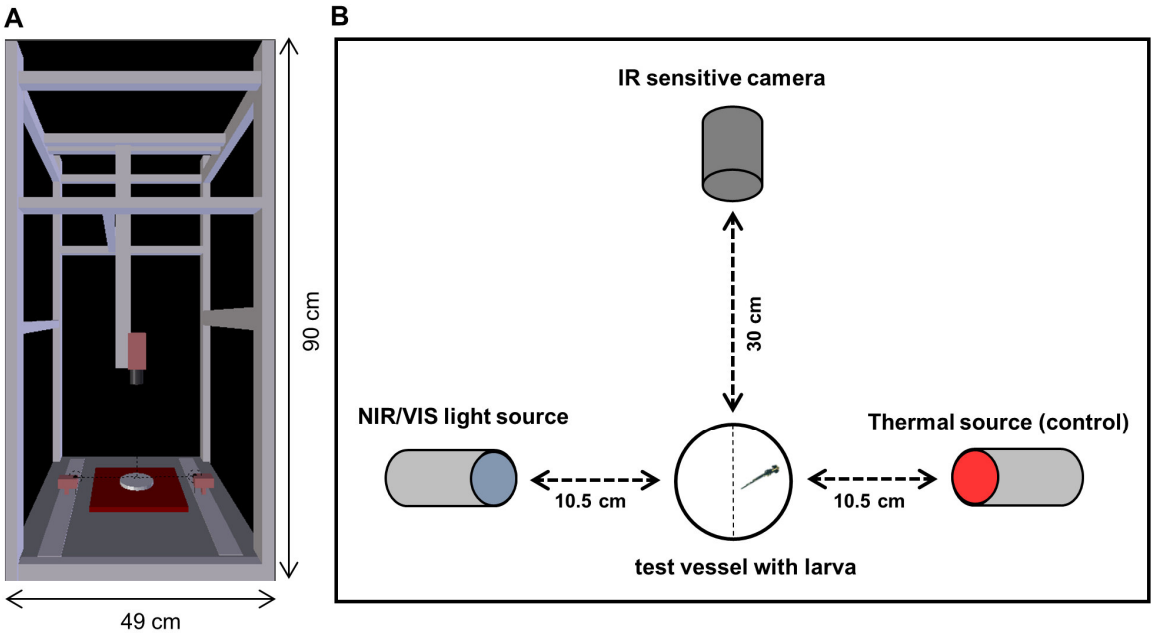


Figure 7-1: Scheme of the experimental set-up of the NIR light experiments. (A) The custom-built computer vision system shows the position of the test vessel in the middle of the set-up, the camera above the test vessel and the position of the light sources. The computer vision system was totally covered so that no light from the surrounding area could enter the system. (B) A detailed view of the set-up: For each experiment, one larva was placed into the test vessel. The IR sensitive camera was set 35 cm above the vessel, and both light sources were 10.5 cm away from the test vessel. A thermal source opposite to the NIR/VIS light served as a control. We switched the position of light and thermal sources after each trial to exclude side biases. The vessel was divided into 2 halves with an imaginary line, one half illuminated by the light source (VIS/NIR), one half radiated by the thermal source (control side) (drawing of zebrafish larva by Kimmel et al. 1996).

irradiance [µWatt/cm²] and the radiated spectrum of all applied light sources and of the thermal source covered by used filters, respectively, with an AvaSpec-2048 spectrometer covering a range of 220 - 1100 nm (Avantes BV, Apdeldoorn, Netherlands, Europe) in 2 cm distance to the source.

We transferred one larva per trail to a 35 mm petri dish filled with 3 mL formulated water (294 mg/l $CaCl_2 \cdot 2\ H_2O$, 123.3 mg/l $MgSO_4 \cdot 7\ H_2O$, 63 mg/l $NaHCO_3$, 5.5 mg/l KCl (ISO, 1996). The vessel was placed under the IR sensitive camera (Figure 7-1 B). Since the transfer of larvae was performed in light, we chose an acclimation time for each larva of 5 min within the device in darkness. Thereafter, light sources (VIS/NIR) and the thermal source were switched on and the swimming activity was recorded for 5 min. It was previously reported that the most stable activity period in zebrafish larvae ranges between 1:00 and 4:30 pm (MacPhail et al., 2009, Vignet et al., 2013). Therefore, all experiments were carried out within this time period.

7.4.3.1 **Phototactic experiments under VIS**

In the first experiment, we tested the phototactic behaviour of zebrafish larvae under blue-white light (blue: LED type: 151053BS04500, Würth Eletronic, Waldenburg, Germany, spectral peak at 460 nm; white: LED type: LW340-A, Soeul Semiconducter Co., Ltd, Ansan, South Korea, spectral peak at 460 nm and 560 nm) which served as a visible light (VIS) source. A blue-white light source was used because zebrafish are able to see blue light (Brockerhoff et al., 1997, Robinson et al., 1993). In order to ensure that the VIS light source did not reflect shorter or longer wavelengths, a UV-IR-cut filter (HD2130, Ningbo Haida Photo supplies Co., Ltd., Ningbo China) was attached to the device that absorbed ultraviolet and infrared wavelength (Table 7-1 and Figure S7-1). The measured spectral range of the VIS light source was 420 - 680 nm, with a maximum absorption at 460 nm and a light intensity of 2.49 µWatt/cm² (Table 7-1 and Figure S7-1). Opposite to the VIS light source, we used the same thermal source as described above to provide thermal radiation only (Figure S7-1). In total, we tested 30 larvae of each larval stage and exposed 15 larvae of each larval stage with VIS light from the right and 15 larvae with VIS light from the left side to exclude side biases.

Table 7-1: Spectral characteristics of the used light sources (VIS, NIR 860 nm and NIR 960 nm) for phototactic experiment.

Light spectrum	$\lambda_{(max)}$ [nm]	measured spectral range [nm]	80 % of the spectral range [nm]	Irradiance [µWatt/cm²]
VIS	460	420 - 680	455 - 645	2.49
NIR 860 nm	860	830 - 910	845 - 885	16.09
NIR 960 nm	960	890 - 1050	912 - 998	31.98

7.4.3.2 Phototactic experiments under NIR 860 nm light condition

In the second experiment, zebrafish larvae of 96 hpf or 120 hpf were illuminated with a light source with the peak illumination at 860 nm (LED type (850 nm): HE1-240AC, Harvatek Corp., Hsinchu City, Taiwan). The light source was covered with an IR filter (IR-filter 850 nm, Delamax, Germany) to eliminate visible light components (below 850 nm) and long wavelengths (above 910 nm) and to shift the maximum to the requested illumination (Table 7-1 and Figure S7-1). Due to the applied filter, the measured spectral profile ranged from 830 to 910 nm with a maximum absorption at 860 nm and has a light intensity of 16.09 µWatt/cm² (Table 7-1 and Figure S7-1). Therefore, we refer this light source as 860 nm source throughout the text. A thermal source was placed opposite to the NIR light source (see above) to provide thermal radiation only (Figure S7-1). We tested 30 larvae of each larval stage as described above.

7.4.3.3 Phototactic experiments under NIR 960 nm light condition

In the third experiment we used an NIR source with peak emission at 960 nm (LED type (940 nm): HE3-245AC, Harvatek Corp., Hsinchu City, Taiwan) which was equipped with an high-pass IR-filter at 950 nm (IR filter 950 nm, Delamax, Germany) to remove those visible light components with shorter wavelengths and to shift the maximum to the peak illumination of 960 nm (Table 7-1 and Figure S7-1). Due to the applied filter measured spectral profile ranged from 890 to 1050 nm with a maximum absorption at 960 nm, with a light intensity of 31.98 µWatt/cm² (Table 7-1 and Figure S7-1). Therefore, we refer this light source as a 960 nm source throughout the text. We tested 30 larvae of each larval stage as described above.

7.4.4 Behaviour analysis

Recordings of the zebrafish movements were analysed by a tracking software developed by the Institute of Real-Time Learning Systems, University of Siegen. The position of the larva (eye position) in the vessel was analysed every 2 s for 5 min and was performed manually through a marking between the eyes (Shcherbakov et al., 2012). According to Shcherbakov et al. (2013) the following default parameters were calculated: (I) swimming activity [%], with the maximum number of possible changes (= 150 within 300 s) in position (x- and y-value) in all analysed pictures set to 100 % within a 5 min test period; (II) absolute allocation time [s] on each side of the test vessel; (III) relative allocation time [%] to illustrate the preferred area in the test vessel; (IV) preferred head orientation in relation to the corresponding light source measured as mean angle [°] (Figure S7-2); and (V) head orientation as the length of the mean directional vector *R* as a scale of the concentration of data points around a circle (Pewsey et al., 2013). The mean head orientation, as a directional parameter, is a good factor to measure the location of circular data and it is correlated to the direction of the length of the mean directional vector *R* of the data (Pewsey et al., 2013). A value of $R = 1$ indicates, that all data points are located around the mean direction, a value near to 0 means evenly distributed data around the circle (Pewsey et al., 2013).

7.4.5 Data analysis

Statistical analyses were carried out using R 3.2.4 for windows (R Core Team, 2016). Before analysing allocation time, we defined an activity threshold excluding those larvae showing a swimming activity lower than 20 % in the vessel during the recording. The threshold was set due to the known freezing behaviour of zebrafish larvae. We wanted to avoid a bias in our results due to freezing larvae on one side of the vessel. To test for differences in swimming activity [%] within a larval stage and between larvae of the two different larval stages under different light sources, respectively, we used a non-parametric Kruskal-Wallis rank sum test followed by the Wilcoxon rank sum test for unpaired samples. To test for differences in allocation time (control side vs. NIR/VIS light side) within one larval stage we used a Wilcoxon signed rank test. All P-values are two tailed and were adjusted with Bonferroni correction. A Rayleigh test was performed to test directional uniformity, to analyse the mean directional vector (*R*) and to assess directional preferences of larvae to a light source. Significance level was set to $\alpha = 0.05$.

7.5 Results

7.5.1 Swimming activity under NIR and VIS light

The swimming activity of 96 hpf zebrafish larvae differed between the experiments with different light sources (Kruskal-Wallis test, χ^2 = 29.339, P ≤ 0.001, Figure 7-2). They showed a significantly higher swimming activity under exposure of 860 nm or 960 nm NIR light (52.83 and 54.38 %, respectively) than under exposure of VIS light (8.59 %) (Wilcoxon rank sum test for unpaired samples, 860 nm: W = 169.5, P ≤ 0.001; 960 nm: W = 102, P ≤ 0.001, Figure 7-2, left panel). The 120 hpf larvae showed at NIR 860 nm and 960 nm sources a swimming activity of 76.26 % and 74.65 %, respectively, therefore, the 96 hpf and 120 hpf old larvae did not differ significantly in swimming activity, neither at 860 nm (Wilcoxon rank sum test for unpaired samples, W = 602.5; P = 0.072, Figure 7-2) nor at 960 nm NIR light (Wilcoxon rank sum test for unpaired samples, W = 318; P = 0.155, Figure 7-2). Thus, the swimming activity of 120 hpf zebrafish larvae did not differ under the different light sources (Kruskal-Wallis test, χ^2 = 4.8308, P > 0.089, Figure 7-2).

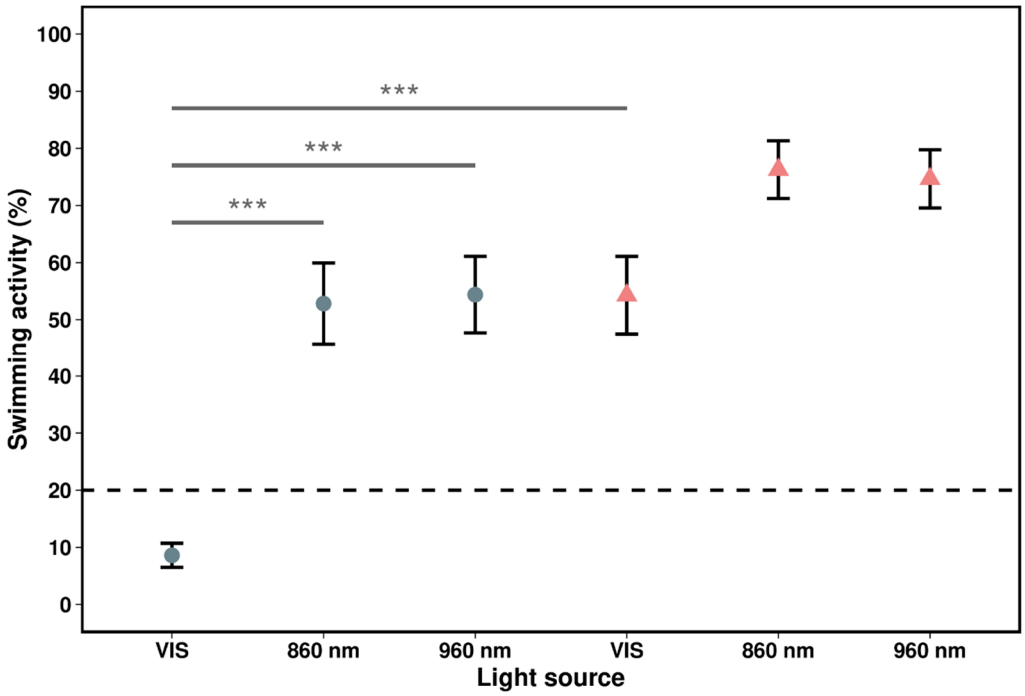


Figure 7-2: Swimming activity [%] of 96 hpf (blue circles) and 120 hpf (red triangles) zebrafish larvae under different light sources. The x-axis indicates the three tested light sources and the y-axis shows the swimming activity (%) of the zebrafish larvae. Shown are mean ± standard error. Sample size in each experiment: N = 30. *** P < 0.001. (VIS = visible light (blue-white-light, 440-700 nm, 860 nm = NIR light with peak illumination at 860 nm; 960 nm = NIR light with peak illumination at 960 nm).

Under VIS light, however, the mean swimming activity of 120 hpf zebrafish larvae was 54.27 % and thus 6.3 times higher than mean swimming activity of the 96 hpf larvae under the same light condition (Wilcoxon rank sum test for unpaired samples, W = 815; P ≤ 0.001, Figure 7-2).

7.5.2 Allocation time under NIR and VIS light

When a 860 nm NIR light source and the thermal source (control) were simultaneously presented opposite to each other, both larval stages spent significantly more time [s] on the control side with the thermal source than on the NIR half (Wilcoxon signed rank test, 96 hpf: V = 120; P ≤ 0.001; 120 hpf: V = 385; P ≤ 0.001, Figure 7-3 A + B). In contrast to this, we found no preference for either side in larvae of both larval stages, when exposed to 960 nm NIR light and the thermal source (Wilcoxon signed rank test, 96 hpf: V = 169.5, P = 0.691, 120 hpf: V = 216.5; P = 0.449, Figure 7-3 A + B). Under VIS light, the 120 hpf larvae spent significantly more time [s] on the side with the thermal source than on the side exposed with VIS light (Wilcoxon signed rank test, V = 211; P = 0.01, Figure 7-3 B). Due to a swimming activity below the pre-defined threshold we could not analyse allocation time [s] in 96 hpf larvae under VIS light (Figure 7-3 A).

When test vessels were divided into 24 sectors of 15 degrees each (Shcherbakov et al., 2013) to visualise the relative allocation time in the test vessel per sector, we found a similar pattern (Figure 7-4). Larvae of both larval stages spent more time on the control side when the light side was illuminated with VIS light and 860 nm NIR light (Figure 7-4 and 7-5). Larvae of both larval stages did not discriminate between both sides when the 960 nm NIR light source was provided in combination with the thermal source (Figure 7-4 and 7-5).

Table 7-2: Length of the mean directional vector *R*. Missing data result from activity level below threshold (for sample size see Figure 7-4 and 7-5).

light spectrum	Exposure from the left side		Exposure from the right side	
	96 hpf	120 hpf	96 hpf	120 hpf
VIS	-	0.32 $^{n.s}$	-	0.53 *
NIR 860 nm	0.75 ***	0.53 **	0.78 ***	0.81 ***
NIR 960 nm	0.67 **	0.29 $^{n.s.}$	0.55 *	0.37 $^{n.s}$

*P < 0.05; **P < 0.01; ***P < 0.001; n.s. = not significant.

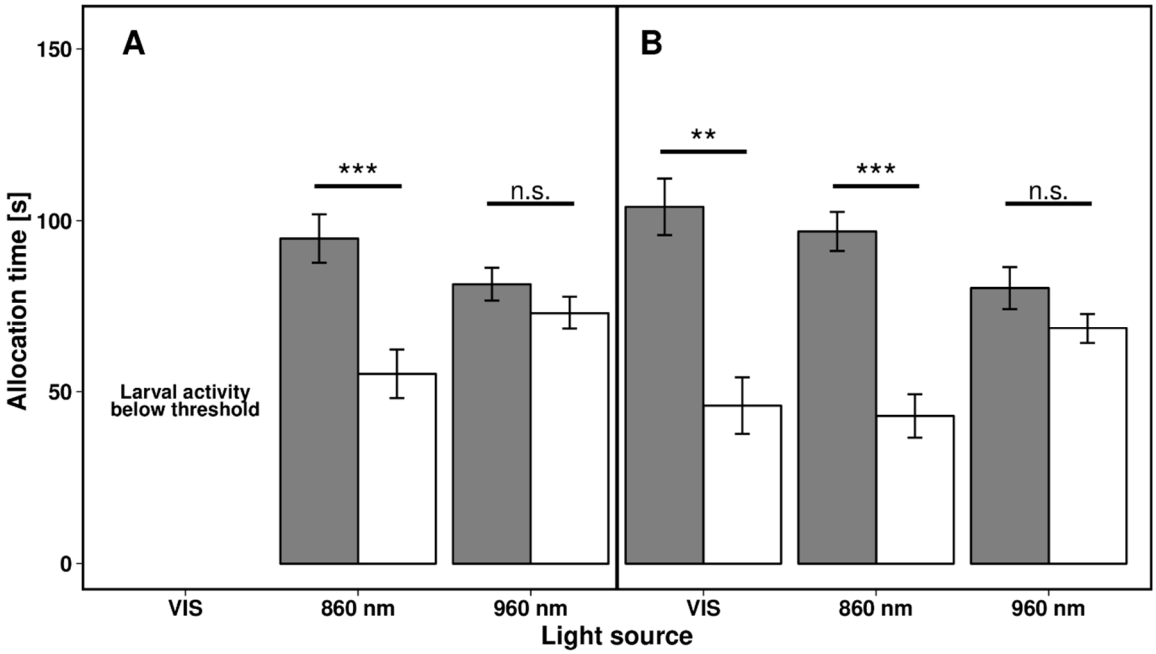


Figure 7-3: Mean allocation time [s] of both larval stages of zebrafish larvae under different light conditions. Bars present mean allocation time [s] ± standard error (which sum up to 150 s) of zebrafish larvae (A: 96 hpf, B: 120 hpf) in halves of vessels illuminated with NIR light (white bars) and radiated by the thermal control (dark grey bars) ($N_{96hpf/VIS}$ = 3, $N_{96hpf/860\ nm}$= 20, $N_{96hpf/960\ nm}$ = 22, $N_{120hpf\ /VIS}$ = 20, $N_{120hpf\ /860\ nm}$ = 28, $N_{120hpf\ /960\ nm}$ = 28). The x-axis represents the wavelengths of the three different used light sources (VIS (440 - 700 nm), 860 nm; 960 nm) and asterisks indicate significant differences compared to thermal control (**P < 0.01, ***P < 0.001, n.s. = not significant).

7.5.3 Head orientation

The 96 hpf and 120 hpf larvae directed their heads significantly more often towards the control side than to the 860 nm NIR light, irrespective of whether the thermal source was presented on the right or on the left side (Table 7-2; 96 hpf: R = 0.75, P < 0.001 (left), R = 0.78, P < 0.001 (right); 120 hpf: R = 0.53, P < 0.01 (left), R = 0.81, P < 0.001 (right)). Surprisingly, under NIR at 960 nm, the 96 hpf larvae showed also a clear preferred head position, since the head of the larvae was pointed significantly more often to the control side (Table 7-2; R = 0.67, P < 0.01 (left), R = 0.55, P < 0.05 (right)) whereas the head position of 120 hpf larvae did not point more often to the control side (Table 7-2; R = 0.29, P = 0.26 (left), R = 0.37, P = 0.12 (right)). Under VIS light head orientation of 120 hpf larvae pointed significantly more often to the control side when it was on the right but not when it was on the left (Table 7-2; R = 0.32, P = 0.35 (left); R = 0.53, P < 0.05 (right)). The analysis of the mean angle [°] did not provide additional information and is listed in supplemental material (Table S7-1).

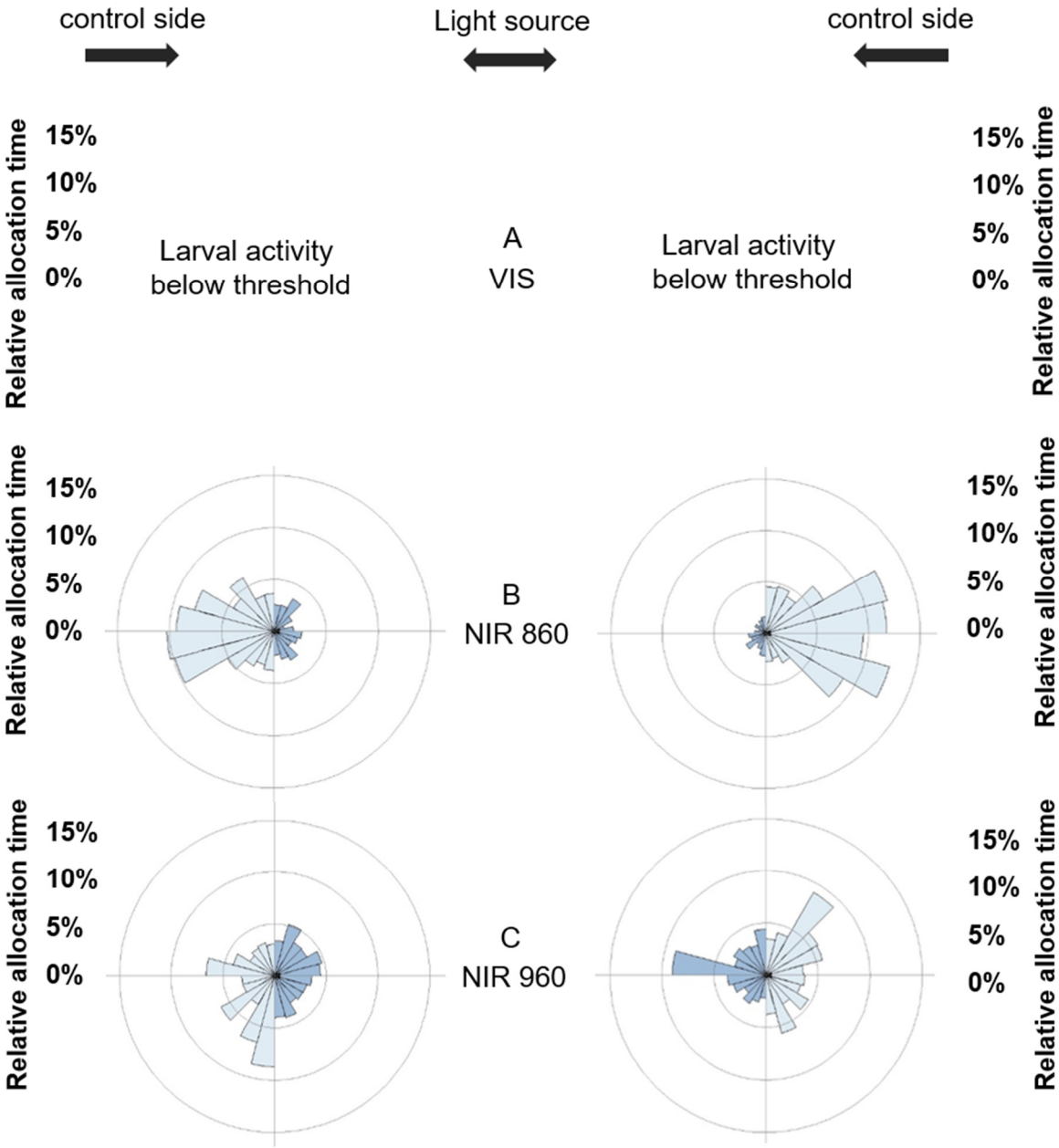


Figure 7-4: Sector diagram of allocation preference for 96 hpf zebrafish. The blue bars represent the mean allocation time [%] of zebrafish larvae in the test vessel under VIS light (A), NIR light at 860 nm (B) and under NIR light at 960 nm (C). The diagram is divided into 24 sectors, whereby each sector illustrates 15° (Left side: $N_{96hpf/VIS}$ = 2, $N_{96hpf/860\ nm}$= 10, $N_{96hpf/960\ nm}$ = 9; Right side: $N_{96hpf/VIS}$ = 1, $N_{96hpf/860\ nm}$ = 12, $N_{96hpf/960\ nm}$ = 11; except larvae below 20 % activity threshold).

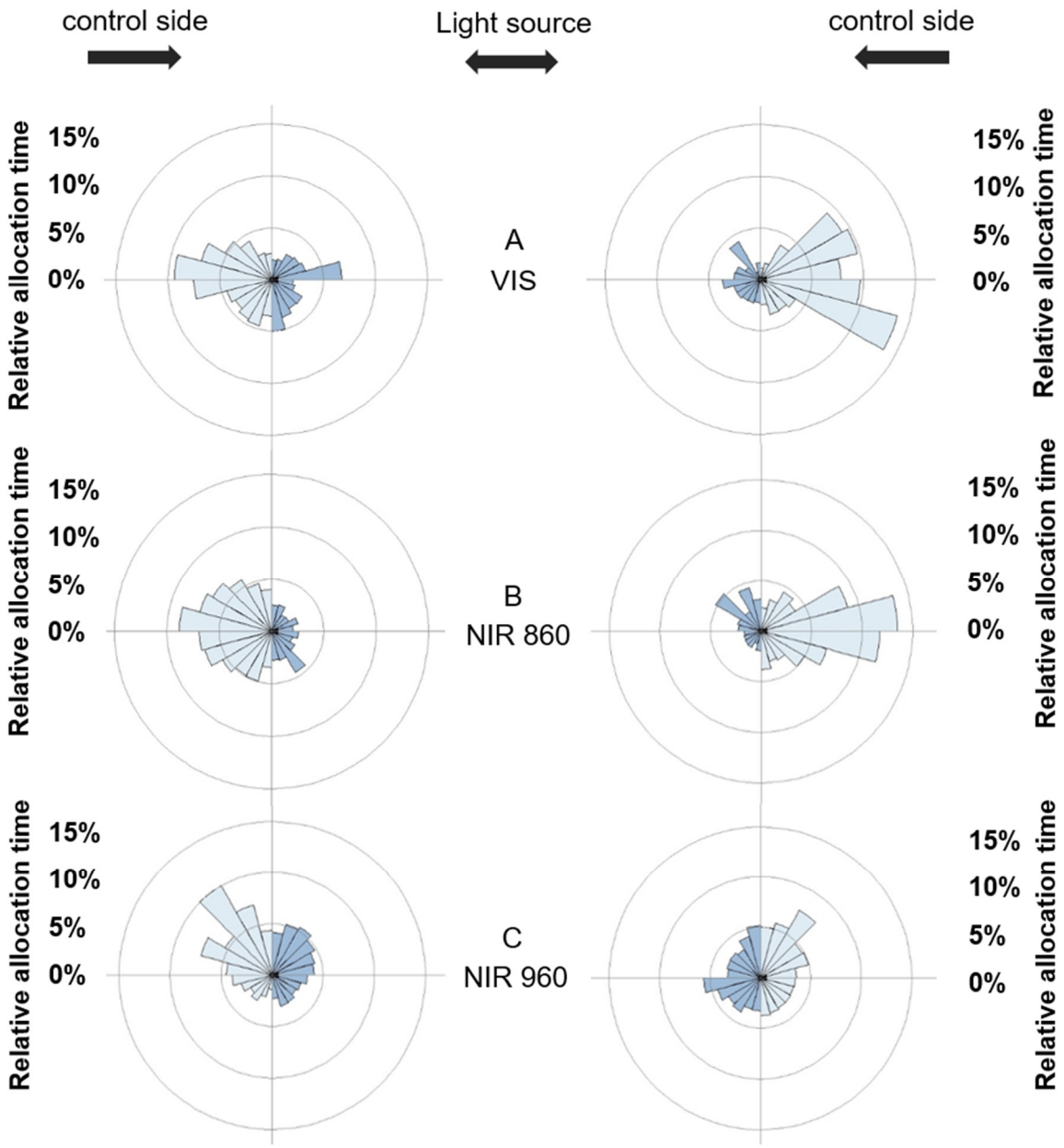


Figure 7-5: Sector diagram of allocation preference for 120 hpf zebrafish. The blue bars represent the mean allocation time [%] of zebrafish larvae in the test vessel under VIS light (A), NIR light at 860 nm (B) and under NIR light at 960 nm (C). The diagram is divided into 24 sectors, whereby each sector illustrates 15° (Left side: $N_{120hpf/VIS}$ = 10, $N_{120hpf/860\ nm}$ = 15, $N_{120hpf/960\ nm}$ = 15; Right side: $N_{120hpf/VIS}$ = 10, $N_{120hpf/860\ nm}$ = 13, $N_{120hpf/960\ nm}$ = 13; except larvae below 20 % activity threshold).

7.5.4 Temperature of light and thermal sources

The thermal source had a mean temperature of 30.36 ± 0.43 °C, the VIS light source had a mean temperature of 30.58 ± 0.45°C, and the 960 nm NIR light source had a mean temperature of 30.82 ± 0.44 °C (Table 7-3).The temperature of the 860 nm NIR light source was on average 33.28 ± 0.18 °C. The temperature of the thermal source differed from the one of the VIS light source by 0.22 °C, and from the NIR 960 nm light source by 0.46 °C. The difference between the 860 nm NIR and the thermal source was on average 2.92 °C.

The temperature measured at 5 positions inside the vessel did not differ neither under VIS (440 - 700 nm), NIR 860 nm, NIR 960 nm light source nor under the thermal source (Table S7-2).

Table 7-3: Mean temperature [°C] ± standard deviation of the used light sources (N = 30) and the temperature differences between the thermal source and the light source.

light spectrum	Mean temperature [°C] after the onset of 5 min (N = 30)	Difference between the thermal source and the corresponding light source [°C]
VIS	30.58 ± 0.45	0.22
NIR 860 nm	33.28 ± 0.18	2.92
NIR 960 nm	30.82 ± 0.44	0.46
Thermal source	30.36 ± 0.43	-

7.6 Discussion

We investigated whether zebrafish larvae of two different larval stages (96 hpf and 120 hpf) were sensitive to NIR light at a peak illumination of 860 nm or 960 nm, and to VIS light (440-700 nm), respectively. Regarding swimming activity (I), allocation time (II), relative allocation time (III), head orientation (IV), and length of the mean directional vector R (V), our results demonstrate that zebrafish larvae of both larval stages showed a clear negative phototactic response towards NIR light with a peak illumination at 860 nm and VIS light, but not to NIR light at 960 nm wavelength. Therefore, we conclude that zebrafish larvae are able to sense NIR light at 860 nm wavelength. This is the first time that a solid study was performed to discriminate the perception towards different NIR wavelengths in zebrafish larvae of two different larval stages. Thus, our findings are crucial to all

experiments using zebrafish larvae in standard testing procedures under NIR light conditions because most experimental devices use 850 nm light sources to provide a presumed dark environment (Liu et al., 2015).

In general, the response pattern to light conditions depends on the age of zebrafish larvae (Padilla et al., 2011, Colwill et al., 2011, de Esch et al., 2012). Younger larvae (96 hpf) are less active than older larvae (> 120 hpf) and the mean resting time decreased with increasing age (Colwill et al., 2011, de Esch et al., 2012). We found similar results in our experiments. The 120 hpf larvae showed a higher swimming activity under VIS light than the 96 hpf larvae (54.27 % versus 8.59 %). Due to the fact that the observed swimming activity in our study is similar to those found in previous studies (Padilla et al., 2011, Colwill et al., 2011, de Esch et al., 2012), the results based on our custom-built computer vision system were comparable to commercially available systems used in other studies.

Since all light sources emitted radiation, we simultaneously provided a non-illuminated thermal source to control for a possible effect of temperature on the swimming activity. The heat distribution within the test vessel due to emitted radiation of the thermal source did not differ from heat distribution due to radiation emitted by the VIS (440 - 700 nm), NIR 860 nm and NIR 960 nm light source (Table S7-2). No thermal gradient was detectable within the petri dishes based on the small size of the test vessel (35 mm diameter) and the distance to the light sources (10.5 cm) (Table S7-2). Thus, thermal radiation or a thermal gradient could not explain the observed behavioural differences in zebrafish larvae in our tests (Shcherbakov et al. 2013, Shcherbakov et al. 2012). Similar to previous studies that exclude thermotaxis as an explaining factor, there are indications that our allocation preferences were not due to thermal radiation but were based on the perception of NIR light possibly due to photoreceptors (Shcherbakov et al. 2013, Shcherbakov et al. 2012).

The hypothesis that zebrafish larvae are not sensitive to NIR light is based on the study with zebrafish larvae of 120-168 hpf by Brockerhoff et al. (1995). They detected no eye movement (optomotor response; OMR) of larvae fixed with a needle to the petri dish in response to illuminated rotating stripes (750 nm NIR light). Optomotor experiments using fixed fish, therefore, do not allow for properly analysing visual perception in fish. In our study, we used a state-of-the-art experimental set-up, which was designed to detect the NIR sensitivity of fish species under controlled conditions (Shcherbakov et al. 2012). Obviously, the set-up of the experiment plays an important role regarding visible sensitivity

of light conditions as shown by the following studies: Kobayashi et al. (2002) found no reaction to NIR over 800 nm by studying the OMR of two strains of Nile tilapia (*Oreochromis niloticus*). When the cardiac-conditioning technique was used, in which the fish learned to associate a NIR or green light stimulus (conditioning stimulus) with a mild electric shock (unconditioning stimulus), however, the same species showed a visual sensitivity to NIR over 850 nm (Matsumoto et al. 2005). Moreover, Shcherbakov et al. (2012) demonstrated the sensation of NIR light at a spectral range of 850 nm - 950 nm in Nile tilapia by using an appropriate behavioural experiment.

Adult zebrafish (6 months old) exhibit a positive phototaxis towards light sources with a maximum wavelength in the range of 845 - 910 nm NIR light (Shcherbakov et al., 2013). They spent 3.4 times more time in the half illuminated with NIR of 825 - 890 nm than in the non-illuminated control side. The same was true when adult zebrafish could choose between exposure to VIS light or no light. It is known that adult zebrafish show a different reaction to light than zebrafish larvae (Colwill and Creton, 2011a). These results are supported by our findings, because in contrast to adult zebrafish, zebrafish larvae exhibited a negative phototactic behaviour towards the side illuminated with NIR light at 860 nm wavelength. Shcherbakov et al. (2013) defined a threshold for NIR sensitivity in adult zebrafish of wavelengths up to 910 nm, as the exposure to longer wavelengths resulted in no behavioural response. Thus, adult zebrafish are able to react to NIR light of a range of 825 nm - 910 nm. We found similar findings in zebrafish larvae at 96 hpf and 120 hpf. They did not show a behavioural reaction to NIR 960 nm. The lack of response might be due to an underrepresentation of photoreceptors with a sensitivity to wavelengths longer than 910 nm (Shcherbakov et al. 2013). To investigate if the reaction is retina related or whether non-visual photoreceptors are involved, further experiments have to be conducted e.g. with blind or eyeless fish. Fernandes et al. (2012) showed that eyeless zebrafish larvae swam towards a light stimulus as light perception was mediated by deep brain photoreceptors. The authors identified neurons of the preoptic region of the hypothalamus as photoreceptors for dark photokinesis (Fernandes et al., 2012). Such findings are very important for the current study, since the mechanism for the sensitivity of NIR perception could not be clarified with our experimental set-up. The NIR perception in zebrafish larvae might be an adaptation to the characteristics of the natural preferred habitat (Meuthen et al., 2012, Shcherbakov et al., 2013). Fish species living in highly

transparent aquatic habitats often show a low sensitivity to NIR (Shcherbokov et al., 2013). In clear water, NIR light over 930 nm only passes through the water surface by a few cm, however, NIR light (between 806 - 847 nm) passes through water up to 2 m (Shcherbakov et al., 2013). *D. rerio* lives in shallow waterbodies around the Ganges and Brahmaputra river basins in north-eastern India, Nepal and Bangladesh (Spence et al., 2006; Spence et al., 2008). In their natural habitats, they are found in relatively clear waters with a depth up to 103 cm and a transparency higher than 15 cm (Lawrence, 2007; McClure et al., 2006; Spence et al., 2006). The preferences for this kind of water quality is correlated to a low NIR spectral sensitivity (Shcherbakov et al., 2013). As zebrafish inhabit slow moving and shallow waters, NIR wavelengths are presented in the natural habitat of this species and may have shaped the sensitivity of their visual system (Shcherbakov et al., 2013, Spence et al., 2006). The optical properties in the natural water habitat of zebrafish seem to correlate with the visual pigments and photo pigment spectral sensitivity in this fish species (Lythgoe, 1984; Wootton, 2012). Based on the ecological adaptation zebrafish have evolved in their natural habitats, it is not surprising that our study confirms that 96 hpf and 120 hpf zebrafish larvae are sensitive to NIR light at 860 nm.

7.7 Conclusion

Opposite to previous knowledge, our results provide evidence that 96 hpf and 120 hpf zebrafish larvae are sensitive to light of 860 nm wavelength within the NIR spectrum. They exhibited a clear negative phototaxis to an 860 nm light source and to VIS light. Our study is highly relevant to all studies using zebrafish larvae as test organism, because most of these studies are by default performed under NIR at 850 nm to mimic a dark environment. Thus, previous results should be re-interpreted due to the negative phototactic response in zebrafish larvae under NIR at 860 nm.

7.8 Supporting Information

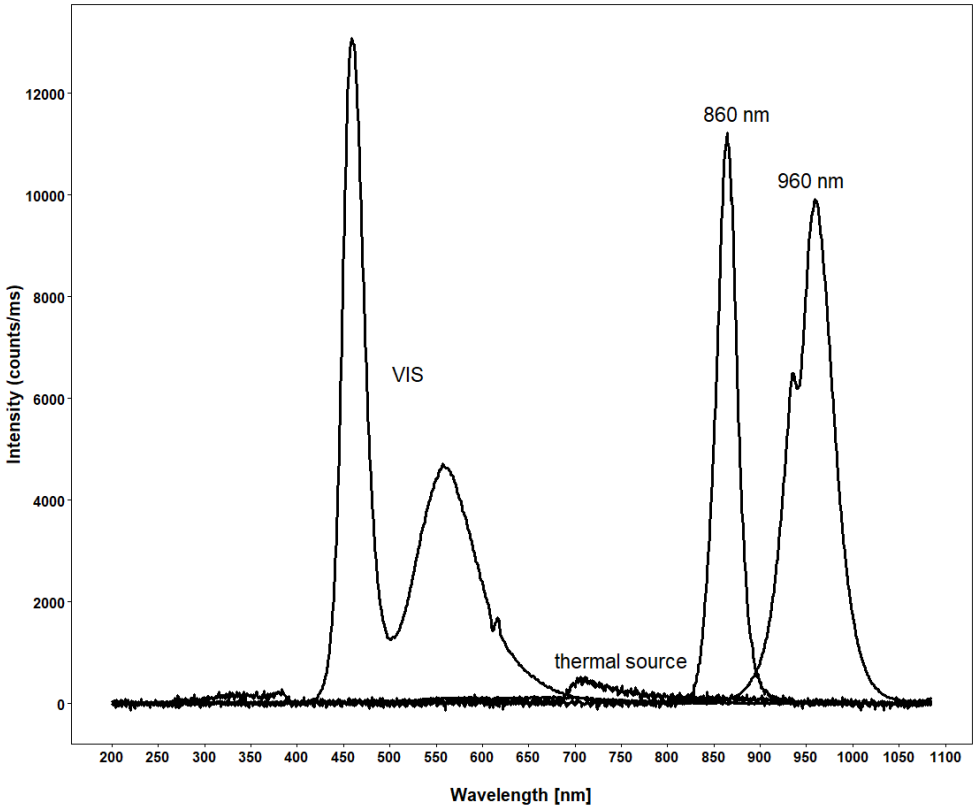


Figure S7-1: Profiles of the spectrum of the used VIS, NIR 860 nm and NIR 960 nm light sources and thermal source.

Table S7-1: Mean angle [°] of the fish head position regarding the side of the exposure.

Light source	Exposure from the left side		Exposure from the right side	
	96 hpf	120 hpf	96 hpf	120 hpf
VIS	-	206.33 ± 121.13	-	206.20 ± 130.56
IR 860 nm	167.50 ± 45.76	177.60 ± 146.67	185.17± 41.96	153.68 ± 160.63
IR 960 nm	174.11 ± 60.39	176.09 ± 124.52	152.67 ± 64.35	179.83 ± 132.36

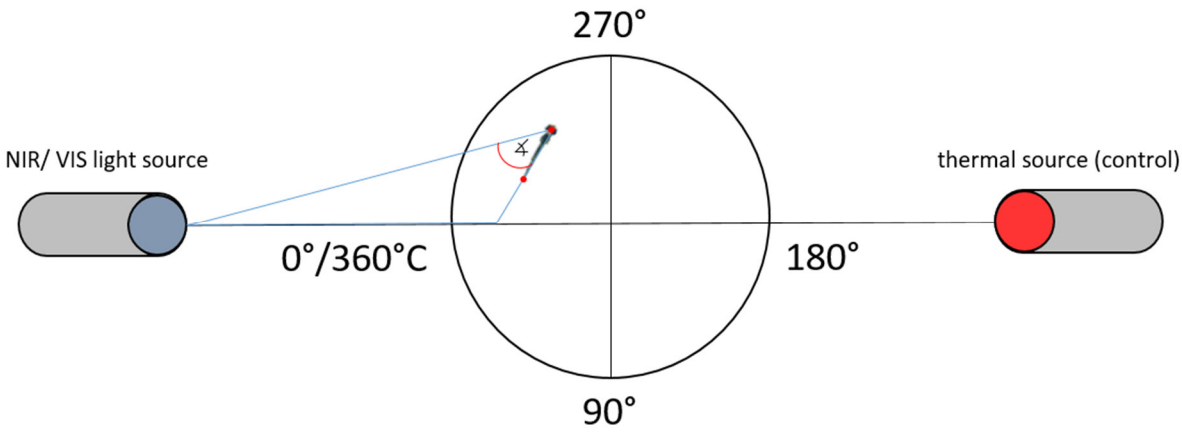


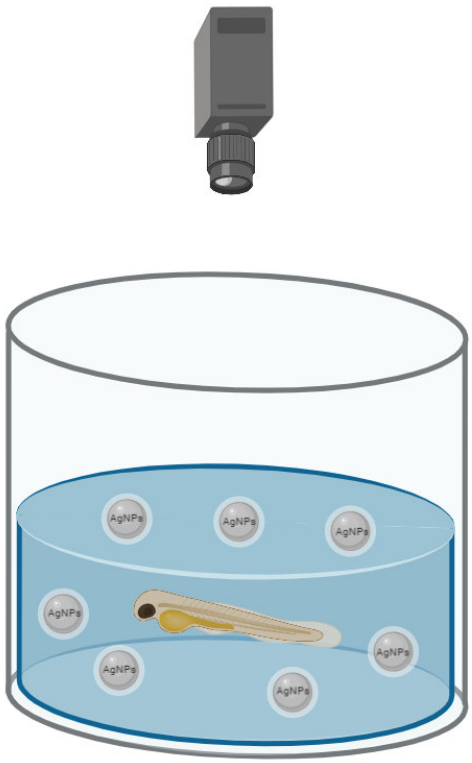
Figure S7-2: Graphic illustration of the determination of the position of the larva in the test vessel.

Table S7-2: Water temperature profiles at different positions within the test vessel (35 mm in diameter) 5 minutes after the onset of the respective light source.

Light source	exposed side	positions within the test vessel (mean + sd)				
		left	center	right	top	bottom
VIS	right	26.14 ± 0.14	26.30 ± 0.25	26.40 ± 0.15	26.42 ± 0.16	26.46 ± 0.16
	left	26.38 ± 0.16	26.06 ± 0.19	26.06 ± 0.11	25.98 ± 0.08	26.06 ± 0.11
IR 860 nm	right	26.16 ± 0.11	26.14 ± 0.11	26.18 ± 0.08	26.12 ± 0.08	26.06 ± 0.08
	left	26.28 ± 0.08	26.26 ± 0.13	26.12 ± 0.10	26.16 ± 0.12	26.18 ± 0.10
IR 960 nm	right	26.28 ± 0.09	26.24 ± 0.11	26.30 ± 0.18	26.32 ± 0.14	26.30 ± 0.12
	left	26.30 ± 0.11	26.26 ± 0.23	26.22 ± 0.16	26.22 ± 0.13	26.20 ± 0.18
IR 860 nm + filter	right	26.14 ± 0.11	26.12 ± 0.13	26.08 ± 0.08	26.10 ± 0.07	26.04 ± 0.15
	left	26.24 ± 0.09	26.18 ± 0.13	26.14 ± 0.08	26.10 ± 0.00	26.12 ± 0.08

Chapter 8

The impact of pristine and wastewater-borne AgNPs on the locomotion behaviour of zebrafish larvae



Anna-Katharina Rauschert from the University of Siegen helped to collect some of the data analysed in this chapter. Darya Mozhayeva provided the analytical data analysed in this chapter.

8.1 Abstract

The increasing use of engineered nanoparticles (NPs) in various consumer products leads to a rising risk for harmful effects of aquatic environment. NPs, such as silver nanoparticles (AgNPs), undergo several transformation processes as they pass sewage treatment plants (STPs) and mostly enter the aquatic environment as sulphide species with reduced bioavailability for aquatic organisms. The aquatic vertebrate *Danio rerio* (zebrafish) is a widely used test species in behavioural ecotoxicology. Automated tracking systems can be used to record movement patterns of zebrafish larvae and produce behavioural-related endpoints, which are helpful for the identification of toxicologically induced behavioural changes. In this study, we investigated if and how the locomotion behaviour of zebrafish larvae (96 hpf (hours post fertilisation)) were affected by environmental realistic concentrations of wastewater-borne AgNPs. The effects of pristine AgNPs (NM-300K) on total swimming distance [mm] and velocity [mm/s] were measured with the common light/dark-transition and compared to those caused by wastewater-borne AgNPs. The exposure with pristine AgNPs indicated the strongest behavioural changes for both investigated endpoints with a significant hypoactivity compared to the control group. These results confirm that the exposure with pristine AgNPs leads to neurotoxic dysfunction during the development of zebrafish larvae. In contrast, the exposure with wastewater-borne AgNPs did not lead to any abnormal behaviour pattern. The current study shows that the exposure scenario is crucial for assessing behavioural effects of nanoprticles, and that the consideration of transformation processes are essential for a reliable risk assessment of NPs.

8.2 Introduction

In the previous chapter we showed the influence of AgNPs to the behaviour and important life cycle parameters to aquatic invertebrates, with *Daphnia magna* as model species. Here, we focused on potential effects of pristine and wastewater-borne AgNPs on vertebrates by using the zebrafish *Danio rerio* as model species. The assessment of the ecotoxicological impact of AgNPs on all tropic levels within the aquatic ecosystem, including invertebrate and vertebrates, is important as it is known that AgNPs can accumulate within the food web, implying a potential risk for humans (Zhang et al., 2019).

The toxic potential of pristine AgNPs to aquatic organisms along the aquatic food chain, including algae, zooplankton and fish, have already been studied (Walters et al., 2014; Zhang et al., 2019). The release of silver ions (Ag^+) from the surface of AgNPs is addressed to be the most important mechanism of toxicity of AgNPs leading to DNA damage, oxidative stress due to reactive oxygen species (ROS) formation or cell membrane permeability (Guo et al., 2019; Zhang et al., 2019). AgNPs cause a disruption of ion regulation due to a reduction of Na^+/K^+ ATPase. This is particularly important for fish, since the main uptake route is via the gills, gut epithelia and skin (Handy et al., 2008; Walters et al., 2014). AgNPs can accumulate in the fish brain, gill and liver tissue (Asharani et al., 2008; Scown et al., 2010), lead to defects in fin regeneration (Yeo and Pak, 2008), increasing the oxidative stress response in gills (Scown et al., 2010) and affect gene expression profiles related to the central nervous system (CNS) development (Xin et al., 2015).

However, studies investigating the ecotoxicological impact of NPs for fish are still limited (Walters et al., 2014). The most common fish species used for aquatic toxicity testing is the zebrafish *Danio rerio* (Pereira et al., 2019) which is recommended for the embryo toxicity test (FET) (OECD, 2013). Benefits, like easy culture conditions, high egg production, fast embryo development (Kimmel et al., 1995) and the similarity of zebrafish´s genetic material to humans and other vertebrates (Guo, 2009; Howe et al., 2013), make it favourable as a model organism in ecotoxicology and other research areas. Furthermore, the research with zebrafish embryos is a good alternative method compared to acute toxicity testing using adult fish, due to a robust correlation of the obtained sensitivity (Lammer et al., 2009). Using zebrafish larvae is in line with the 3R-principles (Replacement, Reducement, Refinement) and therefore not restricted by the Animal Protection Law. In addition to the investigation of the acute and chronic toxicity of chemical compounds, the monitoring of locomotion behaviour serves as a powerful tool for the assessment of chemicals (Gruber et al., 1994; Legradi et al., 2015). The analysis of behavioural changes leads to a 10-100 times higher sensitivity to acute and chronic LC_{50} values (Gerhardt, 2007). Due to technical progression, high-throughput tracking systems allow the recording of behavioural-related endpoints of zebrafish larvae in order to identify possible toxic effects of environmental pollutants.

Recently, only a few researchers focused on the investigation of behavioural-related effects of zebrafish larvae triggered by AgNPs under realistic environmental conditions, including transformation processes and environmentally relevant concentrations. For instance, a significant concentration-dependent hyperexcitability in zebrafish was found after the development exposure to AgNPs in the range of expected environmental AgNP levels (González et al., 2018). Contrary, Ašmonaité et al. (2016) found a reduced swimming distance (hypoactivity) after exposure with low concentrations of AgNPs in developing zebrafish (0 hpf until 120 hpf). So far, to the best of our knowledge, no studies are available that take transformation processes of AgNPs while passing a STPs before they enter the aquatic environment into consideration, while evaluating the ecotoxicological potential. This is essential for a reliable risk assessment for nanoparticles, demonstrated by studies with several other aquatic invertebrate species. The toxicity of AgNPs significantly decreased in comparison to pristine AgNPs while passing a model STP (Georgantzopoulou et al., 2018; *Hartmann et al., 2019*; Kühr et al., 2018). Our multi-generational study over six generations of the freshwater invertebrate *Daphnia magna* showed that wastewater-borne AgNPs do not affect important life cycle parameters, including mortality and reproduction. Pristine AgNPs however, led to a decrease in the reproductive success in a dose-response pattern (*Hartmann et al., 2019*). The acute and chronic toxicity was reduced when the freshwater crustaceans *D. magna* and *Hyalella azteca* were exposed with wastewater-borne AgNPs (Georgantzopoulou et al., 2018; Kühr et al., 2018).

In the present study we, therefore, aimed to evaluate the ecotoxicological potential of pristine AgNPs and wastewater-borne AgNPs on the locomotion behaviour of zebrafish larvae based on important behavioural-related endpoints like velocity and total swimming distance. This experimental approach with transformed Ag allows a more realistic assessment of the toxicity of AgNPs to aquatic organisms and their environment

8.3 Materials and Methods

8.3.1 Study species

In this study, we used the tropical freshwater fish *Danio rerio* as the test organism. The conditions of the zebrafish facility at the Witte lab and the procedure of the egg collection are described in detail in Chapter 2.2.2. For further testing, fertilized and healthy fish eggs

with a cell stage of a least 4 – 8 were chosen (OECD, 2013). Prior to exposure, the selected fish eggs were transferred, using a pipette, into 60 mL crystallisation vessels (60 x 35 mm²) containing 50 mL formulated water (ISO-medium; 294 mg/l $CaCl_2 \cdot 2\ H_2O$, 123.3 mg/l $MgSO_4 \cdot 7\ H_2O$, 63 mg/l $NaHCO_3$, 5.5 mg/l KCl).

8.3.2 Silver nanoparticles

As AgNPs we used the NM-300K particles (polyvinylpyrrolidone- (PVP-) coated) from the OECD Working Party on Manufactured Nanomaterials (WPMN) Sponsorship (Klein et al., 2011). Detailed information of the chemical properties of NM-300K are given in Chapter 3.3.1. For the preparation of the test media, a working stock with a nominal concentration of 100 mg AgNPs /L was prepared in ISO-medium. The AgNP-free stabilization agent NM-300K DIS was used as the dispersant control. A dispersant stock solution was prepared with a nominal concentration of 100 mg NM-300K DIS/L.

8.3.3 Model sewage treatment plant (STP)

The method for the model sewage treatment plant (STP) and the procedure of the production of effluent enriched with AgNPs is described in detail in Chapter 5.3.3. In total, one model STP was run without AgNPs and four model STPs with AgNPs in May 2017. The effluent was collected and stored under the same conditions as described in Chapter 5.3.3. The respective nominal inlet concentrations of the STP units and the measured total Ag concentration are given in Table 8-2. The analysis of total Ag concentrations of the effluents enriched with AgNPs were collected and prepared for ICP-MS analyses. For details on the experimental procedure see Chapter 3.7 and Chapter 5.3.6.

8.3.4 Collection and determination of total Ag concentrations of effluent and test media

Aqueous test samples of the STP effluents were collected directly after each run. A single set (N = 1) of media samples from the behavioural study were taken from the freshly prepared media (fresh media) and after 96 h at the end of the experiments (aged media) to verify the concentrations of total Ag in the different treatments.

The determination of total Ag concentration in STP effluents and in media samples was done by ICP-MS (iCAP Qc, Thermo Fisher Scientific, Bremen, Germany). Sample preparation and measurement conditions are explained in detail in Chapter 5.3.6. The instrumental parameters of ICP-MS used in this study are shown in Table S8-1.

8.3.5 Video Tracking System

For the locomotion experiments with zebrafish larvae we used the same video tracking system as described in detail in Chapter 7.4.3 with a modified experimental set-up. All experiments were performed in a climate-controlled room with a temperature of 26 ± 1°C. We constructed an IR-backlit holder for multi-well plates (12 x 18.5 x 18 cm^3) to record the movement of the zebrafish larvae in the 24-well plates during day and night cycles. To simulate night cycles (NIR light), the backlit light source was equipped with LED lights with a peak illumination at 960 nm (Type: IR-Emitter 940 nm 30° 5mm, Kingbright, Taiwan). Zebrafish larvae are unable to perceive wavelength up to 960 nm (*Hartmann et al., 2018*) thus, darkness is imitated. The light source for the day simulation (visible (VIS) light) was equipped with white LED lights (Type: NSPW515DS Sel. b2V/W LED White, Nichia, Japan) with a peak illumination at 460 nm and 560 nm. The backlit light source was provided with a switch, so that a shifting between light (day) and dark (night) could take place during the experiments. A high-quality IR-sensitive camera (Manta G-235C, Allied Vision, Stadtroda, Germany) was fixed 100 cm above the 24-well plate. Two plexiglass panes served as a diffuser. Furthermore, to reduce heating, the backlit light source was cooled from below with a fan. The video tracking system was connected to a PC to start and control the experimental settings. The recordings of the behaviour of zebrafish larvae were analysed with a tracking software developed by the Institute of Real-Time Learning Systems, University of Siegen. We calculated total distance [mm] and velocity [mm/s] of unexposed and exposed zebrafish larvae as endpoints for movement.

8.3.6 Behavioural assay

8.3.6.1 General test design

To analyse changes in the locomotion behaviour of zebrafish larvae based on the exposure of pristine AgNPs or wastewater-borne AgNPs we used the well described method of light/dark transition testing (Legradi et al., 2015). During light conditions, the activity of zebrafish larvae is relatively low while turning off the light leads to a rapidly increase in the larval activity (Ali et al., 2012; de Esch et al., 2012; Emran et al., 2008; Padilla et al., 2011). No standard guideline exists regarding the dark and light time periods of the light/dark transition test and varies among studies (Legradi et al., 2015). Therefore, we used a customized study protocol and each of the experiments began with a ten-minute

acclimation phase to minimize water movements and effects of the handling procedure. Based on our own preliminary tests, the selected protocol of the light/dark transition test followed a six interchanging dark (NIR light): light cycle (VIS light) over 10 minutes each for a total time period of 60 min (Figure 8-1). The most stable natural activity of zebrafish larvae is between 12:00 PM and 04:00 PM (Vignet et al., 2013). Therefore, all experiments were performed within this time frame. Only one concentration was tested per day.

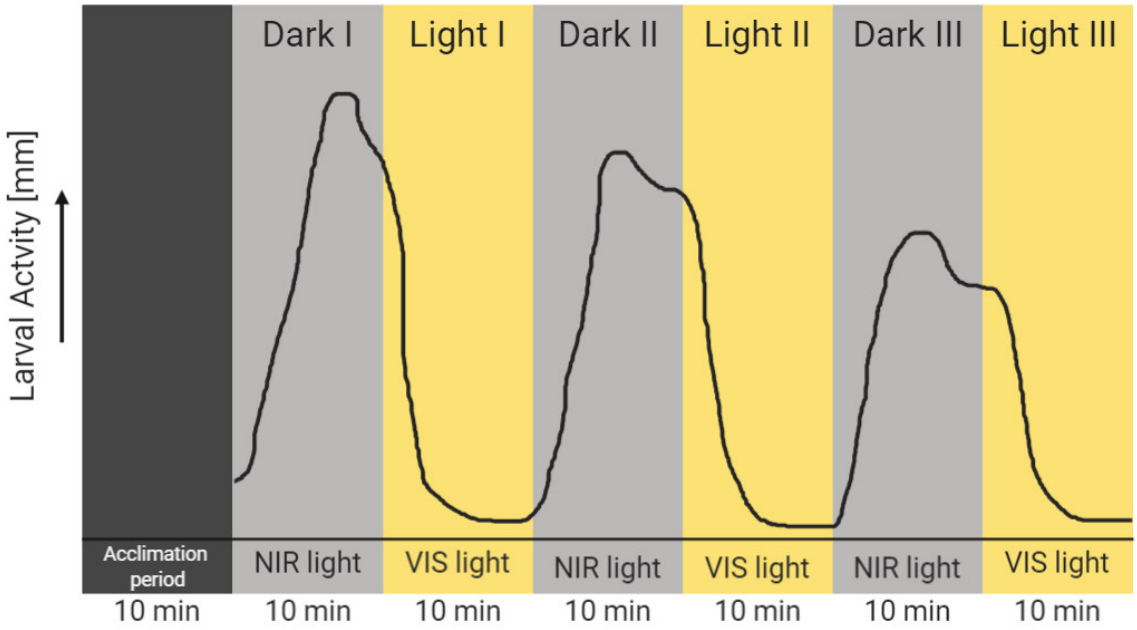


Figure 8-1: The experimental setup of the behavioural assay with a standard larval activity [mm] of untreated zebrafish larvae. The cycle of each experiment starts with 10 min acclimation period (dark grey panel), followed by a 10 min dark phase (light grey panel) and a 10 min light phase (yellow panel), which was repeated three times. The total recording time was 60 min. The black line in the graph shows the activity pattern of an untreated control fish.

The selected fish eggs were exposed to the respective treatment in the prepared crystallisation vessels and were afterwards covered with Parafilm® (Parafilm, Menasha, WI, USA) to prevent evaporation and maintained on a 14:10 light/dark cycle and incubated at 26 ± 1°C for 96 h until further usage. No water exchange took place during the experiments. Dead or abnormal embryos were removed once a day to avoid bacterial infection of the living organisms. After 96 h of exposure, the free swimming (hatched) zebrafish larvae were transferred with a plastic pipette in the morning of the tracking experiments to a 24-well plate (TPP Techno Plastic Products AG, Trasadingen, Switzerland). To generate high quality recordings, six exposed larvae and two unexposed larvae, which serve as internal plate controls, were individually placed into 200 mL test

media in one well of the test vessels as shown in Figure 8-2. In total, we performed four independent trails (replicates), resulting in 24 zebrafish larvae per treatment. At the end of the test, the larvae were anesthetized in accordance with animal welfare regulations.

8.3.6.2 Experiment 1: pristine AgNPs

The exposure with pristine AgNPs was performed with ISO-medium spiked with NM-300K (AgNPs). The experiment included the following treatments: ISO-medium control (control), a dispersant control (DIS) and five Ag-treatments (Ag-1 to Ag-5) with different concentrations of AgNPs (Table 8-1). The used concentrations are based on the predicted environmental concentrations (PEC) in freshwater systems which are calculated as 0.088 to 10.000 ng/L (Gottschalk and Nowack, 2011; Maurer-Jones et al., 2013). All zebrafish were exposed in the 4- to 8-cell stage, 1 hour post fertilisation (hpf).

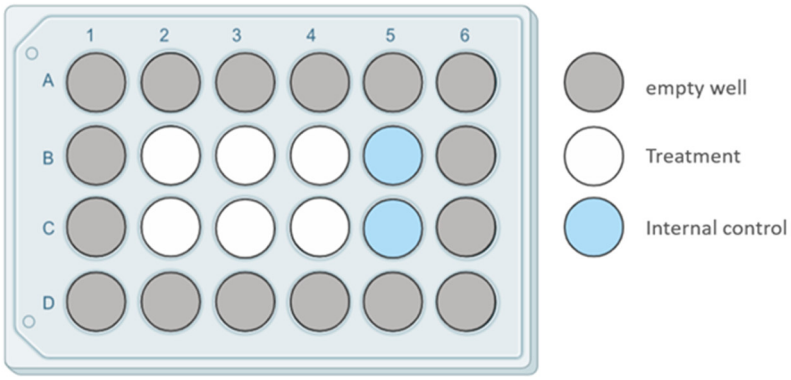


Figure 8-2: Layout of the 24-well plate for analysing the locomotion behaviour of zebrafish larvae.

8.3.6.3 Experiment 2: wastewater-borne AgNPs

The collected effluents from the model STP were diluted with ISO-medium based on the measured total Ag concentration of the effluent to achieve the respective test concentrations similar to Experiment 1. Prior to use, the effluents were shaken for two minutes to obtain a homogenous suspension. The effluent from the model STP unit without AgNPs served as the control treatment (STP-control) and was diluted with the lowest dilution factor used for the treatment preparation (Table 8-2). We tested five treatments with different concentrations of wastewater-borne AgNPs (Table 8-1, STP-1 to STP-5). Since only four model STPs were available, the treatments STP-4 and STP-5 were set

with the same effluent but different dilution factors. All zebrafish embryos were exposed, in the 4- to 8-cell stage, 1 hpf.

8.3.7 Statistical analysis

The raw data of the behaviour tracking was sorted individually for each zebrafish larva and total values for every parameter of locomotion was estimated per second. Based on these values, the average and total response for each dark/light cycle was calculated and analysed (Ašmonaitė et al., 2016). Changes in the locomotion behaviour of zebrafish larvae due to AgNPs exposure was analysed separately in each dark/light cycle and in the combined dark/light phase by using linear mixed effect (LME) models the *lmer* function of the package "*lme4*" (Bates et al., 2014) with total distance [mm] or velocity [mm/s] as the outcome variable. Treatment was included as fixed factor and the identity of test fish (ID) as the random factor. We used the statistics program R version 3.5.0 for Windows (R Core Team, 2016) for all analyses.

Table 8-1: Overview of the performed experiments with the corresponding treatments and the respective total Ag concentration [µg/L + U] measured with ICP-MS (N = 1, n = 10).

Experiment	Treatment	measured total Ag concentration ± U [µg/L]	
		fresh media	aged media
Exp. 1: pristine AgNPs	Control	< 0.19	< 0.19
	DIS	< 0.19	0.28 ± 1.21
	Ag-1	49.93 ± 6.74	15.95 ± 2.37
	Ag-2	62.38 ± 8.35	36.79 ± 5.02
	Ag-3	70.39 ± 9.30	41.39 ± 5.47
	Ag-4	85.68 ± 11.23	49.71 ± 6.43
	Ag-5	88.39 ± 11.65	51.91 ± 6.76
Exp. 2: wastewater-borne AgNPs	STP-control	< 0.19	< 0.19
	STP-1	41.58 ± 5.70	19.88 ± 2.90
	STP-2	67.01 ± 8.78	11.63 ± 1.96
	STP-3	151.03 ± 19.22	41.85 ± 5.45
	STP-4	187.21 ± 24.43	79.28 ± 10.31
	STP-5	262.96 ± 91.07	56.23 ± 7.43

U= uncertainty; N=number of biological replicates; n= number of technical replicates

Table 8-2: Preparation of test media for exposure with wastewater-borne AgNPs (Exp. 2) (N = 1, n = 10).

Experiment	Treatment	Nominal sewage inlet Ag conc. [mg/L]	Effluent total Ag conc. [µg/L] ± U	Effluent dilution factor
Exp. 2: wastewater-borne AgNPs	STP-control	-	0.26 ± 0.16	1:1.10
	STP-1	1	58.15 ± 2.25	1:1.16
	STP-2	5	133.61 ± 6.87	1:1.34
	STP-3	2.5	164.39 ± 7.30	1:1.10
	STP-4	6.5	302.69 ± 10.23	1:1.51
	STP-5	6.5	302.68 ± 10.23	1:1.21

U= uncertainty; N=number of biological replicates; n= number of technical replicates

8.4 Results

8.4.1 Total Ag concentration in STP effluent and test media samples

The analysis of the total Ag content of the STP effluent confirms previous studies, which found out that 90 – 95 % of Ag was removed during the clarification process of a STP and ends up in wastewater biomass (*Hartmann et al., 2019*; Kaegi et al., 2011). The results of the total Ag concentration in the STP effluent and in test media samples are given in Table 8-2. The total Ag content of the fresh samples of ISO-control, DIS-control and STP-control were below < 0.19 µg/L, whereby the treatments with pristine Ag achieved concentrations of 49.93 µg/L (Ag-1), 62.38 (Ag-2), 70.39 µg/L (Ag-3), 85.68 (Ag-4) and 88.39 (Ag-5). The Ag concentration of the effluent spiked with AgNPs was even higher with 41.58 µg/L (STP-1), 67.01 µg/L (STP-2), 151.03 µg/L (STP-3), 187.21 µg/L (STP-4) and 262.96 µg/L (STP-5). The analysis of the Ag concentration after 96 h (aged medium) showed a major decrease in all test media samples.

8.4.2 Behavioural assay

8.4.2.1 Distance

8.4.2.1.1 Experiment 1: pristine AgNPs

Zebrafish larvae exposed to the dispersant control DIS swam a significantly higher total distance [mm] within Dark II and Dark III. Thus, the effects of pristine AgNPs were analysed in comparison to DIS and no combination of DIS and ISO-Control was done. The

strongest response to the exposure with pristine AgNPs was found in Dark III, where all treatments showed a significant lower total distance [mm] in comparison to DIS (LMER: for test statistic see Table S8-2; Figure 8-3 A). The swimming distance of larvae exposed in Dark I and Light I did not differ. We found some significant differences in total distance [mm] in the phases Dark II, Light II and Light III but no constant pattern. For example, larvae exposed to 49.93 µg/L or 85.68 µg/L swam a lower total distance [mm] in Light II compared to DIS (LMER: for test statistic see Table S8-2; Figure 8-3 A).

With the combined dark cycles (I-III) fish larvae moved a smaller total distance [mm] in the control group in comparison to DIS (LMER: Estimate: -262.52; t = -2.712, P = 0.007; Figure 8-3 A). The same could be observed in the treatment with 49.93 µg/L (LMER: Estimate: -232.33; t = -2.400, P = 0.017) and 85.68 µg/L (LMER: Estimate: -216.26; t = -2.234, P = 0.026). Total distance [mm] did not differ between pristine AgNPs and DIS in the combined light cycle (Figure 8-3 B).

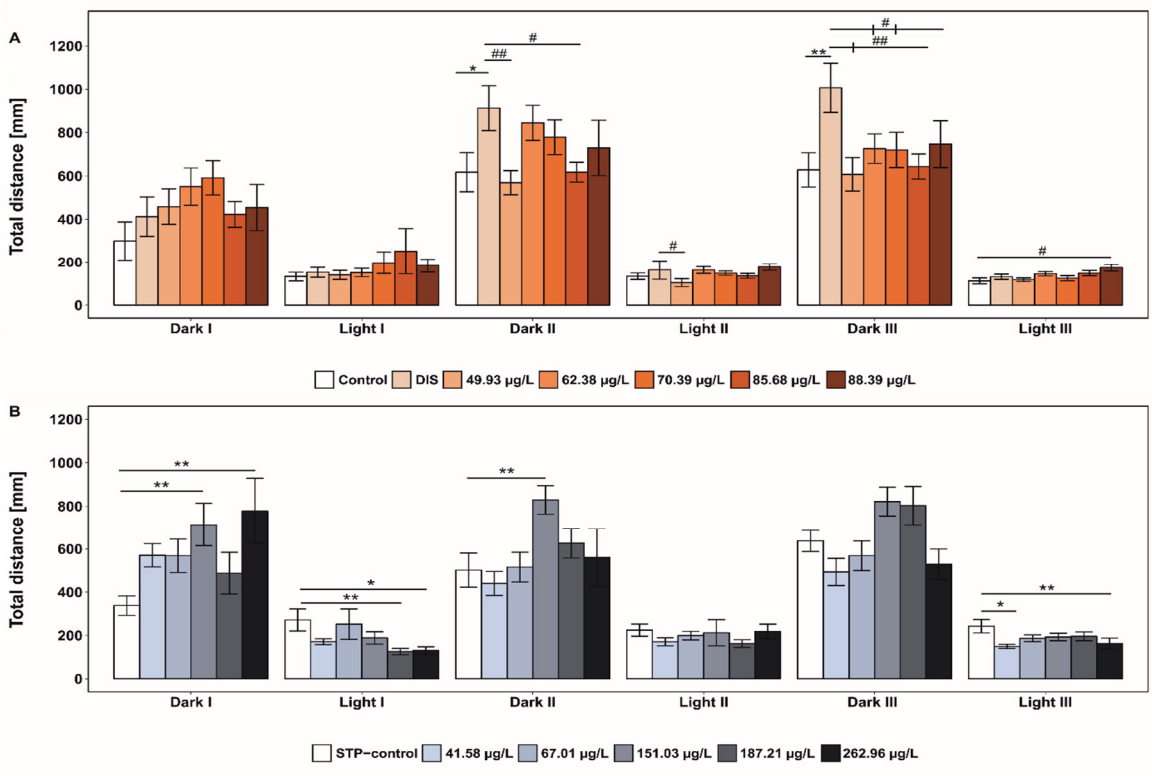


Figure 8-3: Total distance [mm] of zebrafish larvae ($n_{for\ each\ treatment}$ = 24) exposed to pristine AgNPs (Experiment 1, panel A) and wastewater-borne AgNPs (Experiment 2, panel B) separated in each dark and light cycle. * indicates significant differences between control and DIS. # shows differences between treatments groups and DIS in Experiment 1.

8.4.2.1.2 Experiment 2: wastewater-borne AgNPs

Zebrafish larvae exposed to wastewater-borne AgNPs showed no consistent behavioural response for the endpoint total distance [mm] (Figure 8-3 B). In Dark I, Light II, Dark II and Light III we found significant differences, but more inconsistently without any clear dose-response pattern. For example, treating larvae with 151.03 µg/L (LMER: Estimate: 376.324; t = 2.837, P = 0.005) and 262.96 µg/L (LMER: Estimate: 440.187, t = 3.318, P = 0.001) in Dark I led to a significant higher distance in comparison to STP-control (Figure 8-3 B). However, this pattern could not be confirmed for Dark II and Dark III. No differences were found for Light II and Dark II. We detected differences in total distance [mm] for combined dark cycles for zebrafish larvae exposed to 151.03 µg/L wastewater-borne AgNPs compared to STP-control (LMER: Estimate: 295.867, t = 3.185, P = 0.001; Figure 8-4 C). The analysis of the combined light cycles indicated significantly lower total distance [mm] values for treating larvae with 41.58 µg/L (LMER: Estimate: -82.824, t = -2.810, P = 0.005), 187.21 µg/L (LMER: Estimate: -85.026, t = -2.885, P = 0.004), and 262.95 µg/L (LMER: Estimate: -75.503, t = -2.562, P = 0.011) wastewater-borne AgNPs (Figure 8-4 D).

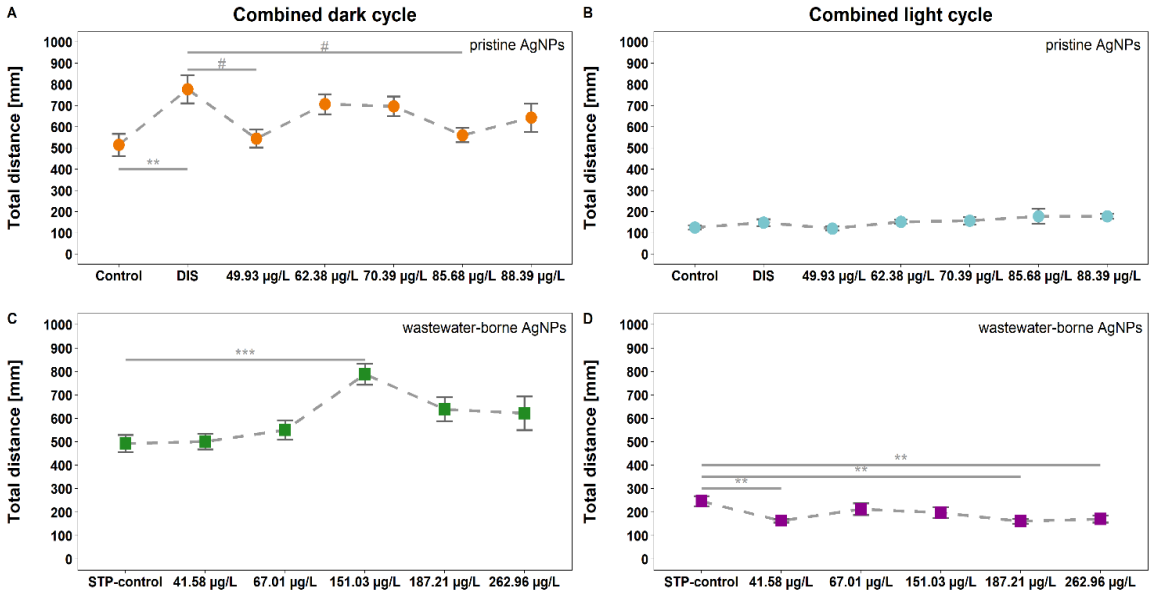


Figure 8-4: Total distance [mm] of zebrafish larvae (n=24) exposed to pristine AgNPs (Experiment 1, panel A + B) and wastewater-borne AgNPs (Experiment 2, panel B + D) for combined dark (A + C) and light (B + D) cycle. * indicates significant differences between control and DIS (Experiment 1) or STP-control and treatments (Experiment 2), respectively. # shows differences between treatments groups and DIS for Experiment 1.

8.4.2.2 **Velocity**

8.4.2.2.1 Experiment 1: pristine AgNPs

The velocity [mm/s] differed significantly between the control and DIS in Dark II, Dark III and in the combined dark cycles (LMER: for test statistic see Table S8-2, Figure 8-5 A). Hence, pristine AgNPs treatments were compared with DIS to identify differences. In Dark III, zebrafish larvae in all treatments with pristine AgNPs showed a significantly lower velocity compared to DIS (LMER: for test statistic see Table S8-2, Figure 8-5 A). Some significant, but rather inconsistent, effects were found for treating larvae with 49.93 µg/L (LMER: Estimate: -0.436, *t* = -0.623, P = 0.016) and 85.68 µg/L (LMER: Estimate: -0.436, *t* = -0.623, P = 0.016) in Dark II and for 85.68 µg/L (LMER: Estimate: -0.436, *t* = -0.623, P = 0.016) of pristine AgNPs in Light III. However, no clear correlation between the increasing concentration of pristine AgNPs and the response pattern could be identified. Velocity [mm/s] of exposed zebrafish larvae was not affected in Dark I, Light I and Light II. The analysis of the combined dark cycle showed that zebrafish larvae of 49.93 µg/L (LMER: Estimate: -0.436, *t* = -0.623, P = 0.016) and 85.68 µg/L (LMER: Estimate: -0.388, *t* = -2.631, P = 0.032) pristine AgNPs swam with a lower velocity [mm/s] compared to animals with DIS. During light cycles, pristine AgNP treatments had no effect on swimming velocity in zebrafish larvae (Figure 8-6 B).

8.4.2.2.2 Experiment 2: wastewater-borne AgNPs

The exposure to wastewater-borne AgNPs led to significantly lower swimming speed in Dark I, Light I, Dark II, Dark III and Light III but no dose-response pattern could be found. In the treatments 41.58 µg/L (LMER: Estimate: 1.099, *t* = 3.819, P < 0.001), 151.03 µg/L (LMER: Estimate: 0.592, *t* = 2.059, P = 0.041) and 262.96 µg/L (LMER: Estimate: 0.730, *t* = 2.537, P = 0.012) of wastewater-borne AgNPs zebrafish larvae moved faster compared to those in STP-control in Dark I (Figure 8-5 B). However, for Dark II this could only be shown for 151.03 µg/L (LMER: Estimate: 0.440, *t* = 2.186, P = 0.030) wastewater-borne AgNPs. No differences were found in Light II (Figure 8-5 B).

Combining all dark cycles showed that zebrafish larvae in treatment with 151.03 µg/L (LMER: Estimate: 0.442, *t* = 2.533, P = 0.012) and 187.21 µg/L of wastewater-borne AgNPs (LMER: Estimate: 0.354, *t* = 2.032, P = 0.044) moved significantly faster compared to those in the STP-control (Figure 8-6 C). When all light cycles are considered zebrafish larvae moved significantly slower when treating with 41.58 µg/L (LMER: Estimate: -0.161

t = -2.974, P = 0.003), 187.21 µg/L (LMER: Estimate: -0.165, *t* = -3.039, P = 0.002) and 262.96 µg/L of wastewater-borne AgNPs (LMER: Estimate: -0.148, *t* = -2.737, P = 0.007) compared to those in the STP-control (Figure 8-6 D).

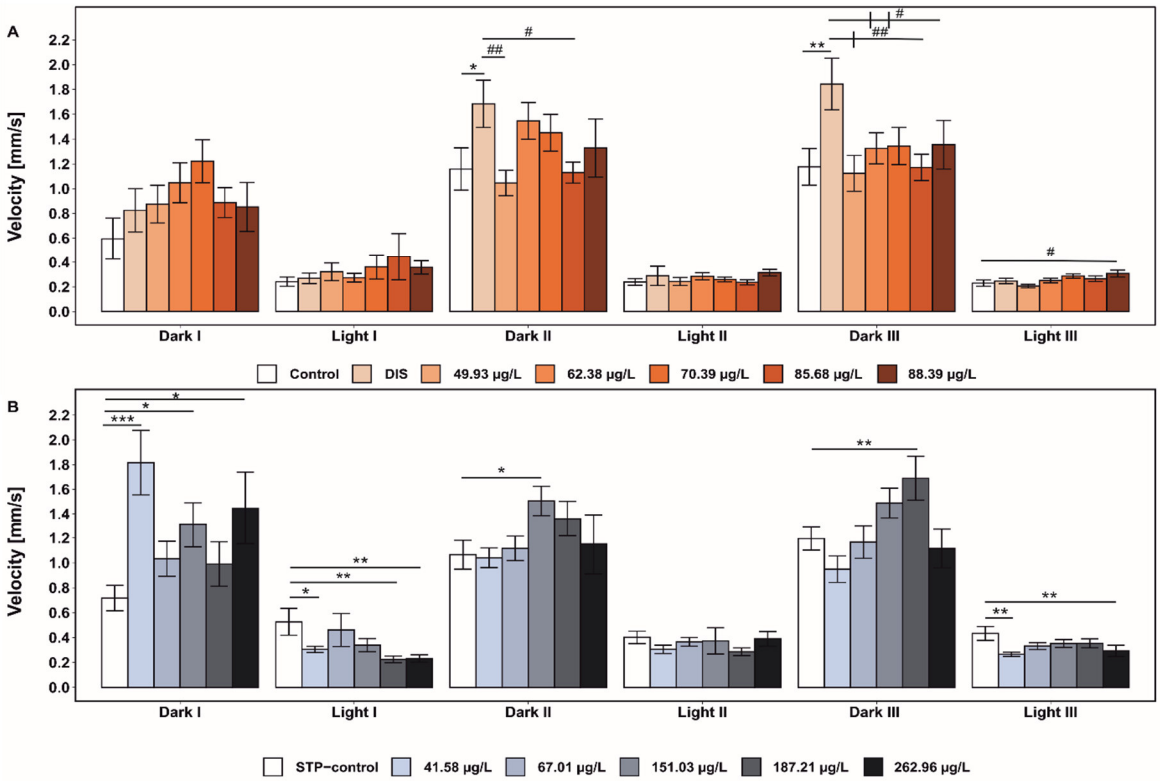


Figure 8-5: Velocity [mm/s] of zebrafish larvae ($n_{for\ each\ treatment}$ = 24) exposed to pristine AgNPs (Experiment 1, panel A) and wastewater-borne AgNPs (Experiment 2, panel B) separated for each dark and light cycle. * indicates significant differences between control and DIS. # shows differences between treatments groups and DIS for Experiment 1.

8.5 Discussion

This study focussed on the ecotoxicity of wastewater-borne AgNPs to the locomotion behaviour of zebrafish larvae in comparison to pristine AgNPs at environmentally realistic concentrations according to Maurer-Jones et al. (2013). Zebrafish larvae treated with wastewater-borne AgNPs showed no dose-response pattern for the endpoints total swimming distance and velocity, neither under Dark nor under Light conditions. However, this study shows that low concentrations of pristine AgNPs cause a significant hypoactivity in zebrafish larvae. Our study provides evidence that low concentrations of pristine AgNPs in the range of realistic environmental conditions lead to changes of the locomotion behaviour of zebrafish larvae.

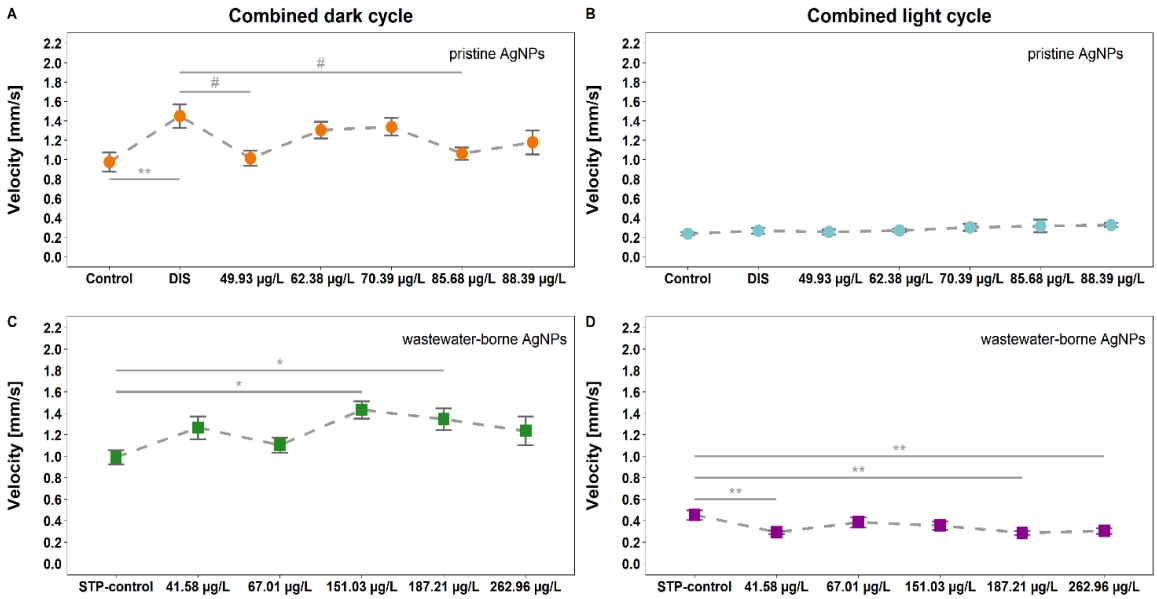


Figure 8-6: Velocity [mm/s] of zebrafish larvae ($n_{\text{for each treatment}}$=24) exposed to pristine AgNPs (Experiment 1, panel A + B) and wastewater-borne AgNPs (Experiment 2, panel B + D) for combined dark (A + C) and light (B + D) cycle. * indicates significant differences between control and DIS (Experiment 1) or STP-control and treatments (Experiment 2), respectively. # shows differences between treatments groups and DIS for Experiment 1.

Behaviour is one of the most sensitive endpoints in assessing the toxicity of chemicals to aquatic vertebrates such as the zebrafish (Gerhardt, 2007). In our study, the AgNPs were stabilized with two dispersing agents to improve the solubility and to reduce the agglomeration potential of NPs. The locomotion activity of larvae treated with DIS was significantly affected, with increasing swimming distance, compared to the ones exposed to ISO-medium (control). Lipophilic dispersant agents can increase the toxicity of NPs when they interfere with the lipophilic lipid bilayer of the cell membrane and thus promote the penetration of NPs into the cell (Deng et al., 2017; Handy et al., 2012). An ecotoxicological study with *Daphnia magna* indicated an increased toxicity of the dispersing agent compared to the culture medium ASTM by analysing the swimming behaviour of the animals *(Galhano et al., Under Review),* which supports the results of the current study. The possibility that the dispersant may have a direct influence on the toxicity mechanism of NPs cannot be excluded and therefore may have possible effects on aquatic organisms. (Handy et al., 2012). In this study, all effects were therefore rated compared to DIS since all AgNP treatments were stabilized with the dispersant agents, and consequently the effects of the AgNPs cannot be considered alone.

It is known that the locomotion activity of zebrafish larvae is disrupted by concentrations of AgNPs that cause no morphological abnormalities, no mortality or delayed hatching rate (González et al., 2018). The current study confirms these findings, since zebrafish larvae exposed to environmentally relevant concentrations of AgNPs show a significant hypoactivity for total distance and velocity during Dark III, while the 48-h-LC_{50} value for AgNPs (NM-300K) is 1.26 mg/L (Muth-Köhne et al. 2013). González et al. (2018) were able to show that the strongest behavioural response towards AgNPs is delayed and visible at a later recording time. They found a significant hyperactivity of zebrafish larvae at 3 dpf when exposed to 300 µg/L, 1 mg/L and 3 mg/L AgNPs (25 nm; stabilized with alginate). Interestingly, both, hyper- and hypoactivity as a result of low-concentration exposure of chemicals can be found in the literature. For example, exposure to ethanol (Irons et al., 2010), lead (Chen et al., 2012) or bisphenol A (Saili et al., 2012) triggered hyperactivity, while studies show abamectin (widely used insecticide) (Raftery and Volz, 2015), TiO_2NPs (Chen et al., 2011) or AgNPs (Ašmonaitė et al., 2016) indicate hypoactivity. Ašmonaité et al. (2016) found that zebrafish larvae follow a dose-response pattern while significantly decreasing the total swimming distance at concentrations > 470 µg/L during dark phases. Similar results were found in the current study with 10 times lower concentrations. To the best of our knowledge, this is the first study showing that low concentrations of AgNPs, which are related to PEC values, lead to an impairment of the swimming behaviour of zebrafish larvae at an age of 96 hpf. The ecological relevance of behavioural changes caused by neurotoxic dysfunctions is important, for instance, for mating or escape behaviour (Legradi et al., 2015). However, linking changes in the behaviour of zebrafish with neurotoxicity is very difficult and complex (Walker, 2003). A study with zebrafish larvae showed that AgNPs act as neurobehavioral disruptors and interfere with neuronal cell replication and differentiation leading to negative effects on survival rate, morphology and behaviour (Powers et al., 2011). Furthermore, the exposure to gold nanoparticles (AuNPs) in zebrafish larvae leads to clear changes in swimming behaviour due to visual impairments as a result of eye defects (Kim et al., 2013). The authors reported injured axon development in AuNP exposed fish and suppose a relationship between behavioural changes and neurotoxic effects since the disruption of axons and neurons caused by chemicals during the embryonic development is a key driver of neuronal connectivity and leads to neurobehavioral abnormalities (Kim et al., 2013;

Sylvain et al., 2010; Yang et al., 2011). However, within the present study, neither gene expression analysis of the neuronal system nor immunohistochemical tools were used to observe molecular and morphological impairments of zebrafish larvae. To confirm the hypothesis, that the behavioural effects of AgNPs observed in this study are linked to neural damage, analyses such as the uptake of Ag in tissues are essential. In general, NPs can enter the blood brain barrier (BBB) of rats due to their small size (< 100 nm) (Oberdörster et al., 2004), thus the used NPs are a potential candidate to cause neurotoxic damage in the larval brain of zebrafish. The BBB of zebrafish is similar to those in mammals and developed between 3 dpf and 10 dpf (Fleming et al., 2013). While we exposed the zebrafish larvae with AgNPs at 1 hpf, the BBB does not act as a physical barrier for NPs. Additionally, the protective function of the embryo's chorion (Kimmel et al., 1995) is reduced by AgNP exposure as they are able to penetrate into the chorion of zebrafish embryos (Lee et al., 2007). Hence, further and detailed research is needed to confirm the hypothesis that AgNPs injured the nervous system of zebrafish larvae and affected the locomotion behaviour of *D. rerio*.

This study aims to compare the toxicity of pristine AgNPs to those induced by wastewater-borne AgNPs. The present study found that wastewater-borne AgNPs only minor affect the locomotion activity of 96 hpf zebrafish larvae indicating a decreased toxicity in contrast to pristine AgNPs. The study of Muth-Köhne et al. (2013) reported a significantly lower 48 hpf-EC_{50} value for zebrafish embryo toxicity for wastewater-borne AgNP from a lab-scale STP in comparison to pristine AgNPs. The observed EC_{50} value of 142 µg/L for wastewater-borne AgNPs resembled the toxicity of silver nitrite ($AgNO_3$) with an EC_{50} of 73 µg/L (Muth-Köhne et al., 2013), one of the most toxic compounds for aquatic organisms (Ratte, 1999). However, these results could not be confirmed by the current study even though the study by Muth-Köhne et al. (2013) did not investigate any behavioural endpoints. Both studies used the OECD reference material NM-300K as AgNPs and exposed the embryos to a similar concentration range (41.58 µg/L – 262.96 µg/L in the current study compared to 90 µg/L - 270 µg/L in the study of Muth-Köhne et al. 2013). These two very important factors of nanomaterial research cannot be taken as an explanation for the contradictory results of the two studies. Especially for the model organism *D. rerio*, age-related and genetic factors could influence the zebrafish behaviour to a high extent (MacPhail et al., 2009; Padilla et al., 2011). The present study and Muth-

Köhne et al. (2013) exposed embryos at 4 – 8 cell stages to wastewater-borne AgNPs and kept the same genetic strain of zebrafish, thus age-related and genetic factors can also be excluded. However, the hypothesis of Muth-Köhne et al. (2013), that the exhaustion of sulphide and chloride in the sewage sludge is a possible factor for the increased toxicity of wastewater-borne AgNPs, cannot be used as an explanation for the results of the current study. Based on microscope technique, the characterisation of AgNPs in the effluent of STP showed that AgNPs are co-localized with sulphur and are present as Ag_2S (Adam et al., 2018; *Hartmann et al., 2019*; Kaegi et al., 2011; Ma et al., 2014). The study of *Galhano et al. (Under Review)* showed that wastewater-borne AgNPs do not affect the behaviour of the aquatic invertebrate *Daphnia magna*. The swimming height and the time spent within specific areas within the test vessel of *D. magna* were not affected while treating with low concentration (25 µg/L – 125 µg/L) of wastewater-borne AgNPs. Furthermore, the results of the current study are in accordance with several other studies, indicating that the toxicity of AgNPs to aquatic and terrestrial organisms decreased after passing a model STP (Georgantzopoulou et al., 2018; *Hartmann et al., 2019*; Kampe et al., 2018; Kühr et al., 2018). Based on the physical-chemical properties of Ag_2S, the reduced release of Ag^+ and the low water solubility (Bianchini et al., 2002; Kaegi et al., 2011; Levard et al., 2012; Ratte, 1999), the transformation process during passage through a STP is the most plausible and responsible factor of the observed reduced toxicity of wastewater-borne AgNPs to the locomotion activity of zebrafish larvae. Hence, the ecotoxicological potential of wastewater-borne AgNPs towards the natural behaviour of *Danio rerio* and the resulting ecological interference of fish can be considered as low. Nevertheless, *Galhano et al. (Under Review)* indicated, that biochemical markers can act as important warning traits for the detection of negative effects caused by low-concentrated wastewater-borne AgNPs (25 µg/L – 125 µg/L) at the subcellular level *(Galhano et al., Under Review).* To date, effects on lipid peroxidation, oxidative stress, cytotoxic effects and energy metabolism have been identified for several aquatic species because of NP-exposure (Hackenberg et al., 2011; Klaper et al., 2009; Metzler et al., 2012; Yang et al., 2009). Thus, the suggestion of an integrated approach, combining behaviour related endpoints and biochemical markers *(Galhano et al., Under Review)*, may be useful to get a more realistic picture of the ecotoxicological potential of wastewater-borne NPs to aquatic and terrestrial organisms.

8.6 Conclusion

This study affirms that the analysis of the locomotion behaviour of zebrafish larvae under chemical exposure acts as a sensitive endpoint to detect negative effects. However, there was a clear difference in response pattern between pristine AgNPs, where a hypoactivity of the locomotion activity was observed, and wastewater-borne AgNPs that did not have any effect. Thus, this provides further evidence that the risk of wastewater-borne AgNPs to aquatic organisms and their environment is lower than assumed when investigating the toxicity of pristine AgNPs under environmentally relevant conditions. Therefore, performing ecotoxicological studies under realistic conditions, such as environmentally relevant concentrations and transformation processes, is essential for a reliable risk assessment of NPs, as also suggested by *Hartmann et al. (2019).* In addition, there is currently no standard test guideline for testing the effects of chemicals on the locomotion activity of adult and larval *Danio rerio,* although they act as an important model organism in biological science, especially in ecotoxicology. For a better comparability and the relevance of further studies, the development of a test guideline, like OECD TG or ISO norm, is really important and should be aimed for. However, further research is needed to investigate the neurotoxic potential of wastewater-borne AgNPs and the potential long-term impact they may have on population dynamics.

8.7 Supporting Information

Table S8-1: Main ICP-MS Instrumental Parameters.

	ICP-MS iCAp Qc (Thermo Fisher Scientific, Bremen, Germany)
Nebulizer	C400d (Savillex, Eden Prairie, MN, USA)
Spray chamber	Peltier-cooled cyclonic quartz
Radio-frequency power	1550 W
Torch injector inner diameter	2.5 mm
Cooling flow	14 L/min
Auxiliary flow	0.8 L/min
Nebulizer flow	1.1 or 1.0 L/min
Sampling position	5 mm
Dwell time	10 ms
Number of sweeps	30

Table S8-2: LMER estimates for the effects on Total distance [mm] of *Danio rerio* larvae after the exposure of pristine AgNPs. Significant differences (* P < 0.05, ** P < 0.01, *** P < 0.001) within the performed Dark and Light cycles and for separately combined Dark and Light cycles compared to DIS are marked in bold. DIS = dispersant control, control = ASTM control, df = degrees of freedom, *t* = test statistics.

	Treatment	Estimate	Std. Error	df	t	P
Dark I	(Intercept)	411.831	85.780	161.00	4.800	**< 0.001**
	control	-112.933	121.312	161.00	-0.930	0.353
	Ag-1	46.445	121.312	161.00	0.382	0.702
	Ag-2	138.722	121.312	161.00	1.143	0.254
	Ag-3	179.391	121.312	161.00	1.478	0.141
	Ag-4	10.6683	121.312	161.00	0.087	0.930
	Ag-5	42.061	121.312	161.00	0.346	0.729
Light I	(Intercept)	151.634	48.505	161.00	3.126	**0.002**
	control	-19.618	68.597	161.00	-0.285	0.775
	Ag-1	-11.809	68.597	161.00	-0.172	0.863
	Ag-2	-1.160	68.597	161.00	-0.016	0.986
	Ag-3	46.086	68.597	161.00	0.671	0.502
	Ag-4	99.357	68.597	161.00	1.448	0.149
	Ag-5	31.958	68.597	161.00	0.465	0.641
Dark II	(Intercept)	912.965	87.361	161.00	10.450	**< 0.001**
	control	-295.802	123.547	161.00	-2.394	**0.017**
	Ag-1	-344.121	123.547	161.00	-2.785	**0.006**
	Ag-2	-67.830	123.547	161.00	-0.549	0.583
	Ag-3	-133.954	123.547	161.00	-1.084	0.279
	Ag-4	-295.956	123.547	161.00	-2.395	**0.01**
	Ag-5	-183.736	123.547	161.00	-1.487	0.138
Light II	(Intercept)	162.853	21.144	161.00	7.701	**< 0.001**
	control	-29.238	29.903	161.00	-0.977	0.329
	Ag-1	-59.259	29.903	161.00	-2.981	**0.049**
	Ag-2	-0.093	29.903	161.00	-0.003	0.997
	Ag-3	-15.030	29.903	161.00	-0.502	0.615
	Ag-4	-27.770	29.903	161.00	-0.928	0.354
	Ag-5	14.005	29.903	161.00	0.468	0.640

Table S8-2: Continued.

Dark III	(Intercept)	1006.739	85.756	161.00	11.739	**< 0.001**
	control	-378.812	121.277	161.00	-3.123	**0.002**
	Ag-1	-399.311	121.277	161.00	-3.292	**0.001**
	Ag-2	-280.820	121.277	161.00	-2.315	**0.021**
	Ag-3	-286.753	121.277	161.00	-2.364	**0.019**
	Ag-4	-363.465	121.277	161.00	-2.996	**0.003**
	Ag-5	-259.971	121.277	161.00	-2.143	0.033
Light III	(Intercept)	130.664	12.145	161.00	10.758	**< 0.001**
	control	-18.881	17.176	161.00	-1.099	0.273
	Ag-1	-13.441	17.176	161.00	-0.782	0.435
	Ag-2	13.739	17.176	161.00	0.799	0.425
	Ag-3	-6.359	17.176	161.00	-0.370	0.711
	Ag-4	16.888	17.176	161.00	0.983	0.327
	Ag-5	42.717	17.176	161.00	2.486	**0.013**
All Dark	(Intercept)	777.18	68.44	161.00	11.356	**< 0.001**
	control	-262.52	96.79	161.00	-2.712	**0.007**
	Ag-1	-232.33	96.79	161.00	-2.400	**0.017**
	Ag-2	-69.98	96.79	161.00	-0.723	0.470
	Ag-3	-80.44	96.79	161.00	-0.831	0.407
	Ag-4	-216.26	96.79	161.00	-2.234	**0.026**
	Ag-5	-133.88	96.79	161.00	-1.383	0.168
All Light	(Intercept)	148.384	18.779	161.00	7.902	**< 0.001**
	control	-22.580	26.557	161.00	-0.850	0.396
	Ag-1	-28.170	26.557	161.00	-1.061	0.290
	Ag-2	4.162	26.557	161.00	0.157	0.876
	Ag-3	8.232	26.557	161.00	0.310	0.757
	Ag-4	29.492	26.557	161.00	1.113	0.267
	Ag-5	-22.580	26.557	161.00	-0.850	0.396

TableS8-3: LMER estimates for the effects on Total distance [mm] of *Danio rerio* larvae after the exposure of wastewater-borne AgNPs. Significant differences (* P < 0.05, ** P < 0.01, *** P < 0.001) within the performed Dark and Light cycles and for separately combined Dark and Light cycles compared to DIS are marked in bold. DIS = dispersant control, control = ASTM control, df = degrees of freedom, *t* = test statistics.

	Treatment	Estimate	Std. Error	df	t	P
Dark I	(Intercept)	337.860	93.796	138.00	3.602	**< 0.001**
	STP-1	232.342	132.648	138.00	1.751	0.082
	STP-2	230.223	132.648	138.00	1.735	0.084
	STP-3	376.324	132.648	138.00	2.837	**0.005**
	STP-4	149.909	132.648	138.00	1.130	0.260
	STP-5	440.187	132.648	138.00	3.318	**0.001**
Light I	(Intercept)	271.373	38.885	138.00	6.978	**< 0.001**
	STP-1	-100.612	54.992	138.00	-1.829	0.069
	STP-2	-19.629	54.992	138.00	-0.356	0.721
	STP-3	-82.971	54.992	138.00	-1.508	0.1336
	STP-4	-145.963	54.992	138.00	-2.654	**0.008**
	STP-5	-140.912	54.992	138.00	-2.562	**0.011**
Dark II	(Intercept)	501.752	82.920	138.00	6.051	**< 0.001**
	STP-1	-61.999	117.267	138.00	-0.528	0.597
	STP-2	13.841	117.267	138.00	0.118	0.906
	STP-3	327.526	117.267	138.00	2.792	**0.006**
	STP-4	124.660	117.267	138.00	1.063	0.289
	STP-5	58.025	117.267	138.00	0.494	0.621
Light II	(Intercept)	224.710	33.038	138.00	6.801	**< 0.001**
	STP-1	-54.142	46.723	138.00	-1.158	0.248
	STP-2	-25.511	46.723	138.00	-0.546	0.585
	STP-3	-12.317	46.723	138.00	-0.263	0.792
	STP-4	-62.170	46.723	138.00	-1.330	0.185
	STP-5	-5.758	46.723	138.00	-0.123	0.902

Table S8-3: Continued.

Dark III	(Intercept)	637.486	68.814	138.00	9.263	**< 0.001**
	STP-1	-144.135	97.317	138.00	-1.481	0.140
	STP-2	-69.344	97.317	138.00	-0.712	0.477
	STP-3	183.752	97.317	138.00	1.888	0.061
	STP-4	165.263	97.317	138.00	1.698	0.091
	STP-5	-108.782	97.317	138.00	-1.117	0.265
Light III	(Intercept)	242.845	20.937	138.00	11.600	**< 0.001**
	STP-1	-93.717	29.610	138.00	-3.165	**0.001**
	STP-2	-56.125	26.610	138.00	-1.865	**0.006**
	STP-3	-49.922	26.610	138.00	-1.686	0.094
	STP-4	-46.944	26.610	138.00	-1.585	0.115
	STP-5	-79.838	26.610	138.00	-2.696	**0.007**
All Dark	(Intercept)	492.367	65.685	288.00	7.495	**< 0.001**
	STP-1	8.736	92.863	138.00	0.009	0.925
	STP-2	58.240	92.863	138.00	0.626	0.531
	STP-3	295.867	92.863	138.00	3.185	**0.001**
	STP-4	146.610	92.863	138.00	1.578	0.116
	STP-5	129.810	92.863	138.00	1.397	0.164
All Light	(Intercept)	246.319	20.834	288.00	11.822	**< 0.001**
	STP-1	-82.824	29.464	138.00	-2.810	**0.005**
	STP-2	-33.755	29.464	138.00	-1.145	0.253
	STP-3	-48.404	29.464	138.00	-1.642	0.102
	STP-4	-85.026	29.464	138.00	-2.885	**0.004**
	STP-5	-75.503	29.464	138.00	-2.562	**0.011**

Table S8-4: LMER estimates for the effects on velocity [mm/s] of *D.rerio* larvae after the exposure of pristine AgNPs. Significant differences (* P < 0.05, ** P < 0.01, *** P < 0.001) within the performed Dark and Light cycles and for separately combined Dark and Light cycles compared to DIS are marked in bold. DIS = dispersant control, control = ASTM control, df = degrees of freedom, *t* = test statistics.

	Treatment	Estimate	Std. Error	df	t	P
Dark I	(Intercept)	0.825	0.165	161.00	4.994	**< 0.001**
	control	-0.231	0.233	161.00	0.212	0.832
	Ag-1	0.049	0.233	161.00	0.948	0.344
	Ag-2	0.221	0.233	161.00	1.691	0.092
	Ag-3	0.395	0.233	161.00	0.265	0.790
	Ag-4	0.062	0.233	161.00	0.113	0.909
	Ag-5	0.026	0.233	161.00	-0.988	0.324
Light I	(Intercept)	0.268	0.091	161.00	2.933	**0.003**
	control	-0.027	0.129	161.00	0.410	0.834
	Ag-1	0.053	0.129	161.00	0.038	0.682
	Ag-2	0.004	0.129	161.00	0.719	0.969
	Ag-3	0.093	0.129	161.00	1.376	0.472
	Ag-4	0.178	0.129	161.00	0.688	0.170
	Ag-5	0.089	0.129	161.00	-0.209	0.492
Dark II	(Intercept)	1.684	0.161	161.00	10.441	**< 0.001**
	control	-0.525	0.228	161.00	-2.799	**0.022**
	Ag-1	-0.638	0.228	161.00	-0.603	**0.005**
	Ag-2	-0.137	0.228	161.00	-1.022	0.547
	Ag-3	-0.233	0.228	161.00	-2.429	0.308
	Ag-4	-0.554	0.228	161.00	-1.561	**0.016**
	Ag-5	-0.356	0.228	161.00	-2.301	0.120
Light II	(Intercept)	0.289	0.038	161.00	7.545	**< 0.001**
	control	-0.049	0.054	161.00	-0.854	0.394
	Ag-1	-0.046	0.054	161.00	-0.072	0.942
	Ag-2	-0.003	0.054	161.00	-0.537	0.591
	Ag-3	-0.029	0.054	161.00	-0.972	0.332
	Ag-4	-0.052	0.054	161.00	0.476	0.634
	Ag-5	0.025	0.054	161.00	-0.921	0.357

Table S8-4: Continued.

Dark III	(Intercept)	1.843	0.157	161.00	11.694	**< 0.001**
	control	-0.667	0.223	161.00	-2.991	**0.003**
	Ag-1	-0.719	0.223	161.00	-3.227	**0.001**
	Ag-2	-0.518	0.223	161.00	-2.324	**0.021**
	Ag-3	-0.499	0.223	161.00	-2.240	**0.026**
	Ag-4	-0.672	0.223	161.00	-3.014	**0.003**
	Ag-5	-0.489	0.223	161.00	-2.192	0.029
Light III	(Intercept)	0.247	0.021	161.00	11.404	**< 0.001**
	control	-0.017	0.030	161.00	-1.348	0.179
	Ag-1	-0.041	0.030	161.00	0.126	0.899
	Ag-2	0.003	0.030	161.00	1.315	0.190
	Ag-3	0.040	0.030	161.00	0.590	0.555
	Ag-4	0.018	0.030	161.00	1.983	**0.049**
	Ag-5	0.060	0.030	161.00	-0.563	0.573
All Dark	(Intercept)	1.451	0.1274	336.00	11.382	**< 0.001**
	control	-0.474	0.180	161.00	-0.802	**0.009**
	Ag-1	-0.436	0.180	161.00	-0.623	**0.016**
	Ag-2	-0.144	0.180	161.00	-2.152	0.423
	Ag-3	-0.112	0.180	161.00	-1.513	0.533
	Ag-4	-0.388	0.180	161.00	-2.631	**0.032**
	Ag-5	-0.272	0.180	161.00	-2.419	0.132
All Light	(Intercept)	0.268	0.035	336.00	7.585	**< 0.001**
	control	-0.031	0.050	161.00	0.032	0.530
	Ag-1	-0.011	0.050	161.00	0.696	0.818
	Ag-2	0.001	0.050	161.00	0.958	0.974
	Ag-3	0.034	0.050	161.00	1.171	0.487
	Ag-4	0.047	0.050	161.00	-0.628	0.339
	Ag-5	0.058	0.050	161.00	-0.229	0.243

Table S8-5: LMER estimates for the effects on velocity [mm/s] of *D.rerio* larvae after the exposure of wastewater-borne AgNPs. Significant differences (* P < 0.05, ** P < 0.01, *** P < 0.001) within the performed Dark and Light cycles and for separately combined Dark and Light cycles, compared to DIS are marked in bold. DIS = dispersant control, control = ASTM control, df = degrees of freedom, *t* = test statistics.

	Treatment	Estimate	Std. Error	df	t	P
Dark I	(Intercept)	0.716	0.203	138.00	3.518	**< 0.001**
	STP-1	1.099	0.287	138.00	3.819	**< 0.001**
	STP-2	0.316	0.287	138.00	10.99	0.273
	STP-3	0.592	0.287	138.00	2.059	**0.041**
	STP-4	0.273	0.287	138.00	0.951	0.342
	STP-5	0.730	0.287	138.00	2.537	**0.012**
Light I	(Intercept)	0.526	0.075	138.00	6.970	**< 0.001**
	STP-1	-0.221	0.106	138.00	-2.073	**0.039**
	STP-2	-0.065	0.106	138.00	-0.611	0.541
	STP-3	-0.187	0.106	138.00	-1.752	0.081
	STP-4	-0.300	0.106	138.00	-2.817	**0.005**
	STP-5	-0.294	0.106	138.00	-2.758	**0.006**
Dark II	(Intercept)	1.065	0.142	138.00	7.483	**< 0.001**
	STP-1	-0.025	0.201	138.00	-0.124	0.900
	STP-2	0.051	0.201	138.00	0.254	0.799
	STP-3	0.440	0.201	138.00	2.186	**0.030**
	STP-4	0.295	0.201	138.00	1.466	0.144
	STP-5	0.086	0.201	138.00	0.430	0.667
Light II	(Intercept)	0.401	0.058	138.00	6.876	**< 0.001**
	STP-1	-0.096	0.082	138.00	-1.164	0.246
	STP-2	-0.035	0.082	138.00	-0.435	0.663
	STP-3	-0.028	0.082	138.00	-0.340	0.733
	STP-4	-0.115	0.082	138.00	-1.396	0.164
	STP-5	-0.011	0.082	138.00	-0.142	0.886

Table S8-5: Continued.

Dark III	(Intercept)	1.194	0.133	138.00	8.931	**< 0.001**
	STP-1	-0.245	0.189	138.00	-1.300	0.195
	STP-2	-0.028	0.189	138.00	-0.150	0.880
	STP-3	0.293	0.189	138.00	1.552	0.122
	STP-4	0.495	0.189	138.00	2.617	**0.009**
	STP-5	-0.079	0.189	138.00	-0.422	0.673
Light III	(Intercept)	0.432	0.0376	138.00	11.490	**< 0.001**
	STP-1	-0.167	0.053	138.00	-3.143	**0.002**
	STP-2	-0.101	0.053	138.00	-1.902	0.059
	STP-3	-0.080	0.053	138.00	-1.509	0.133
	STP-4	-0.079	0.053	138.00	-1.494	0.137
	STP-5	-0.140	0.053	138.00	-2.629	**0.009**
All Dark	(Intercept)	0.991	0.123	288.00	8.036	**< 0.001**
	STP-1	0.276	0.174	138.00	1.581	0.116
	STP-2	0.113	0.174	138.00	0.647	0.518
	STP-3	0.442	0.174	138.00	2.533	**0.012**
	STP-4	0.354	0.174	138.00	2.032	**0.044**
	STP-5	0.245	0.174	138.00	1.407	0.161
All Light	(Intercept)	0.453	0.038	288.00	11.798	**< 0.001**
	STP-1	-0.161	0.054	138.00	-2.974	**0.003**
	STP-2	-0.067	0.054	138.00	-1.242	0.216
	STP-3	-0.098	0.054	138.00	-1.813	0.072
	STP-4	-0.165	0.054	138.00	-3.039	**0.002**
	STP-5	-0.148	0.054	138.00	-2.737	**0.007**

Chapter 9

Conclusion and Further Perspectives

9.1 Synopsis

The rapid development of nanotechnology and the related rising production volume of engineered manufactured nanoparticles (NPs) lead to an increasing risk for the aquatic environment. The major entry path of NPs are sewage treatment plant (STPs) and significant concentrations of NPs enter the aquatic environment and may have negative effects on living aquatic organisms and their ecosystems. One study found that the toxicity of AgNPs to zebrafish larvae significantly increased after they pass the STPs (Muth-Köhne et al., 2013), indicating a higher potential risk of the aquatic environment than assumed. Thus, the aim of the project FENOMENO was to investigate the ecotoxicological potential of NPs from lab-scale STPs to aquatic key species, potential transformation process of NPs while passing the STPs and the development and improvement of new and existing techniques to measure low concentrations of NPs in aqueous samples.

In this thesis, as a part of the EU-project FENOMENO, I studied the ecotoxicological impact of wastewater-borne AgNPs and TiO_2NPs on the behaviour, physiology and reproduction of two aquatic key species, *Daphnia magna* and *Danio rerio*. This project investigated the risk of NPs to the aquatic ecosystem under more realistic conditions, including environmentally relevant concentrations within the experiments and contributed to the understanding of the effects of transformation processes of NPs and their related toxicity of wastewater-borne NPs. Additionally, my work gave insights into the perception and sensitivity *of D. rerio* larvae towards NIR light, an important aspect for all ecotoxicological studies using changes in swimming behaviour of *D. rerio* larvae for an risk assessment. The aquatic organisms *D. magna* and *D. rerio* larvae play an important key role in the aquatic ecosystem, making them the most suitable model species in the context of this project. The water flea *D. magna* acts as a primary consumer as it feeds on primary producers (e.g. algae), and is prey for secondary consumers (e.g. small fishes) (Ebert, 2005). *Danio rerio* is a well-studied model organism in various disciplines due to specific characteristics, like genetic similarities with humans, short generation time or transparent chorion (Kimmel et al., 1995). The model substances used in this study, AgNPs and TiO_2NPs, are two well-investigated chemicals and their ecotoxicological impact of the pristine form has been described in various publications (reviewed by Guo et al. (2019) and Zhou et al. (2019)). Since the major entry path of these NPs to the aquatic ecosystem are STPs, transformation processes of NPs may take place and could have an important

influence on toxicity of NPs. In the performed experiments, I tested the ecotoxicological impact of wastewater-borne AgNPs and TiO_2NPs and pristine form towards *D. magna* and *D. rerio* and compared their toxicity. As described in the literature, the fate of AgNPs in STPs is building of the less toxic compound Ag_2S (Kaegi et al., 2011). To provide further knowledge about the toxicity and the risk of wastewater-borne NPs to the aquatic environment, I used two important key species and two well investigated NPs to answer open questions in this context.

Changes in an animal's behaviour due to a stressor can have negative effects towards reproduction, growth and survival and therefore can influence the whole population and the respective ecosystem (Fent, 2013). Hence, in **Chapter 3** I studied whether the exposure of wastewater-borne AgNPs and TiO_2NPs might have an influence on acute toxicity, behaviour and biochemical markers on *D. magna* as an integrated approach. I found that only *Daphnia* exposed to pristine AgNPs resulted in a 96-h EC_{50} for immobilization of 113.8 µg/L and neither for wastewater-borne AgNPs nor wastewater-borne and pristine TiO_2NPs an EC_{50} could be calculated because the test specimen were still mobile. Furthermore, analysing behaviour with the endpoint allocation time for preferred zones showed that animals exposed to pristine AgNPs spent more time at the surface and bottom at the beginning of exposure (0 h) and in the middle and bottom after 96 h of exposure. This effect was observed neither with pristine TiO_2NPs nor with wastewater-borne AgNPs or TiO_2NPs. Biochemical analyses showed that wastewater-borne AgNPs had the biggest influence on subcellular level. Interestingly is the fact that a reaction on subcellular level is detectable but not measurable at behavioural level. The approach of this study, namely using behavioural and biochemical marker, went further than the state of the art. It showed that acute toxicity as well as behavioural-related effects of pristine AgNPs are much stronger compared to wastewater-borne AgNPs, while at the subcellular level, wastewater-borne AgNPs are more toxic than pristine AgNPs, justifying the necessity of an integrated approach. Thus, the chosen behavioural-related markers and the battery of selected biochemical markers can effectively function as important warning indicators for the detection of adverse effects caused by wastewater-borne NPs. With this study, I provide for the first time important information (i) on the decreased acute toxicity of wastewater-borne AgNPs and TiO_2NPs on behavioural level and (ii) provide essential and early warning background information for environmental risk assessment on

the effects of wastewater-borne NPs in aquatic environments using a combined integrated approach.

In addition to Chapter 3, the aim of **Chapter 4** was to develop new behavioural-related endpoints to use *D. magna* as a sensitive and reliable biomarker for NP contamination in water systems. The analysis showed that the highest sensitivity for the endpoint were “cross backs” during exposure with pristine AgNPs. The next reliable parameter was “crossings” indicated the first significant behavioural change after 3 h of exposure with pristine AgNPs. Both parameters were sensitive towards pristine TiO_2NPs as well, however, but with a delayed in reaction. Interestingly, wastewater-borne AgNPs affected the swimming direction to the same extent as pristine AgNPs. Hence, in this study I developed new behavioural-related endpoints that are suitable to detect both, wastewater-borne NPs and pristine NPs in water systems, like effluents or freshwater, show a high sensitivity for low concentrations of NPs contaminations and indicate behavioural changes of *D. magna* with a rapid response time. Thus, the new developed endpoints in this study can therefore be useful and integrated as a measurement parameter for a biological early warning system (BEWS) with *D. magna* to protect the aquatic environment and human health.

Experiments with *D. magna* exposed to high concentrations of pristine AgNPs and TiO_2NPS have demonstrated that an exposure over multiple generations led to extinction of the whole population (Jacobasch et al., 2014), increased toxicity of AgNPs (Völker et al., 2013) or a significantly higher acute toxicity in the next generation (Bundschuh et al., 2012). All these studies indicate transgenerational effects. However, a still unanswered question that I investigated in **Chapter 5** was whether the exposure with *environmentally relevant concentrations* of pristine and wastewater-borne AgNPs and TiO_2NPS over six continuous generations led to (i) negative effects towards key life cycle parameters on *D. magna* and (ii) maternal effects that are visible in the next generations. I found that the exposure neither with wastewater-borne AgNPs nor with pristine and wastewater-borne TiO_2NPs affects the *Daphnia* life cycle over six generations. In contrast, pristine AgNPs led to a high toxicity with a significantly reduced reproductive success for all six generations. Still, transgenerational effects were not observed in any of the tested exposure scenarios. Hence, the toxicity of NPs to *D. magna* significantly decreased after passing STPs. AgNPs were transformed to the less toxic compound Ag_2S, which is not

bioavailable for aquatic species due to the reduced water solubility and furthermore, the formation of the most toxic compound for aquatic organisms, silver ions (Ag^+), is significantly reduced. It seems that *environmentally relevant concentrations* of wastewater-borne and pristine TiO_2NPs did not have any effect on *D. magna* reproduction. With this study, I provided further evidence that the risk of NPs to the aquatic organism *D. magna* under realistic conditions, including transformation processes, exposure scenarios with *environmentally relevant concentrations* tend to be much lower as assumed by the current literature and should be considered for a reliable risk assessment of NPs and aquatic pollutions in general.

Since phenotypic plasticity is known to be responsible for the induction of anti-predator defence mechanism in *Daphnia* species (Laforsch et al., 2009; Tollrian and Harvell, 1999; Weiss et al., 2012), I tested first time whether adults and offspring of *D. magna* develop defensive traits if they were exposed to chemicals cues of fish (kairomones) in combination with NPs. That is why I tested in **Chapter 6** the induction of kairomone-mediated anti-predator defence mechanism of adult *D. magna* and their offspring under the exposure of *environmental relevant concentrations* of pristine AgNPs and TiO_2NPs. I found that the offspring of adult *D. magna* exposed to AgNPs did not show any anti-predator defence mechanism, indicating that pristine AgNPs have a clear influence on the phenotypic plasticity of juvenile *D. magna.* Interestingly, pristine AgNPs did not affect the induction of anti-predator defence mechanism in adult *D. magna,* since I found no differences in morphological parameters compared to animals treated with kairomones only. Pristine TiO_2NPs did not influence the anti-predator defence mechanism neither of adult *D. magna* nor of their offspring. Hence, I showed for the first time that under the exposure of AgNPs adult *D. magna* produce neonates without any defence mechanism, despite the presence of predators. Different gene expression programs are involved in kairomone-mediated anti-predator defence mechanism in *D. ambigua* within a generation and in transgenerational responses (Hales et al., 2017). This might explain, why adult female *Daphnia* responded to kairomones in the presence of AgNPs and their offspring did not. Hence, with this study, I provided important information on the interaction of environmental and anthropogenic pollutants on defensive traits. The lack of defence mechanisms in the next generation of *Daphnia* can have dramatic consequences for *Daphnia* population structure in the aquatic ecosystem in the presence of predation risk, and thus could

influence the whole aquatic food web. However, further research is needed on this topic to explain the mechanism behind the lack of anti-predator defence mechanism in the offspring of adult *D. magna* exposed to pristine AgNPs. This should be one focus for further projects, since also Trotter et al. (2019) found that microplastic led to significantly reduction in defensive traits of *D. longicephala*.

As a pre-study for analysing behavioural changes under the exposure of AgNPs, I investigated in **Chapter 7** the perception of zebrafish larvae at two larval stages (96 hpf and 120 hpf) towards two wavelengths of NIR light and analysed their phototactic behaviour. Even though investigations on behavioural changes of zebrafish larvae in ecotoxicology became popular in the last few years and the recording is by default performed in darkness with NIR light and NIR sensitive cameras (Legradi et al., 2015), no study has ever investigated the sensitivity of zebrafish larvae to different wavelengths within the NIR spectrum. Contrary to expectations, both larvae stages (96 hpf and 120 hpf) showed a negative phototactic behaviour towards NIR with a wavelength of 860 nm, by spending more time in the unexposed side of the test vessel. This behaviour is also present with visible light (VIS) which was previously reported by Padilla et al. (2011) and Colwill et al. (2011). However, zebrafish larvae illuminated with 960 nm did not show a phototactic response. Zebrafish larvae, therefore, are able to perceive wavelength of 860 nm but did not react to 960 nm. These results were supported by the study of Shcherbakov et al. (2013) who found that adult zebrafish show positive phototactic behaviour towards NIR light of 850 nm and 950 nm and the reaction towards light differs between adult and larval zebrafish (Colwill and Creton, 2011a). Hence, this study indicates that the applied darkness with 860 nm in common behaviour tracking systems with zebrafish larvae does not mimic a dark environment for the specimens. Further tests are recommended using NIR illumination of 960 nm. Furthermore, the performed ecotoxicological studies with NIR illumination at 860 nm should be re-interpreted and considered with caution, taking into account the results of this study.

Monitoring systems that use behavioural changes in fish as a biosensor have great advantages and are especially useful to monitor effluents from STPs (Bae and Park, 2014). Finally, for **Chapter 8**, an own-built tracking system was built to detect movement patterns of zebrafish larvae, wherefore 960 nm NIR light was used to record the behaviour in real darkness based on the results of Chapter 7. I performed a behaviour assay with

zebrafish larvae (96 hpf) and tested the influence of wastewater-borne AgNPs and pristine AgNPs to the locomotion behaviour. The exposure with pristine AgNPs indicated a significant hyperactivity of zebrafish larvae while no changes of the locomotion were observed while treating zebrafish larvae with wastewater-borne AgNPs. With this study, I showed for the first time that the toxicity of AgNPs towards fish swimming behaviour significantly decreased after they passed the STP due to the transformation to Ag_2S and the significantly reduced formation of silver ions. Even more important is the gained knowledge from this study that *environmentally relevant concentrations* of wastewater-borne AgNPs did not affect the swimming behaviour of fish and therefore, no negative consequences on important behaviour-associated mechanism are assumed. Hence, the risk of wastewater-borne AgNPs for aquatic vertebrate and the aquatic ecosystem is to be expected as low.

The results of this thesis clearly demonstrate that the toxicity of AgNPs and TiO_2NPs to the aquatic organism *D. magna* and *D. rerio* depends on the used concentrations of NPs and the consideration of possible transformation processes of NPs prior to their discharge into the aquatic environment. The investigation of the ecotoxicological impact of NPs under *environmentally relevant concentrations* and conditions leads to a much more realistic risk assessment for the aquatic environment. Based on the results of this thesis, the risk of NPs to the aquatic environment can be considered lower than assumed in the recent literature. Due to this important gained knowledge, the current existing assessment of the toxicity of NPs needs to be reconsidered and enhanced with the new findings of this study, taking into account the decreased toxicity of NPs after they pass the STPs and using of *environmentally relevant concentrations*. Furthermore, I could provide new important knowledge about the sensitivity of *D. rerio* larvae towards NIR light and provide results that must be considered in all new and previous ecotoxicological or neurotoxic behavioural studies with zebrafish.

9.2 Further Perspectives

The performed experiments in this thesis have answered important questions in regard to the ecotoxicity of wastewater-borne AgNPs and wastewater-borne TiO_2NPs to the aquatic organisms *D. magna* and *D. rerio*, but at the same time, new questions and ideas have emerged.

Transformation processes have an important influence on the toxicity of wastewater-borne NPs. Although the main entry route of NPs to the aquatic environment are STPs, this thesis has shown that only AgNPs undergo transformation processes by building the less toxic compound Ag_2S, whereas TiO_2NPs remain in their initial state. As a first step for further research, the reason for the different fates of the two tested NPs while they pass the STP has to be investigated. Does the degree of transformation and the corresponding toxicity depend on the chemical properties, ion-releasing and non-ion-releasing, of the NPs? This hypothesis can be addressed by the investigation of transformation processes during STP of other known ion-releasing NPs like CuO-NPs and non-ion-releasing like Au-NPs by known microscopic techniques (TEM and EDX) and ecotoxicological test assays with organism of different trophic levels. Based on that, for a reliable risk assessment, the investigation of possible transformation processes, and related toxic effects of other anthropogenic introduced chemical pollutants, like pharmaceutical products, pesticides, heavy metals or microplastic while they pass STPs should be investigated. One more question raised by this study was: do wastewater-borne NPs biomagnify within the aquatic food chain and lead to negative effects towards organisms of higher levels? To answer this, further investigations are needed in determining the bioaccumulation potential and resulting adverse effects of wastewater-borne NPs within the aquatic food chain, algae-*Daphnia*-fish. In addition, since sewage sludge contains nanoparticles and represents an important source for engineered nanomaterials into the aquatic and terrestrial environment, the investigation of the mediated effects should be a major concern for further research. Until now, the investigation of behavioural changes did not take place in the risk assessment of chemicals, although this thesis has clearly showed that, the two aquatic organisms *Daphnia magna* and *Danio rerio* act as suitable biosensors for nanoparticles contaminated water systems. Hence, the establishment of own OECD guidelines is recommended to analyse the toxicity and the risk of further anthropogenic pollutants based on the high sensitivity of behavioural-related endpoints. Especially zebrafish larvae act as a good alternative test species for ecotoxicological risk assessment under REACH. Moreover, the establishment of an own guideline (e.g. OECD) for testing nanoparticles is advisable, as recommended within the project proposal of FENOMENO and which is as well initiated by the Malta Initiative, an international self-organised group of ECHA (European Chemical Agency), EU member states, industry and academics. So

far, test guidelines for the testing of chemical substances are applied to assess the risk of MNMs. However, the specific characteristics and properties of NMs were not met, wherefore the application of those are not considered as suitable for testing NMs. In the future, the use of *environmentally relevant concentrations* based on PEC values should be an important issue for a reliable risk assessment of NPs and all further anthropogenic pollutants in order to reflect the environment under realistic conditions and should be included within the REACH regulations.

Summary

Nanoparticles (NPs), especially silver (Ag) NPs and titanium dioxide (TiO_2) NPs, are found in a variety of consumer products and are discharged into urban wastewater and into the aquatic environment through daily use. The toxicity of pristine AgNPs and TiO_2NPs to the aquatic ecosystem is well investigated. However, this does not reflect a realistic situation of pollution in aquatic ecosystems. During the wastewater treatment process in municipal wastewater sewage treatment plants (STPs) and before NPs are introduced into the environment, transformation processes take place that can have a major influence on the toxicity of NPs. An ecotoxicological assessment of so-called wastewater-borne NPs has not yet been sufficiently performed. The project "FENOMENO - Fate and effect of wastewater-borne manufactured nanomaterials in aquatic ecosystems" therefore aimed to analyse and characterise the fate of NPs with possible transformation processes and to investigate the related ecotoxicological potential of wastewater-borne AgNPs and TiO_2NPs along the aquatic food chain, algae-*Daphnia*-fish.

As part of this project, I have investigated the effects of wastewater-borne AgNPs and TiO_2NPs on two key organisms of the aquatic ecosystem, *Daphnia magna* and *Danio rerio*. In particular, I conducted studies on the behaviour of *D. magna* and *D. rerio* and the reproductive success of six consecutive generations of *D. magna* under the influence of wastewater-borne AgNPs and TiO_2NPs. All studies were performed in environmental relevant concentrations (based on PEC values) and in comparison, with pristine AgNPs and TiO_2NPs in order to compare the toxicity of NPs after they pass the STP. Furthermore, I investigated the formation of anti-predator defence mechanisms of *D. magna* under the influence of pristine AgNPs and TiO_2NPs and the perception of near infrared (NIR) light of *D. rerio* larvae.

I found that the toxicity of wastewater-borne AgNPs was significantly reduced compared to pristine AgNPs. No negative effects on important life cycle parameters such as reproductive success, body length or day to first brood were found in multi-generation study with *D. magna*. The exposure with pristine AgNPs led to a significant reduction in reproductive success in all six generations studied. This result was confirmed by the evaluation of behavioural-related endpoints, such as swimming height, allocation time,

crossings and cross backs for *D. magna* and swimming speed and total distance for *D. rerio* larvae. The reduced toxicity of wastewater-borne AgNPs can mainly be explained by the transformation of AgNPs to silver sulphide (Ag_2S). Due to the low water solubility and the reduced formation of Ag^+ ions, the bioavailability of Ag has been significantly reduced, which decreased the toxic potential of Ag for aquatic organisms. Further experiments with *environmentally relevant concentrations* of wastewater-borne and pristine TiO_2NPs showed no influence neither on behavioural nor on life-cycle parameters on both key organisms.

In addition, I was able to show that anti-predator defence mechanisms in the next generation were not developed when adult *D. magna* were exposed with pristine AgNPs, although they themselves showed defensive traits. Offspring of female *Daphnia* treated with pristine AgNPs showed a significantly reduced relative spine in comparison to offspring the female animals from the control group. This effect could not be shown with pristine TiO_2NPs, since the anti-predator defence mechanisms was not negatively affected in the next generation. In contrast to all previous assumptions, I was able to show that *D. rerio* larvae can perceive near infrared light (NIR) up to a wavelength of 860 nm and show negative phototactic behaviour. This has never been tested before although zebrafish larvae are used in ecotoxicological studies as a standard model species. This pattern can also be seen in visual light. Wavelength from 960 nm are no longer perceived by the larvae and can be used as a "dark" light source for behavioural experiments.

In this work, I performed a realistic risk assessment of AgNPs and TiO_2NPs to two aquatic key species by including natural transformation processes in the life cycle of NPs and environmentally relevant concentrations. In summary, I found that the ecotoxicological potential of wastewater-borne NPs seems to be very low and the risk to the aquatic ecosystem has been significantly overestimated in the current literature. These aspects should be examined and taken into account in the risk assessment and authorisation of NPs due to their specific chemical properties. The perception of NIR light from *D. rerio* larvae is also a very important finding for further ecotoxicological behavioural experiments to display a dark environment for the test organisms.

Zusammenfassung

Nanopartikel (NP), darunter vor allem Silber (Ag) NPs und Titandioxid (TiO_2) NPs sind heutzutage in einer Vielzahl von Konsumgütern enthalten, und werden durch den täglichen Gebrauch in das urbane Abwasser und damit in die aquatische Umwelt eingeleitet. Die Toxizität von unbehandelten („pristine“) AgNPs und TiO_2NPs auf das aquatische Ökosystem ist hinreichend bekannt. Diese Betrachtung spiegelt aber kein realistisches Bild der Belastung von aquatischen Ökosystemen wider. Während des Klärprozesses in kommunalen Kläranlagen und damit vor dem Eintrag von NPs in die Umwelt, finden Transformationsprozesse statt, die einen großen Einfluss auf deren Toxizität haben können. Eine ökotoxikologische Analyse von sogenannten „wastewater-borne“ NPs wurde bisher nicht ausreichend durchgeführt. Das Projekt „FENOMENO - Fate and effect of wastewater-borne manufactured nanomaterials in aquatic ecosystems” hatte sich daher zum Ziel gesetzt das Verhalten, mit möglichen Transformationsprozessen von NPs zu analysieren und zu charakterisieren und das ökotoxikologische Potential von „wastewater-borne“ AgNPs und TiO_2NPs entlang der aquatischen Nahrungskette, Alge-*Daphnia*-Fisch, zu untersuchen.

Im Rahmen dieses Projektes, habe ich in meiner Doktorarbeit die Auswirkungen von „wastewater-borne“ AgNPs und TiO_2NPs auf zwei Schlüsselorganismen des aquatischen Ökosystems, *Daphnia magna* und *Danio rerio*, untersucht. Im Speziellen habe ich dabei Studien zum Verhalten von *D. magna* und *D. rerio*, zum Reproduktionserfolg von sechs aufeinander folgenden Generationen von *D. magna* unter dem Einfluss von „wastewater-borne“ AgNPs und TiO_2NPs durchgeführt. Alle Studien wurden in Konzentrationsbereichen im umweltrelevanten Bereich (basierend auf PEC Werten) und im Vergleich mit „pristine“ AgNPs und TiO_2NPs durchgeführt, um eine Aussage über die Toxizität der NPs im Ausfluss der Kläranlage zu treffen. Darüber hinaus habe ich die Ausbildung von Abwehrmechanismen gegenüber Fressfeinden bei *D. magna* unter dem Einfluss von „pristine“ AgNPs und das Nahinfrarot Sehen von *D. rerio* Larven untersucht. Ich konnte mit den durchgeführten Experimenten zeigen, dass die Toxizität von „wastewater-borne“ AgNPs im Vergleich zu „pristine“ AgNPs signifikant reduziert ist. So zeigten sich in der Mehrgenerationsstudie mit *D. magna* keine negativen Effekte auf wichtige Parameter des Lebenszyklus, wie Reproduktionserfolg, Körperlänge oder Tag der ersten Nachkommenschaft. Die Behandlung mit „pristine“ AgNPs führte dagegen zu einer signifikanten Reduktion des Reproduktionserfolgs in allen sechs untersuchten

Generationen. Dieses Ergebnis konnte durch die Auswertung von verhaltensrelevanten Endpunkten in den durchgeführten Verhaltensstudien, wie Schwimmhöhe, Ortswechsel und Aufenthaltszeit für *D. magna* und Schwimmgeschwindigkeit und zurückgelegte Distanz für *D. rerio* Larven, untermauert werden. Die reduzierte Toxizität von „wastewater-borne“ AgNPs kann hauptsächlich durch die Transformation von AgNPs zu Silbersulfid (Ag_2S) erklärt werden. Durch die geringe Wasserlöslichkeit und die verringerte Bildung von Ag^+ Ionen ist die Bioverfügbarkeit von Ag deutlich reduziert worden, wodurch das toxische Potential von Ag für aquatische Organismen deutlich sinkt. Weitere Experimente mit umweltrelevanten Konzentrationen an „wastewater-borne“ und „pristine“ TiO_2NPs zeigten keinen Einfluss auf Verhaltensrelevante- und Lebenszyklusparameter in beiden untersuchten Schlüsselorganismen. Darüber hinaus konnte ich zeigen, dass keine Ausbildung von Abwehrmechanismen gegenüber Fressfeinden in der nachfolgenden Generation stattfindet, wenn adulte *D. magna* mit „pristine“ AgNPs behandelt wurden, obwohl sie diese selber zeigen. Die Nachkommen von adulten *Daphnien,* die mit „pristine“ AgNPs behandelt wurden, zeigten signifikant verringerte Endpunkte, wie z.B. verkürzte Stachelspitze im Verhältnis zur Körperlänge. Dieser Effekt konnte bei „pristine“ TiO_2NPs nicht nachgewiesen werden, da hier die Ausbildung der Abwehrmechanismen auch in der nächsten Generation nicht negativ beeinflusst wurde. Entgegen aller bisherigen Annahmen konnte ich zeigen, dass *D. rerio* Larven nahinfrarotes Licht (NIR) bis zu einer Wellenlänge von 860 nm wahrnehmen können und ein negativ phototaktisches Verhalten zeigen. Dieses Muster ist auch bei visuellem Licht zu erkennen. Wellenlängenbereiche ab 960 nm werden von den Larven nicht mehr wahrgenommen und können für Verhaltensversuche als „dunkele“ Lichtquelle verwendet werden. Durch die Einbeziehung von natürlichen Transformationsprozessen im Lebenszyklus von NPs und von umweltrelevanten Konzentrationen kann meine Arbeit eine realistischere Risikoabschätzung von AgNPs und TiO_2NPs für aquatische Organismen liefern. Zusammenfassend habe ich herausgefunden, dass das ökotoxikologische Potential von „wastewater-borne“ NPs als sehr gering einzuschätzen ist und das Risiko für das aquatische Ökosystem bislang deutlich überschätzt wurde. Diese Aspekte sollten in der Risikobewertung und Zulassung von Nanopartikeln, aufgrund ihrer besonderen chemischen Eigenschaften untersucht und berücksichtig werden. Das Wahrnehmen von NIR-Licht von *D. rerio* Larven stellt zudem eine wichtige Erkenntnis für ökotoxikologische Untersuchungen dar.

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Abbreviations

AcH	acetylcholinesterase
Ag^+	ionic silver (silver ions)
AgNP	silver nanoparticle
Al	aluminium
ANOVA	analysis of variance
ASTM	American Society of Testing and Materials
Au	gold
BEWS	biological early warning systems
C_{60}	fullerene
$CaCl_2$	calcium chloride
CAT	catalase
CNT	carbon nanotubes
CT	control medium ASTM
DA	control medium dispersant Agent NM-300K DIS
DNA	deoxyribonucleic acid
DOM	dissolved organic matter
dpf	days post fertilisation
DVM	diel vertical migration
ECHA	European Chemicals Agency
EDX	energy-dispersive x-ray analysis
EFF	control medium of STP, effluent without NPs
ENP	engineered nanoparticles
EPA	U.S. Environmental Protection Agency
EU	European Union
Fe	iron
FENOMENO	Fate and effect of wastewater-borne manufactured nanomaterials in aquatic ecosystems
GLMM	generalized mixed effect model
GST	glutathione *S*-transferase
H_2O	water
hpf	hours post fertilisation
K^+	potassium
KCl	potassium chloride
LDH	lactate dehydrogenase
LMER	linear mixed effect model

LPO	lipid peroxidation
$MgSO_4$	magnesium sulfate
mM	millimolar
mMol	millimol
MNM	manufactured nanomaterials
MOA	Mode of Action
Na+	sodium
NM	nanomaterial
NNI	National Nanotechnology Initiative
NP	nanoparticle
OECD	Organization for Economic Co-operation and Development
ORF	open reading frames
PEC	predicted environmental concentrations
PMR	Photomotor Response test
PMS	post-mitochondrial supernatant
PP	polypropylene
PSD	particle size distribution
PVP	polyvinylpyrrolidone
REACH	European law: Registration, Evaluation, Authorisation and Restriction of Chemicals
ROS	reactive oxygen species
Si	silicon
SP-ICP-MS	single particle inductively coupled plasma mass spectrometry
STEM	Scanning Transmission Electron Microscopy
STP	sewage treatment plant
TBARS	thiobarbituric acid reactive substances assay
TiO_2NP	titanium dioxide nanoparticle
WPMN	Working Party on Manufactured Nanomaterials
WWTP	wastewater treatment plant
Zn	zinc

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