Universität Bremen

The Ecotoxicity of Iron Oxide Nanoparticles

- Acute, Chronic and Mixture Effects on *Daphnia magna* -

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DECLARATION

I herewith declare that this thesis is the result of my independent work. All sources and auxiliary materials used by me in this thesis are cited completely. This work was not submitted elsewhere. Furthermore, I declare that I have submitted three identical printed exemplars of the thesis.

ERKLÄRUNG

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Kriftel/Bremen, August 2014.

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I Abstract

As industrial products and wastes tend to end up in surface waters, it is inevitable that – with a rising production volume – nanomaterials (NM) and their by-products will enter the aquatic environment. Although we are increasingly gathering information about potential risks from NM for human and nature health, there is still a serious lack of knowledge about the environmental concentration, fate, bioavailability, biocompatibility, distribution in biota and food webs, and the hazard potential of NM in aquatic organisms. In order to assess potential risks for aquatic ecosystems, data from laboratory studies is important. However, classical, standardized test systems must be critically analyzed and adopted where necessary.

This PhD thesis focuses on the investigation of iron oxide nanoparticles (IONP). Due to the increasing (experimental) application of iron-based NM in medicine, but especially in environmental remediation of contaminated groundwater and soil, tons of these NM will consequently be released to the environment with unknown risks to biota. For the assessment of effects of IONP, the big water flea *Daphnia magna* was used. *Dahpnia* was chosen since there are several standardized test protocols available. Furthermore, due to its sensitivity against most pollutants and its filter-feeding way of life it is a preferred organism for testing NM in aquatic ecotoxicology.

A first study concerned the miniaturization of the *Daphnia* standard acute test, which allowed an enormous economization of the test due to reduced animals and substances needed. Furthermore, the possible prolongation of the acute test from 48 to 96 h was investigated and proven, since some NM are being presumed to affect organisms with a delay compared to dissolved chemicals. Another study focused on the influence of different surface coatings on the impact of IONP in *daphnia*. Each coating caused individual effects. Inhibitory effects could not be correlated to the hydrodynamic diameter or the type of stabilizing forces. Rather, effects were linked to decreasing colloidal stability and the release of iron ions from the core material. The effects of colloidal different IONP on life history parameters of *daphnia* were investigated with chronic tests. Increased mortality was observed indicating that acute test might dramatically underestimate the hazard potential of nanoparticles. Furthermore, colloidal instable IONP had stronger effects than colloidal stable ones. Acute and chronic effects were mainly attributed to physiological inhibitions of the daphnids such as disturbed ecdysis, increased energy demands due to IONP adsorbing to the daphnids' exoskeleton and suppressed nutrient uptake. In another study, the use of IONP for remediation applications and the risk for the environment from this technique was studied. In combinatory acute toxicity tests cadmium, copper, resorcin and glyphosate were added to the IONP and

daphnids were expose to the mixtures. Results indicated high efficiency of IONP for heavy metals by significantly reducing their bioavailability and lower or no effects for the organic compounds.

II Zusammenfassung

Oberflächengewässer sind oft Senken für industrielle Produkte und Abfälle. Mit steigenden Produktionsvolumina werden sich auch Nanomaterialien (NM) und ihre Nebenprodukte in der aquatische Umwelt anreichern. Auch wenn wir immer mehr Informationen über mögliche Risiken für Mensch und Umwelt erhalten, wissen wir noch relativ wenig über das Verhalten von künstlichen NM in der Umwelt, in welchen Konzentrationen diese dort auftreten, über ihre Bioverfügbarkeit, Biokompatibilität und die Anreicherung und Verteilung in Biota und Nahrungsnetzen sowie das Gefährdungspotential für aquatische Organismen. Um mögliche Risiken für aquatische Ökosysteme ermitteln zu können, sind Daten aus Laborstudien wichtig. Jedoch müssen die klassischen, standardisierten ökotoxikologischen Testsysteme kritisch analysiert und möglicherweise für die Testung von NM angepasst werden.

Diese Dissertation fokussiert sich auf die Untersuchung von Eisenoxidnanopartikeln (IONP). Auf Grund ihrer zunehmenden (experimentellen) Anwendung in der Medizin, jedoch vor allem in der Sanierung von kontaminierten Grundwasser und Böden, werden künftig Tonnen eisenbasierter NM in die Umwelt entlassen – mit unbekannten Risiken für Biota. Für die Ermittlung der Effekte von IONP wurde der große Wasserflohe *Dapnia magna* gewählt, da es mehrere standardisierte Testprotokolle für diesen Organismus gibt. Daphnien ernähren sich durch filtrieren des Umgebungsmediums und reagieren sehr sensibel gegenüber vielen (toxischen) Substanzen, weshalb sie ein bevorzugter Organismus für die Testung von suspendierten NM sind.

Die erste Untersuchung hatte die Miniaturisierung des akuten Daphnien-Standardtests zum Ziel, durch welche der Test erheblich ökonomisiert wurde, da weniger Tiere und Testsubstanzen benötigt wurden. Außerdem wurde die mögliche Verlängerung des Akuttests von 48 auf 96 h erprobt, da vermutet wurde, dass manche NM eine verzögerte Wirkung im Vergleich zu gelösten Chemikalien zeigen könnten. Die nächsten Untersuchungen konzentrierten sich auf die Einflüsse verschiedener Oberflächenstabilisatoren auf die Wirkung von IONP auf Daphnien. Inhibitorische Effekte konnten weder dem hydrodynamischen Durchmesser noch der Funktionsweise des Stabilisators zugerechnet werden. Die Effekte resultierten eher aus abnehmender kolloidaler Stabilität und der möglichen Freisetzung von Eisenionen. Der Einfluss von zwei IONP mit unterschiedlichen kolloidalen Eigenschaften auf verschiedene Parameter der Daphnienentwicklung wurde in chronischen Studien untersucht. Die Ergebnisse zeigten auf Grund erhöhter Mortalität der Daphnien, dass Akuttests möglicherweise das Gefahrenpotenzial von NM drastisch unterschätzen könnten. Des Weiteren wurden stärke Effekte für die kolloidal instabilen IONP

ermittelt. Akute und chronische Effekte waren hauptsächlich auf eine physiologische Beeinträchtigung der Daphnien, z.B. durch eine gestörte Häutung, erhöhten Energieverbrauch auf Grund von IONP, die an das Exoskelett der Daphnien adsorbierten, sowie reduzierte Nahrungsaufnahme, zurückzuführen. In einer weiteren Studie wurde der mögliche Einsatz der IONP in der Umweltsanierung und damit verbundene Risiken untersucht. In kombinatorischen Akuttests wurde jeweils Cadmium, Kupfer, Resorzin und Glyphosat den IONP-Suspensionen zugegeben und die Daphnien gegenüber den Mixturen exponiert. Die Ergebnisse deuteten auf eine hohe Effizienz der IONP bei den beiden Schwermetallen durch signifikant reduzierte Bioverfügbarkeiten hin. Auf die Wirkung der organischen Verbindungen hatten die IONP hingegen geringen bzw. keinen Einfluss.

III Table of Abbreviations and Symbols

%	percent
°C	degree Celsius
AAS	atomic absorption spectroscopy
Ag^+	silver ions
Ag^0	elementary silver
AgNP	silver nanoparticles
ASC	ascorbate
ASC-IONP	ascorbate fuctionalized iron oxide nanoparticles
ATP	adenosine triphosphate
Au^0	elementary gold
CeO_2	cerium dioxide
CIT	citrate
CIT-IONP	citrate fuctionalized iron oxide nanoparticles
CNT	carbon nanotubes
D. magna	*Daphnia magna*
DEG	diethylenglycol
DEX	dextran
DEX-IONP	dextran fuctionalized iron oxide nanoparticles
DLS	dynamic light scattering
DLVO-theory	theory named after Derjaguin, Landau, Verwey and Overbeek
e.g.	exempli gratia
EC_{10}	10 % effect concentration
EC_{50}	50 % effect concentration
ENP	engineered nanoparticles
Fe	iron
$Fe(acac)_3$	iron (III)acetylacetonate
Fe^0	elementary iron
Fe_3O_4	magnetite

Gd	gadolinium
h	hours
HDD	hydrodynamic diameter
HNO_3	nitric acid
i-IONP	instable iron oxide nanoparticles
ICP-MS	inductively coupled plasma mass spectrometry
INP	iron-based nanoparticles
IONP	iron oxide nanoparticles
ISO	International Organization for Standardization
JRC	Joint Research Centre
K	kelvin
kDa	kilo dalton
L	liters
LC_{10}	10 % lethal concentration
LC_{50}	50 % lethal concentration
m^2/L	square meters per liter
max	maximum
mg/L	milligrams per liter
mL	milliliters
mM	millimole
MRI	magnetic resonance imaging
NM	nanomaterial(s)
nm	nanometers
NOM	natural organic matter
NP	nanoparticle(s)
nZVI	nanoparticular zero valent iron
OECD	Organization for Economic Co-operation and Development
p	p-value
particles/L	particles per liter
PRB	permeable reactive barrier

PTM	paratrophic membrane
PVP	polyvinylpyrrolidone
PVP-IONP	polyvinylpyrrolidone coated iron oxide nanoparticles
REACH	Registration, Evaluation Authorization and Restriction of Chemicals
RMN	representative manufactured nanomaterials
ROS	reactive oxygen species
SE	standard error
TEM	transmission electron microscopy
$Ti0_2$	titanium dioxide
Tween 20	polyoxyethylene (20) sorbitan monolaurate
UFT	Center for Environmental Research and Sustainable Technology
UFZ	Helmholtz Zentrum für Umweltforschung, Leipzig
US EPA	United States Environmental Protection Agency
UV	ultra violet (light)
w/w	wet weight
WPMN	Working Party on Manufactured Nanomaterials (OECD)
ZnO	zinc oxide
µm	micrograms
µm	micrometers

IV List of Figures

V Structure of the Thesis

This thesis is divided into four main chapters: (1) Introduction, (2) Materials and Methods, (3) Publications and Manuscripts and (4) the Summarizing Discussion, Conclusions and Future Perspectives.

The first chapter introduces the reader to basic knowledge about nanomaterials and the application of the two nanomaterials which were investigated in this thesis – iron-based and silver nanomaterials – and their environmental relevance.

The second part reviews “Materials and Methods” used for the investigations within the publications/manuscripts of this thesis to provide the reader with a more detailed background. It describes the synthesis of iron oxide nanoparticles, the applied silver nanoparticles, the analytical methods used for their physicochemical characterization, the biology of daphnids and the applied standardized and adopted tests systems.

The third part presents the results obtained during the laboratory work of this thesis. This chapter is divided into five sub-chapters containing publications/manuscripts that describe a miniaturized and prolonged acute test system with *Daphnia magna*, acute and chronic effects of IONP and combinatory effects of IONP and selected contaminants in daphnids. The sub-chapters 3.1 to 3.3 contain already published articles. The sub-chapter 3.4 contains a manuscript that is under revision for publication. The sub-chapter 3.5 contains a manuscript that was recently submitted to a journal. These manuscripts were included in the thesis in the form in which they have been submitted for publication. However, the format of the text and citations of the manuscripts was adjusted to the layout of this thesis and the figures and tables were positioned together with their legends directly after the respective results.

The fourth part summarizes, discusses and concludes the key findings of the thesis. Furthermore, it presents an outlook and recommendations for future studies.

1 Introduction

1.1 Nanomaterials: Properties and Impacts

Many scientists call nanotechnology the key technology of the 21st century. By some estimates, nanotechnology even promises to far exceed the impact of the Industrial Revolution (Nel et al. 2006). But what is nanotechnology? “Nano” derives from the Greek word “nanos”, which means dwarf. By definition, nanomaterials (NM) have structures with at least one dimension in the range of 1 to 100 nm (e.g. Lespes & Gigault 2011, Moore 2006, Stone et al. 2010, Weinberg et al. 2011, Wiesner et al. 2009). This is a very arbitrary definition since 100 nm do not represent a physicochemical threshold that justifies the distinction of NM and larger (bulk) materials. Therefore, another definition says that, in order to be a NM, it must have properties that are different from the bulk material of the same chemical composition (Zänker & Schierz 2012). These “non-bulk” properties usually only occur in dimensions under 30 nm (Auffan et al. 2009).

The most prominent NM are nanoparticles (NP), often (±) spherical particles with all three dimensions between 1 and 100 nm (ISO 2008) and manifold possible morphologies (Henry 2005). In this dimension, the surface-to-volume ratio is highly increased; more atoms are distributed on the surface in relation to the volume. By this, the reactive surface is highly increased compared to bulk materials, which can substantially modify the materials’ behavior. At the nanoscale the laws of physics seem no longer to apply: materials abruptly change their characteristics, e.g. “opaque substances, like copper, become transparent; stable elements, such as aluminum, burst into flames; normally safe substances, including latex, become poisonous; and gold turns to liquid at room temperature” (Brown 2007). However, the unique advantageous properties of NM also raised concern due to unknown or unexpected effects at the bio-nano interface (Maurer-Jones et al. 2013).

Today, nanotechnology is already present in numerous consumer products, although the risks to humans and the environment have not been fully assessed. In May of 2014, the Nanotechproject recorded 1885 consumer products containing nanomaterials (Nanotechproject 2014). The number of unreported cases might exceed this number many times over.

Important NM are carbon nanotubes (CNT), fullerenes, nanowires, TiO_2, ZnO, CeO_2, and silica NP, Fe^0, Ag^0, and Au^0 NP, and dendrimers (Zänker & Schierz 2012). The most prominent application of NM in consumer products is nano-silver due to its anti-bacterial properties (Rizzello & Pompa 2014) and nano-titanium dioxide as physical UV-blocker in sunscreens (Nohynek et al. 2007, Serpone et al. 2007). NM can also be found in colors/paints, self-cleaning surfaces, scratch-resistant coatings, fibers and fascicles in fabrics,

high-performance insulation and other fillers, tires, catalysts, semiconductors, microelectronics, and medical applications. In the broad field of food technology, NM are important for the optical appearance as well as for the taste of many products (Frimmel & Delay 2010, Wiesner et al. 2006)

The human skin builds a dense barrier through which an uptake into the lymphatic system or the blood seems not to appear. NP only penetrate the first 3-5 corneocyte layers of the stratum corneum (Gontier et al. 2008). Lademann et al. (1999) and Mahe et al. (2009) showed that NP can be accumulated in the hair follicle canals and may pass through the skin on this route (Nohynek et al. 2007). Furthermore, Lee et al. (2013b) found evidence that negatively charged NP might be able to pass the stratum corneum and penetrate into deeper skin cells when the exposure time is increased from 24 to 48 h. Via the air and lung exposure, NP were shown to easily enter the blood system and to be quickly distributed in the whole organism (Borm & Kreyling 2004, Nemmar et al. 2002). In the digestive tract, nano- and microsized particles are known to be absorbed by the epithelial mucus and to pass into the lymphatic or the blood system. NP are known to be able to pass the blood-brain-barrier and even to enter cells (Oberdörster et al. 2005). This raises much concern about their impact on humans and organisms, especially in accidental administration. Depending on their characteristics, NM have a high potential to impact human health.

Possible toxicity mechanisms of NM may include (among others) (1) disruption of membranes or membrane potentials, (2) formation of reactive oxygen species (ROS) and oxidative stress, (3) induction of apoptosis and necrosis and induction of stress-related genes, and (4) oxidation and denaturation of proteins and other biomolecules (Fent 2010, Gwinn & Vallyathan 2006). However, their possible deep penetration into the human body also opens the possibility for new medical applications e.g. as drug delivery system (De Jong & Borm 2008, Wilczewska et al. 2012).

Industrial products and wastes tend to end-up in surface waters. With rising production volumes, also nanoscaled materials and their by-products will consequently enter aquatic systems with unknown risks to biota. In the aquatic milieu there are multiple exposure pathways, which might even occur simultaneously. Nanoparticular matter can be assimilated via the gills and other surface epithelia (Moore 2006, Mueller & Nowack 2008). The same NM can also enter organisms via direct ingestion or passively by eating contaminated food (Oberdörster et al. 2005). Potential environmental exposure routes of NP are shown in **Fig. 1**.

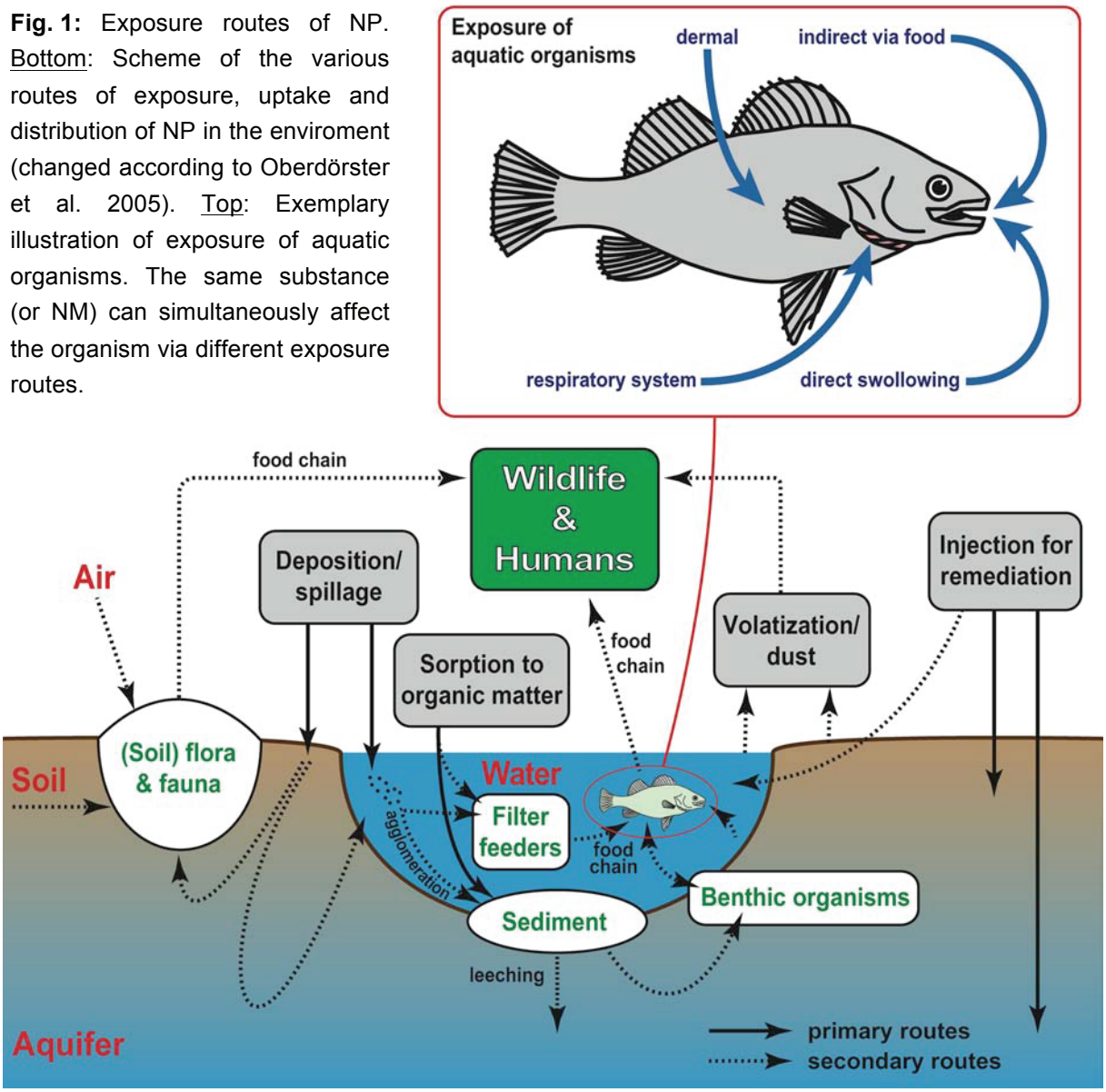

Fig. 1: Exposure routes of NP. Bottom: Scheme of the various routes of exposure, uptake and distribution of NP in the enviroment (changed according to Oberdörster et al. 2005). Top: Exemplary illustration of exposure of aquatic organisms. The same substance (or NM) can simultaneously affect the organism via different exposure routes.

The determination of the (environmental) hazard potential of NP still poses a big challenge, since NP cannot be treated like dissolved chemicals. In general, potential risks are not just related to the substance and the mass concentration as in classic analytical chemistry and (eco)toxicology (Crane et al. 2008). The fate and transport of NM in the environment, as well as their bioavailability and interactions on the bio-nano interface, are determined by their physicochemical properties such as size, size distribution, shape, concentration, material composition, surface charge and functionalization, coating materials, interaction with natural NP, organic matter and other chemicals, and the colloidal stability, which is influenced by photochemical transformation, oxidation and reduction, dissolution, precipitation, agglomeration, adsorption, desorption, combustion, biotransformation and abrasion, among other bio-geochemically driven processes (Nowack et al. 2012, Zänker & Schierz 2012).

Although the modeling of environmental characteristics of engineered NP (ENP) is continuously advancing, there is still a serious lack of knowledge about their actual release rates and their fate in the environment as well as their risks to biota. For the latter more laboratory data is required. However, even on the laboratory scale, the testing of ENP still poses a major challenge to ecotoxicologists. Some considerations concerning standardized (ecotoxicological) tests systems have implicated that they might not be appropriate for the investigation of hazard potentials of NM (Crane et al. 2008), since these classic test systems were designed for the testing of dissolved chemicals. Further detailed analytical data will be needed. For example the way NP are dispersed into, and maintained within, the test medium, the measurement of NP (e.g. size, size distribution, charge) within the tests, and abiotic factors are important additional information for a better understanding of potential implications of NP (Crane et al. 2008, Handy et al. 2012a). Therefore, test operating procedures will have to be critically revised and adopted to the unique characteristics of NM as far as possible.

1.2 nanoToxCom

The nanoToxCom Graduate School was founded in 2008/2009 at the University of Bremen. It provided eight PhD scholarships which were funded by the Hans-Böckler Foundation (of the Affiliation of German Labor Unions). The name nanoToxCom derived from the idea of testing the single and combinatory toxicity of NP. Combinatory toxicity can be understood in different ways: (1) the harmful effects of NP and the materials used to improve their properties such as coating materials; (2) the combined exposure of NP and other toxic substances (secondary stressor); and (3) the harmful effects of products resulting from interaction between NP and additional substances.

nanoToxCom aimed to contribute to a sound hazard assessment for manufactured NP by considering their whole life cycle from synthesis to application and disposal to gain deeper insights into the requirements for environmentally more benign particles. nanoToxCom thus pursued two general objectives:

1) *The hazard assessment of selected metallic and metal-oxidic nanoparticles (silver and iron (oxide) NP) in combination with other physical/chemical stressors in relation to selected exposure scenarios.*

2) *The derivation of recommendations for synthesis, processing and distribution of NP.*

The group activities were based on the special expertise generated by the synergistic interactions of different disciplines such as biochemistry, organic chemistry, physical chemistry, environmental chemistry, risk assessment, process engineering as well as aquatic and soil ecotoxicology (http://www.nanotoxcom.uni-bremen.de).

1.3 Applications of Iron Nanomaterials

Iron-based NM are applied due to their unique characteristics, such as their small size, surface chemistry and magnetic properties. Besides some special applications e.g. in magnetic seals and inks, data storage, and ferrofluids (Teja & Koh 2009), the two most prominent application fields of iron NM are in medicine and in environmental remediation. The following section provides a short introduction to these two application areas.

1.3.1 Medical Application of Iron (oxide) Nanoparticles

In medical applications, usually iron oxide NP (IONP) are used. Due to their superparamagnetic properties and relatively low harmful effects, IONP are under investigation for several medical applications (Pankhurst et al. 2009, Roca et al. 2009).

The most prominent application of IONP might be the use as a contrast agent in magnetic resonance imaging (MRI) (Chaughule et al. 2012, Qiao et al. 2009). Currently, gadolinium (Gd)-based contrast agents dominate in MRI. However, Gd itself is toxic and can induce negative side-effects which are clinically referred to as nephrogenic system fibrosis. This disease has been found primarily in patients with renal insufficiencies (Berry & Green 2010) with symptoms of joint pain, muscle weakness and skin problems. Toxicity is related to the release of Gd-ions from the chelated Gd-complex and metal toxicity (Grobner & Prischl 2007). In contrast, IONP seem to be less toxic and can significantly enhance the contrast in some MRI applications. IONP-based contrast agents have a longer half-life, which is advantageous for repeated imaging without subsequent administration (Winer et al. 2011). IONP are able to pass the blood-brain barrier and can persist in the brain for several days (Murillo et al. 2005), which opens up the possibility for repeated or long-term investigations, e.g. of brain tumors without renewal of the contrast agent (Geppert 2012).

IONP (and other NP) are also tested as controlled drug delivery systems. The nanocarrier can be functionalized with recognition ligands for cell-specific targeting. Here, drugs are precisely transported to the target location, consequently reducing the required doses

(Wilczewska et al. 2012). Having the ability to pass the blood-brain barrier, NP were already shown to serve as a possible vehicle for drug delivery to the brain, e.g., IONP were successfully used as a drug delivery vehicle for MRI-monitored magnetic labeling of brain tumors (Chertok et al. 2008). Combining the possibility of cell targeting with the MRI-enhancing properties, IONP can also be used for precise tissue and tumor labeling and imaging (Cromer Berman et al. 2011).

Another application also uses the superparamagnetic properties of IONP for anti-tumor treatment. IONP are directly injected into the tumor. With a high frequency magnetic field the IONP are set into fast oscillation, which induces hyperthermia of the surrounding tumor tissue (Yu et al. 2013).

1.3.2 Environmental Application of Iron Nanomaterials

Pump-and-treat methods were often applied in the past for the environmental remediation of contaminated groundwater. While they are very effective, they are also very cost-intensive due to their huge impact on the environment. Alternatively, below-ground (*in situ*) remediation with thermal treatment or permeable reactive barriers (PRB) is possible. PRB are composed of materials which degrade or immobilize contaminants when the groundwater passes through the barrier. Currently, injection methods are often preferred due to their high cost efficiency and low impact on the environment compared to classic treatment methods. A reactive substance is injected into groundwater or soil directly into the contamination plume (Crane & Scott 2012, Karn et al. 2009).

The experimental application of (iron) NM for *in situ* injection methods has increased in the past decade. Due to the highly enlarged surface of nanostructures, they provide a much higher reactivity compared to granular materials (Karn et al. 2009, Wang & Zhang 1997). The use of a smaller mass of material to achieve equal or even better results can theoretically conserve both raw materials and energy with significant associated cost savings (Crane & Scott 2012, Masciangioli & Zhang 2003).

In water, the movement of microscale particles is largely controlled by gravity-induced sedimentation because of their size and high density. In contrast, nanoscale particles are so small that their physical movement and transport are dominated by Brownian movement or random motion. The small size of NP allows the material to deeply penetrate into soils, and it can be more easily injected into shallow and deep aquifers (Karn et al. 2009, Noubactep et al. 2012). NM originating from wet synthesis can be directly injected as a liquid into the

contaminated subsurface. Furthermore, NP are often coated with a stabilizing material, e.g. polymers, to increase long-term colloidal stability for higher efficiency (Henn & Waddill 2006). Additionally, the coating material itself can have beneficial catalytic properties, whereby the NP may act as a carrier vehicle (see publication 3 of this thesis). However, in soil systems the risk of uncontrolled NP distribution and drift might be overestimated since transport distances are limited to a few centimeters, primarily due to heteroaggregation with soil surface coatings (Emerson et al. 2014, Gomes et al. 2013, Lin et al. 2010).

For environmental remediation, often iron-based NM are used, which usually consist of zerovalent iron (Fe^0/nZVI) because of their high redox reactivity (Tang & Lo 2013). nZVI was found to be 10 – 1,000 times more reactive than granular iron (Wang & Zhang 1997).

Depending on the contaminant chemistry, various possible contaminant removal pathways have been identified, including sorption, complexation, (co)precipitation and surface mediated chemical reduction (Crane & Scott 2012). The authors summarized that iron NP

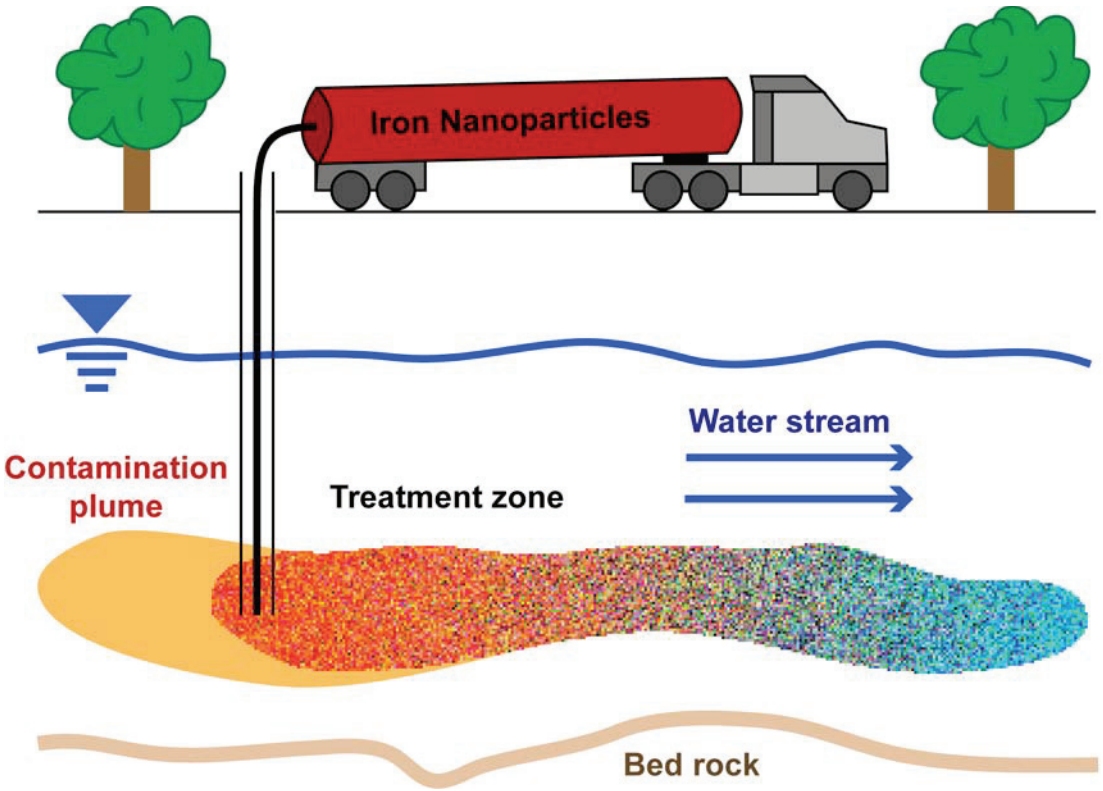

Fig. 2: Simplified scheme of the application of iron-based NP (INP) for the remediation of contaminated groundwater. INP are injected into the plume. Due to their superparamagnetic properties INP may even be recovered later by magnetic treatment.

“are effective for the removal or degradation of a wide range of chemical pollutants, including: ß-lactam and nitroimidazole-based antibiotics; azo dyes; chlorinated solvents; chlorinated pesticides; organophosphates; nitroamines; nitroaromatics; p-chlorophenol; polybrominated diphenl ethers; polychlorinated biphenyls; inorganic anions, including nitrate and perchlorate; alkaline earth metals, including barium and beryllium; transition metals, including chromium, cobalt, copper, lead, molybdenum, nickel, silver, technetium and vanadium; post-transition metals, including zinc and cadmium; metalloids, including arsenic, selenium; and actinides, including uranium and plutonium” (Crane & Scott 2012 and citations therein).

Due to their low toxicity (compared to other substances, e.g. reactive chemical oxidants) and their efficiency in relation to a broad range of pollutants, iron NP are increasingly applied in environmental remediation (Nanotechproject 2014). Injected in the plume, they build a highly efficient reaction zone in the groundwater stream (**Fig. 2**). Due to their superparamagnetic properties, iron NP might even be recovered by magnetic treatment (Rickerby & Morrison 2007). In this way, pollutants adsorbed to the NP can be completely removed even though this technique might be more relevant for the treatment of contaminated surface or sewage water (Brame et al. 2011, Tang & Lo 2013).

Although nZVI is referred to as being metallic, each particle exists in natural conditions with a thin but encapsulating layer of surface Fe oxides (Crane & Scott 2012) due to the following redox reactions:

$$Fe^{0}_{(s)} + 2H_2O_{(aq)} \rightarrow Fe^{2+}_{(aq)} + H_{2(g)} + 2OH^{-}_{(aq)}$$

$$2Fe^{0}_{(s)} + 4H^{+}_{(aq)} + O_{2(aq)} \rightarrow 2Fe^{2+}_{(aq)} + 2H_2O_{(l)}$$

(s = solid; aq = aqueous; g = gas; l = liquid; Matheson & Tratnyek 1994)

The corrosion products can be Fe hydroxides or oxides, which might also impact the retention of contaminants, transformation products, and their colloidal stability (Noubactep et al. 2012). Since Fe oxides are the predominant form of iron-based NM in natural environments, the focus of the investigations in this thesis is on environmentally more relevant iron oxide nanoparticles (IONP).

1.4 Potential Environmental Risks from Iron Nanomaterials

By the application of iron NM for environmental remediation, tons of nanosized material are consequently released to the environment with unknown risks to organisms. “The same

properties that make iron NM useful for the environmental application, in particular their small size and their high redox reactivity, also make them potentially harmful to organisms" (Nel et al. 2006). Their potential environmental risks *in situ* are largely unknown (Kharisov et al. 2012). Most studies have focused on the *in vitro* toxicity of iron NM (Grieger et al. 2010, and references there, Soenen & De Cuyper 2010, Tang & Lo 2013). The predominant mechanisms for cellular damage are considered to result from iron reduction leading to the formation of reactive oxygen species (ROS). Within cells, ROS can cause oxidative stress, lipid peroxidation, and DNA damage (Mahmoudi et al. 2012, Xia et al. 2006).

In nature, several transformation processes are possible, such as: (1) chemical, (2) physical and (3) biological transformation and (4) interaction with macromolecules – all altering the fate, transport, and toxicity of NM (Lowry et al. 2012). Under natural conditions, Fe^0 is quickly reduced to Fe oxides/hydroxides (Crane & Scott 2012). This natural "aging" of nZVI leads to corrosion products (IONP) with changed properties. In general, environmentally processed iron NM present a significantly reduced risk to organisms since iron oxides themselves are less (cyto)toxic than nZVI (Phenrat et al. 2008). Furthermore, the volume of any corrosion product is higher than that of Fe^0 (Noubactep et al. 2012), thus increasing the iron NP size. The (nano)toxicity of voluminous corrosion products might decrease, as the oxidized iron NP might lose their nano properties.

However, NP are often functionalized with a stabilizing surface coating to increase their colloidal properties and longevity, and therefore their mobility (Kim et al. 2009). The coating can significantly influence the physicochemical properties of NP and therefore also their risk to biota. Given the anticipated higher hazard potential of smaller NP, a prolonged size-stabilization might greatly increase their potential impacts. In order to achieve a full risk assessment – not only of iron NM – it is essential to investigate the effects of both the core and the coating materials, both alone and in combination (as the final product). The applications of iron NM for remediation have already proven their efficiency on the laboratory scale (Karn et al. 2009, Khin et al. 2012). Potential risks from iron NM should normally not outweigh their advantages. Nevertheless, effects of the endproducts and by-products of a remediation have not been fully investigated and there is a serious lack of knowledge on the long-term data, including the persistency and migration of iron NM in natural environments (Grieger et al. 2010).

However, IONP are less toxic than other metallic NP due to the low toxicity of Fe (Zhang et al. 2012). This allows for an investigation of the effect of the NPs' size, form, particle concentration, coating material, surface charge, and colloidal properties on organisms

without the influence of a highly toxic core material or other effects, e.g. induced by released toxic metal ions.

1.5 Silver Nanoparticles: Applications and Implications

Due to their antimicrobial properties, nano-silver (AgNP) is already present in many different (consumer) products ranging from biomedical applications, domestic appliances and cleaning products, functional textiles, cosmetics and personal care products (Allen et al. 2010, Arvizo et al. 2012, Chaloupka et al. 2010, Kokura et al. 2010, Marambio-Jones & Hoek 2010, Siripattanakul-Ratpukdi & Furhacker 2014). In May of 2014, the Nanotechproject database reported 424 registered consumer products worldwide containing nano-silver, which is about 25 % of all registered applications (Nanotechproject 2014).

Due to their main application in biocide products, most studies of AgNP focused on the *in vitro* toxicity in microorganisms or cell systems as reviewed by Arvizo et al. (2012). Reidy et al. (2013) concluded that AgNP (cyto-) toxicity can be attributed to different mechanisms: (1) direct interactions (adhesion) of AgNP with cell surfaces, altering the membrane properties or inhibiting cell wall embedded components (Dasari & Hwang 2010, Wong & Liu 2010); (2) AgNP penetrating inside the cell and interruption of the cell metabolism; (active) transport might be easier for uncharged AgNP than for charged Ag^+ (Choi & Hu 2008); (3) dissolution of AgNP releasing highly toxic silver ions (Ag^+) (Asghari et al. 2012, Jo et al. 2012), which can interact with sulphur-containing proteins in the (bacterial) cell wall and phosphorus-containing compounds such as the DNA (Magdolenova et al. 2014, Morones et al. 2005, Samberg et al. 2011, Wong & Liu 2010); (4) the formation of ROS, which can be formed by either AgNP (due to oxidation processes) and Ag^+ inside and outside the cells, inducing oxidative stress (Choi & Hu 2008, Hwang et al. 2008, Marambio-Jones & Hoek 2010, Yang et al. 2013); and (5) the disruption of the transmembrane electrochemical gradient by disturbing the ATPase (and consequently the ATP synthesis), leading to cell death (Bianchini et al. 2005, Cao et al. 2011, Grosell et al. 2002).

The existing results on the toxicity of AgNP are very heterogeneous. Some authors found that AgNP were more toxic than Ag^+ (Amato et al. 2011, Choi & Hu 2008, Lok et al. 2007), others found contrary effects (Levard et al. 2012, Navarro et al. 2008b, Sotiriou & Pratsinis 2010, Yang et al. 2013, Zhao & Wang 2011). The mode of toxicity highly depends on the specific physicochemical properties of AgNP such as size, shape, crystallinity, surface charge, surface coating, elemental composition, solubility, dissolution, adsorption and agglomeration (El Badawy et al. 2011, Levard et al. 2012, Siripattanakul-Ratpukdi &

Furhacker 2014), all of which define both bioavailability and biocompatibility. Furthermore, the (microbial) test species of choice can highly influence the outcomes due to the very different sensitivities against Ag exposure (Guzman et al. 2012, Marambio-Jones & Hoek 2010).

Because of their applications, AgNP are mainly released to waste waters. As a consequence, Ag may significantly damage microorganism communities in sewage treatment plants, leading to the failure of the treatment (Hou et al. 2012, Siripattanakul-Ratpukdi & Furhacker 2014, Yang et al. 2013). Their potential impacts *in vitro* and *in vivo* were compared by Bondarenko et al. (2013). Environmentally relevant test species (crustaceans, algae and fish) were most sensitive, and – surprisingly – AgNP were less toxic to bacteria. This would indicate the need to be careful about the applications of AgNP, since there are still unknown risks for non-target organisms.

1.6 Objectives

The presented studies of this thesis aim to examine several aspects of IONP impacts on the big water flea *Daphia magna*, one of the preferred test organisms in aquatic nanotoxicology. Due to the low toxicity, it was hypothesized that IONP might only significantly harm daphnids at high concentrations. Due to the low toxicity of iron (oxide), IONP are useful for the investigation of nano-effects without the influence of additional toxicity, e.g. toxicity induced by ions decomposed from the metallic cores.

The major objectives where:

1. **Toxicity of Different IONP**

   Due to the low toxicity of iron (oxides), the effects of IONP on organisms are determined by their physicochemical properties such as surface charge or colloidal stability. These properties are influenced and controlled with specific surface functionalizations (coatings). To verify this assumption, acute tests with differently coated IONP have been performed.

2. **Combinatory Toxicity**

   Iron-based NP are applied for the environmental remediation due to their ability to process and bind/complex various toxic contaminates. Not much is known about the risks or benefits of remediation processes for biota. Remediation products might represent an increased risk to the environment. For example, IONP might mobilize

potentially immobile chemicals and serve as a transport vehicle to organisms. For the investigation of combinatory effects, IONP were incubated with four different potential target contaminates and afterwards acute toxicity tests were performed and compared to the toxicity of the single substances.

3. Long-term Toxicity

Often acute tests underestimate the risk from substances. Especially (metallic) NP often induce effects much later than their corresponding dissolved ions. Thus, investigations of long-term effects are important to understand how NP might affect the life history responses of organisms. 21-day chronic exposure tests of *D. magna* with different IONP were conducted and multiple development and reproduction parameters were investigated.

4. Accumulation and Depuration

For filter-feeding organisms such as daphnids, the main exposure route appears via ingestion. It should thus be expected that the effects of NP exposure will mainly be related to the intestines. However, the ingestion of NP is not synonymous with the bioaccumulation of NP. Therefore, NP also have to be enriched in internal compartments such as the organs. In order to investigate the potential bioaccumulation of IONP in *D. magna,* ingestion and depuration tests were performed by measuring the iron contents of exposed and unexposed individuals over a certain period of time.

5. Test Systems

Standardized tests systems were developed for the testing of dissolved chemicals. Often, they are only partially effective for the investigation of NP due to the NP unique characteristics. Additional analytics are needed for a better understanding of NP effects. The *daphnia* tests were performed according to OECD standard protocols and checked for their suitability in NP testing. It was investigated how these tests might be adopted, e.g. by prolonging or miniaturizing the acute toxicity test. Due to their physicochemical characteristics AgNP were used as a reference NM.

2 Methodology

2.1 Synthesis of Iron Oxide Nanoparticles (IONP)

There are various ways of producing NM. They can mainly be divided into two approaches to the synthesis of NM and the fabrication of nanostructures: top down and bottom up. Top-down techniques are typically based on the attrition or milling of materials. Although this is a relative simple production method, it has several disadvantages such as imperfect surface structures and difficult size distribution control. In contrast, bottom-up approaches refer to the building up of material from atoms, molecules, or clusters. These methods are often more complex, but they bring the advantage of highly controlled size distribution, surface structure and particle shape (Cao 2004).

The synthesis of all IONP used within the studies of this thesis were one-pot reactions though bottom-up syntheses. All IONP were produced by Darius Arndt, PhD student and a member of the nanoToxCom graduate school. He developed the synthesis of several differently functionalized, water-soluble IONP (cf. Arndt et al. 2012). The synthesis was based on the thermal decomposition of iron(III) acetylacetonate ($Fe(acac)_3$) in diethylenglycol (DEG). By varying the synthesis temperature between 453 and 523 K, the primary particle diameter could be controlled. Primary particle diameters ranged from 4 to 8 nm. All IONP obtained by this method mainly consisted of magnetite (Fe_3O_4).

In order to guarantee high colloidal stability, the IONP were functionalized with different surface coatings. Most studies in this thesis investigated polyvinylpyrrolidone (PVP)-coated IONP (PVP-IONP). PVP is a polymer by which a steric repulsion of the NP is achieved. It provided the best (long-term) colloidal stability of all stabilizers. By the addition of PVP to the reaction mixture, its binding to the IONP was achieved during the NP formation process.

To separate the IONP from DEG and possible residues, acetone was added after cooling the synthesis product and the mixture was centrifuged. The supernatant was decanted. The precipitated IONP were air-dried and re-suspended in water.

Other ligands were attached to the IONP in a post-synthetic process. After purification, the IONP were re-suspended in a dispersion containing the respective ligand. Residual ligand material was removed by precipitating the IONP with acetone and magnetic treatment of the solution. The ligands ascorbate (ASC), citrate (CIT), and dextrane (DEX) delivered an appropriate colloidal stability of IONP in the *Daphnia* culturing medium (Elendt M7). Other coatings used by D. Arndt were not tested because of their insufficient colloidal stability in the medium. These IONP quickly agglomerated and settled, which would have reduced their bioavailability to (pelagic) organisms such as *Daphnia*. For this reason, they were not relevant to the questions examined in this thesis.

2.2 The Applied Silver Nanoparticles

AgNP were used as a reference material in publication 1. They were chosen, since AgNP are known to show typical (critical) behaviours of metallic NP in the aquatic environment, e.g. agglomeration, adsorption to surfaces and micro particles (such as algae) as well as decomposition and release of (toxic) ions (Lau et al. 2013, Reidy et al. 2013, Siripattanakul-Ratpukdi & Furhacker 2014).

The applied NM-300K AgNP are part of the priority list (NM-Series) of the Representative Manufactured Nanomaterials (RMN) of the European Commission Joint Research Centre (JRC) supported by the Organization for Economic Co-operation and Development (OECD) Working Party on Manufactured Nanomaterials (WPMN) Sponsorship Program. They were purpose-made for measurement and testing for hazard identification, risk and exposure assessment studies. NM-300K is nano-silver with a primary particle diameter <20 nm. NM-300K is purchased as a colloidal dispersion with a nominal silver content of 10 w/w%. The aqueous dispersion contains stabilizing agents consisting of 4% w/w% each of Polyoxyethylene Glycerol Trioleate and Polyoxyethylene (20) Sorbitan mono-Laurat (Tween 20) (Klein et al. 2011).

NM-300K was also used in the “UMSICHT” project partly carried out at the UFT (http://www.umsicht.uni-bremen.de), which gave me the opportunity to rely on existing knowledge – especially on the physicochemical characterization – from the project within the presented account.

2.3 Nanoparticle Analyses

In order to determine and to interpret effects of NP a detailed characterization of the physicochemical properties is essential. Size, size distribution, concentration, colloidal stability and agglomeration, surface charge, and degree of decomposition are parameters that always have to be tracked before, during and after the ecotoxicological testing of NP. The following section will provide a short introduction on the analytical methods applied in this thesis.

2.3.1 Size Determination

Light scattering methods, such as dynamic light scattering (DLS), belong to the most commonly used methods for measuring the size of colloids and NP (Kato et al. 2009, Lopez-Serrano et al. 2014).

DLS allows a noninvasive characterization of particle emulsions and molecules dispersed or dissolved in liquids (Zanetti-Ramos et al. 2010). Usually, no further preparation of a sample is needed. DLS is a sensitive, accurate and reliable method to track the hydrodynamic diameter of colloids between 0.6 nm and 7 µm (Beckman Coulter 2010). The hydrodynamic diameter can be explained as the diameter of the dispersed particle and its surrounding dispersion molecules, which interact due to electrostatic forces. In the case of water, dispersed particles are always surrounded by a hydrate shell. Furthermore, dissolved ions can also interact with the particles surface.

DLS also provides information on the size distribution within a colloidal dispersion, even though it is more powerful in monomodal particle populations. In complex particle systems with multiple size fractions, bigger particles often mask the signal of smaller ones (Zänker & Schierz 2012). At times ultrafiltration of the sample was necessary to measure smaller particle fractions.

During measurement, the particles are irradiated with a laser light, so that the scattered light emitted from the particles is detected. Due to Brownian motion or gravity, particles are always in motion with movement speeds which are dependent on the particles size. Consequently, the relative positions of particles changes in time, and thus the time fluctuations of the scattered light intensity are observed and analyzed using an autocorrelation function (Beckman Coulter 2011).

DLS measures the hydrodynamic diameter, which should not be understood as the NPs' primary/physical diameter. Similarly, it does not provide information about the core-shell structure or the shape of NP. For these measurements, more invasive methods such as transmission electron microscopy (TEM) must be applied. TEM was used by Darius Arndt during the development of the particles' synthesis as a quality control (Arndt et al. 2012). Due to the invasive preparation of the NP, e.g. sample drying, TEM cannot reflect the NPs' *in situ* state (Zänker & Schierz 2012). Since NP underlie multiple transformation processes in ecotoxicological media and tests, such as swelling, agglomeration, sedimentation and dissolution, size-related measurements of this thesis focused on DLS measurements.

2.3.2 Surface Charge

The interactions of NP and biological surfaces and their colloidal properties highly depend on their surface charge. This is determined by the surface composition such as the surface

material and oxidation, the functionalization respectively the coating, and the interacting ions and other molecules.

Dispersed particles usually have a positive or negative surface charge. In order to sustain electric neutrality, charged particles are surrounded by ions with an opposite charge, building an “electrical double layer”. The concentration of the counter-ions gradually decreases with distance from the particles’ surface. In the area far from the surface, positive and negative ions exist in equal numbers to maintain electric neutrality. Thus, the diffuse electrical double layer around a charged particle can be divided into two layers: (1) the layer of ions closely attached to the surface, also called the “Stern layer”; and (2) the “diffuse layer” outside the Stern layer. The zone between the Stern and the diffuse layer is called “Slipping plane” (Beckman Coulter 2011, Handy et al. 2008b).

The surface charge is usually measured via the ζ-potential (zeta). It is defined as the potential at the slipping plane. Particles with a high zeta potential are colloidally stable due to high electrostatic repulsion. On the hand, for particles with a low zeta potential value (approaching zero) the probability of particle collisions increases, thus increasing the possibility of building agglomerates (Beckman Coulter 2011).

When an electric field is applied to a dispersion containing charged particles, the particles move towards the electrode with the opposite charge. Since the particles’ velocity is proportional to their charge, the zeta potential can be estimated from their movement speed. In order to measure the electrophoretic mobility of particles, they are irradiated with a laser beam, which allows to detect the scattered light emitted from the particles. This method is called “Electrophoretic light scattering” and is based on the “Doppler effect” (Beckman Coulter 2011).

2.3.3 Concentration Measurements

To conduct a sound ecotoxicological effect study of NP, exact concentrations have to be determined. NP concentrations can be indicated in particle concentrations (e.g. particles/L), the specific surface (m^2/L), or the substance concentrations (e.g. mg/L). Because they can be directly measured in the latter case, this is often preferred. Furthermore, as in classic ecotoxicological studies, it allows for a direct comparison with the dissolved ionic form of the substance of interest. However, the two other objects of study can also provide important information for the interpretation of physicochemical properties and biological effects.

The most common way to measure NP concentration is atomic absorption spectroscopy (AAS). AAS allows direct measurement of NP dispersions with little or no sample preparation (Lopez-Serrano et al. 2014). However, due to the high adsorption potential of AgNP and Ag ions to the storing container walls, in this case, it was necessary to pretreat all samples with HNO_3.

AAS can be conducted using two different techniques: flame and graphite furnace AAS. Both depend on the same principal: Samples are atomized or vaporized to produce free ground-state atoms. Ground-state atoms are able to absorb energy in the form of light to be elevated to an excited state. Each element absorbs light at characteristic frequencies or wavelengths. The amount of absorbed light is detected and can be correlated to the concentration of the element of interest (US EPA 2014).

Since there is much evidence that metallic ions might be more toxic than their nanoparticular form, the determination of dissolved metal ions in the NP dispersion was one of the greatest analytical challenges in some of the presented studies. Before measuring the concentration, the ions were separated from the NP via ultrafiltration. Samples were transferred to tubes with a 3 kDa cutoff membrane in the middle. Separation was achieved by centrifuging the sample. This is an easy and fast approach with little sample preparation. However, interactions with the filtration membrane might influence actual ion concentrations (Lopez-Serrano et al. 2014).

Concentrations of IONP or iron, respectively, were further detected with a photometric assay. The “iron assay” was used to measure iron contents in (exposed) daphnids. The method was adopted from Riemer et al. (2004) with a detailed description in Manuscript 5. *Daphnia* tissues and IONP were digested. The dissolved iron ions were then stained with ferrozine. The staining signal was detected with a photometric plate reader and compared to standards, allowing a precise detection of iron contents.

2.4 Test Organism Daphnia magna

Daphnia magna, the big water flea on which this thesis has concentrated, is a planktonic invertebrate belonging to the phylum of Arthropods, the subphylum of Crustacea, the class Branchiopoda, the subclass Cladocera and the family of Daphniidae (Ruppert et al. 2004).

An adult of *D. magna* can reach a size of 6 mm. Its phenotype is characterized by its large swimming antennae and the two uncalcified carapace shells building the exoskeleton, an attribute of all Arthropods. The exoskeleton largely consists of chitin, a polysaccharide. In

order to grow, all Arthropods must molt by shedding the old exhuvia in favor of a new exoskeleton (Ruppert et al. 2004). Daphnids have 9 joint appendages. From front to back these are: sensory antennules, swimming antennae, maxillae, mandibles and 5 limbs on the trunk which serve feeding and respiration. At the end of the abdomen they have a pair of claws. At the posterior end of the carapace, daphnids have a distal spike, which can become longer under predatory pressure. Some species are even able to form head spikes under predatory conditions (Ebert 2005, Vollmer 1960). All Cladocerans have an unpaired compound eye, which is a result of a fusion of two eyes during late embryonic development. Located between the compound eye and the mouth, they have an additional unpaired naupliar eye (Ebert 2005). The primary internal cavity, the hemocoel, contains their internal organs and has a hemolymph system (open circulatory blood) (Ruppert et al. 2004).

Daphnids inhabit most types of standing freshwater like ponds and lakes, but they can also be found in small (temporary) pools and puddles as well as slowly flowing water. They colonize the shallow zones of the water body. They prefer the warm and eutroph littoral zones. Due to their size and slow movement, they find cover there from predators such as fish.

Fig. 3: Female daphnids. **Left:** *Daphnia magna*. **Right:** The functional anatomy of *Daphnia* sp. (Vollmer 1960); A = First Antenna (antennule): sensory organ; A* = Second Antenna: locomotion; Au = Compound eye with 22 ommatidia and black pigment: basic vision and orientation; B = Brood chamber (here without eggs); Bf = first limb; D = Digestive tract: Divided into esophagus, mid-, and hindgut; G = Brain (cerebral ganglion); H = Heart: pump of open blood system; K = Gill sac; L = Hepatic caeca (Diverticulum): production of digestive fluids; Md = Mandible: mechanical food processing; N = Nauplius eye (Ocellus); Ov = paired ovary: with parthenogenetic oocyte clusters; S = carapace shell; Sd = shell gland; So = lateral sensory organ.

The pelagic-living daphnids predominantly feed on planktonic algae and detritus. The filter feeders effectively gather food particles from the water body with the help of their filtering apparatuses. With their phylopods they produce a water current flowing from anterior to posterior. At the same time, they collect particles with special setae that transfer the food to the grove. *D. magna* can also feed on settled detritus by actively swirling it up (Ebert 2005, Flößner 2000, Vollmer 1960). *D. magna* can ingest particles and microbes from around 1 µm up to 70 µm (Burns 1968, Scholten et al. 2005).

The digestive tract resembles a tube. The gut is divided into 3 parts, the esophagus, the midgut, and the hindgut. Nutrient assimilation appears via microvilli of the midgut epithelial cells by absorption of molecules. The epithelial cells are protected by the paratrophic membrane (PTM) inside the lumen of the gut. It has a mesh-like structure and only lets particles pass which are smaller than 130 nm. Furthermore, the PTM prevents excessive settling of microorganisms inside the gut. With peristaltic contractions of the gut walls food is passed through the gut. The excretion of feces from the hindgut requires additional pressure of more recently acquired food (Ebert 2005).

Under optimal conditions, the life cycle of *D. magna* is characterized by its asexual reproduction (**Fig. 4**). Female daphnids reproduce via parthenogenesis. After each adult molting, diploid eggs are released to the dorsal brood chamber under the carapace shells. After about 1 day the embryos hatch and remain in the brood chamber for further development for another 1 to 2 days. The development is immediate without a larval stage (Ebert 2005, Sommer 1996). The neonates are released by the mother through ventral movements of the abdomen. This is followed by another molting of the mother and the reproduction circle is repeated (Vollmer 1960). A juvenile daphnid passes 4 to 6 instars before it becomes primipare which is reached after around 5 to 10 days (Ebert 2005).

Apart from the parthenogenic reproduction, daphnids also undergo sexual reproduction (**Fig. 4**). The appearance of parthenogenetic diploid males for self-fertilization of the females is triggered by a complex set of different stimuli, e.g. limited food availability in combination with a high population density. The production of males is followed by the formation of only two haploid eggs, which will be fertilized by the males. Afterwards, the two eggs are encapsulated in a strongly melanized ephippium. With the next molting, these resting eggs sink to the ground or float away with the water stream. Even the transportation by animals or with the wind is possible. Usually, the resting eggs undergo a latency phase to endure unfavorable seasons. Hatching is induced by external stimuli such as an appropriate photoperiod or temperature or simply the presence of water in a previously dry pond. From

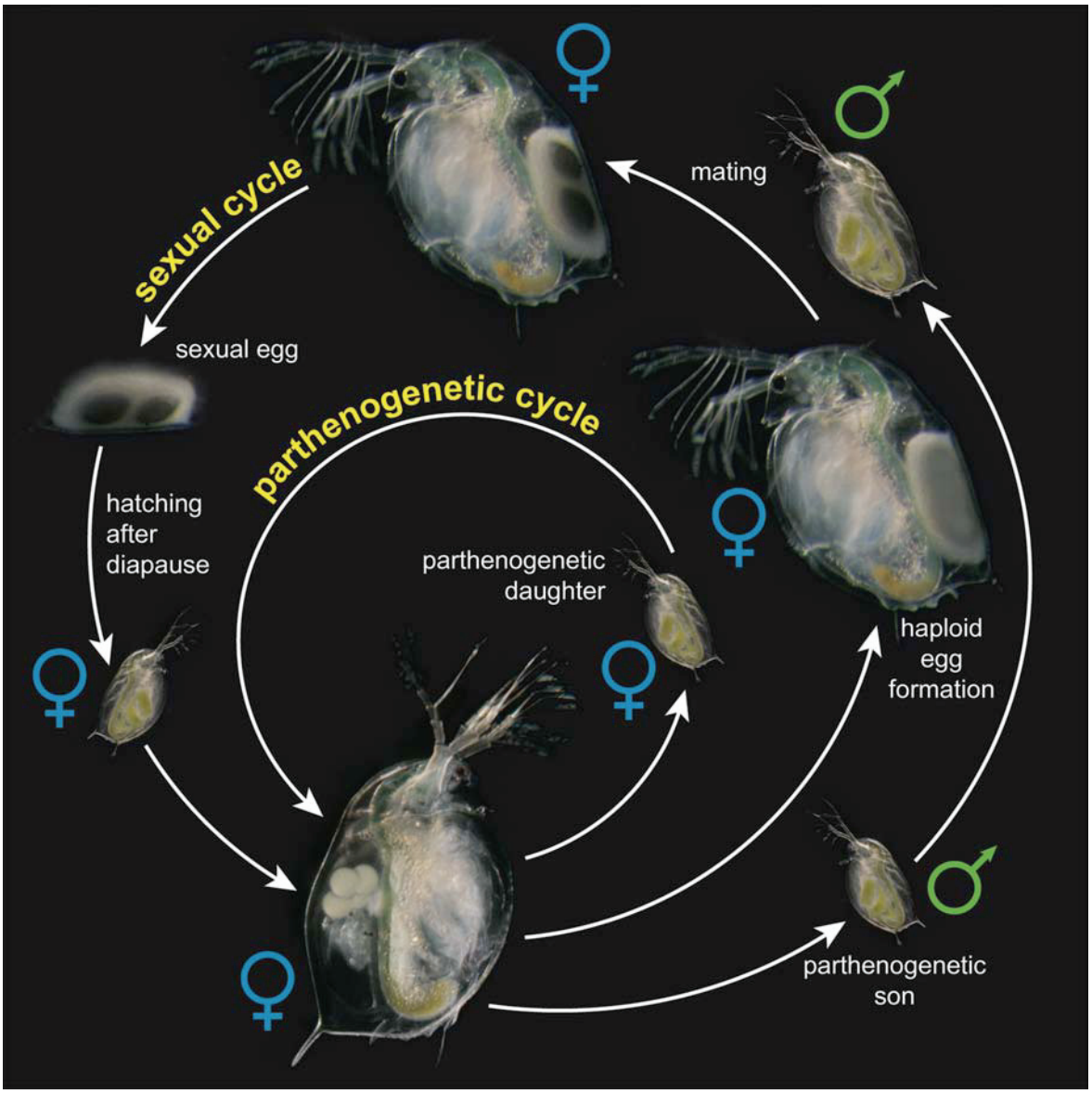

Fig. 4: Scheme of the sexual and asexual (parthenogenetic) life cycle of *Daphnia sp.*.

the resting eggs, only females hatch, which usually continue asexual reproduction (Ebert 2005, Sommer 1996, Vollmer 1960).

Daphnids play an important role in many freshwater ecosystems. Because they are often the predominant form of the zooplanktic biomass, daphnids are an important food source for planktivorus fish, thus playing an essential role in the food web of surface waters (Sommer 1996). Due to their enormous filtering capacities, daphnids are able to significantly influence the equilibrium of surface waters. Especially in eutrophic waters, the development of algae can quickly increase in spring and summer. These algae blooms are often followed by a quickly increasing *Daphnia* population density, consequently decreasing the algae density

and sometimes also resulting in a clear-water stadium (Flößner 2000, Scholten et al. 2005). This phenomenon is explained by the Lotka-Volterra model, which describes predatory-prey relationships (Brauer & Castillo-Chavez 2000, Wittig & Streit 2004). In this way, daphnids can considerably contribute to the self-purification of a surface water.

Daphnids are easy to culture. They have a quick reproduction circle, their biology is extensively investigated and they are sensitive to pollutants. These characteristics make daphnids a predestined organism for ecological testing (Zitova et al. 2009). Due to their filter feeding mode of life, their important role in the food chain, and the fact that they are the most commonly used invertebrate species in regulatory chemical testing, daphnids are often taken into consideration for testing NP (Baun et al. 2008a, Li et al. 2010). The enteral exposure of actively and passively (bond to food particles) ingested NP has already been shown (Alves de Matos et al. 2009, Feswick et al. 2013, Heinlaan et al. 2011, Hu et al. 2012, Lovern et al. 2008, Mendonca et al. 2011, Rosenkranz et al. 2009, Zhu et al. 2010), but further harmful effects via dermal or gill exposure might also be possible.

2.5 Culturing of Daphnia magna

At the beginning of this work, it was necessary to establish cultures of living daphnids. *Daphia magna* was cultured in a semi-static setup. In each case, 30 daphids were elevated in a volume of 1.5 L of Elendt M7 medium (detailed composition see OECD 1998). The same medium was also used for all tests. The cultures were placed in a climate-controlled chamber at 20±1 °C and a 16:8 h day-night rhythm and gentle aeration. The medium was renewed twice a week or when the cultures were synchronized one day before a test to separate mothers and neonates. The daphnids were fed with living algae (*P. subcapitata*) on a basis of 200 µg carbon per daphnid and day.

Two different *D. magna* clones were used. The first one was the Bayer clone B (Bayer, Monheim, Germany) obtained from the Helmholtz Center for Environmental Research (UFZ, Leipzig, Germany). The second clone was the IBACON clone (IBACON, Roßdorf, Germany) obtained from the Aquatic Ecotoxicology group of Prof. Dr. Oehlmann at Goethe University (Frankfurt, Germany). Each clone was cultured in 2 to 4 independent culture strains. Mothers in one culture were all of the same age, but usually mothers of different strains had different ages. This was meant to ensure that neonates were continuously available for the tests. Furthermore, the independent cultures ensured that possible diseases such as fungal infection did not infest all daphnids, which might have led in the worst case to complete extinction.

2.6 Ecotoxicological Test Systems

Environmental chemicals can take effect on many biological levels; from single molecules to complete ecosystems. Despite this, most ecotoxicological studies are performed on the single-organism level. In ecotoxicological tests, representative species from different trophic levels and with different modes of life are usually applied. Typical species are easy to culture in the lab. Although these simplified tests cannot reflect the complex situation in nature, they can provide reliable information on the possible impacts of a substance in the ecosystem. A better estimation of environmental modes of action is only possible with a series of different tests with different species (Fent 2003).

Due to the continuously rising amount of (new) chemicals in the market, the European Commission decided to launch the new European Chemical Regulation for the Registration, Evaluation, Authorization, and Restriction of Chemicals (REACH). Since gathering information on all chemicals would dramatically increase the use of laboratory animals, REACH recommends increasing the use of *in vitro* testing methods (European Commision 2012). These simple tests can quickly deliver reliable results, but they are usually limited to the biochemical level (Fent 2003). Relying solely on such methods can underestimate the potentially hazardous properties of chemicals that could harm humans and the environment (European Commision 2012). Therefore, *in vivo* and single organism tests still represent the main pillar of ecotoxicological testing (Fent 2003).

Most classic *in vivo* tests are performed with aquatic organisms, since the aquatic environment is the ultimate sink for any chemicals (van der Oost et al. 2003). In aquatic ecotoxicology, standard test systems are divided into acute and chronic tests. Test systems are based on static tests with and without renewal of the medium or the test substance and flow-through systems. The simplest tests are static and semi-static acute tests. These are short-term tests with a duration of between a few hours and a few days (max. 96 h). Endpoints are usually survival rate or mortality (Fent 2003). Although acute tests are very cost-efficient, they are the least representative and sensitive tests. In contrast, chronic tests have a usual duration of 21 or 28 days, or at least for a complete life-cycle of the test organism, and can be performed as semi-static or flow-through tests (Fent 2003). Chronic tests aim for more sensitive, sub-lethal endpoints such as individual growth or development, reproduction and population growth rates. Their performance is time-intensive compared to acute tests. However, they are more sensitive, and due to the versatile endpoints being investigated, they provide for far more information on the impact of (harmful) substances on the respective organism. This additional information is important for understanding and interpreting the complex processes in nature and for a consolidated hazard assessment.

In order to ensure standardized testing of chemicals, several ecotoxicological tests methods with a wide range of test species were developed and are prescribed by standard test guidelines. One of the most prominent test organisms are daphids. Artificial test systems with daphnids – mainly with *Daphnia magna* – are described by several guidelines and do not differ much from one another or are even based on one another. The most prominent acute test instructions are given in the OECD (Organization for Economic Co-operation and Development) Guideline 202 (OECD 2004), the ISO (International Organization for Standardization) Norm 6341 (ISO 1996) and the EPA (United States Environmental Protection Agency) Guideline (US EPA 1996). The acute tests are all performed as static or semi-static tests. The tests are conducted with neonates, since this life-stage is believed to be the most sensitive. At the test begin the neonates should be younger than 24 h. After 24 and/or 48 h exposure the immobilization or mortality of the daphnids is investigated. Immobilization means no normal swimming movement within 15 seconds after gentle agitation of the test vessel. From the results, one can calculate dose-response curves and estimate LC/EC_{50} or LC/EC_{10} values. In the standard set-up, the guidelines stipulate a minimum of at least five test concentrations each with 4 replicates containing 5 neonates. Tests should be performed in small beakers or comparable test containers.

Fig. 5: Scheme of the miniaturization of the *Daphnia sp*. acute toxicity test presented in publication 1. Due to the performance in 24-well microtiter plates, 50 % of animals and test substances can be saved compared to the OECD standard test. Alternatively, the performance of the standard test was tested in 6-well microtiter plates, which still allows remarkable time savings.

Within the context of this thesis, the miniaturization of the *Daphnia sp.* acute test was established in order to save material and to be able to perform more tests (simultaneously). Tests were performed in microtiter plates with 10 replicates per concentration and only 1 neonate per replicate (**Fig. 5** and **Fig. 6**A). The detailed adaptations of the test design are described in Publication 1. Nevertheless, it should be pointed out that all acute tests performed in this thesis used the miniaturized test design.

The most commonly used chronic test is prescribed by the OECD Guideline 211 (OECD 1998). The test duration is 21 days. In contrast the US EPA is using a 10 days protocol (US EPA 1994). All chronic tests are performed as semi-static tests, but can also be performed as flow-through systems. They aim to investigate sub-lethal life-cycle parameters of the daphnids such as overall reproduction, day of first offspring, size of first offspring and growth of the daphnids. Thus, they are also entitled “Reproduction Tests”. In contrast to the acute test, the daphnids are fed with living algae, e.g. *Pseudokirchneriella subcapitata*. At the test start, neonates not older than 24 h are placed in prepared test vessels. The daphnids are exposed in volumes of between 50 and 100 mL with one daphnid per replicate and 10 replicates per test concentration. The test medium should be renewed continuously. The renewal interval depends on the test substance. Usually the medium is renewed every 2 to 3 days. For this reason, new test vessels are prepared and the daphnids are carefully transferred with a pipette to the new test vessel. Every replicate is investigated on a daily basis for survivability and reproduction.

All chronic tests of IONP were performed according to Guideline 211 (OECD 1998). Tests were carried out in 100 mL glass beakers covered with a glass lid against evaporation (**Fig. 6**B). In derogation from the Guideline, tests were started in a volume of 30 mL (assuming young daphnids have lesser space requirements), which was gradually increased to 50 mL with the medium renewals. Furthermore, the interval for changing the test medium was extended to 3 or 4 days. Both adaptations aimed to save IONP, since the synthesis of IONP was very time-consuming, and relatively high doses had to be applied to achieve effects.

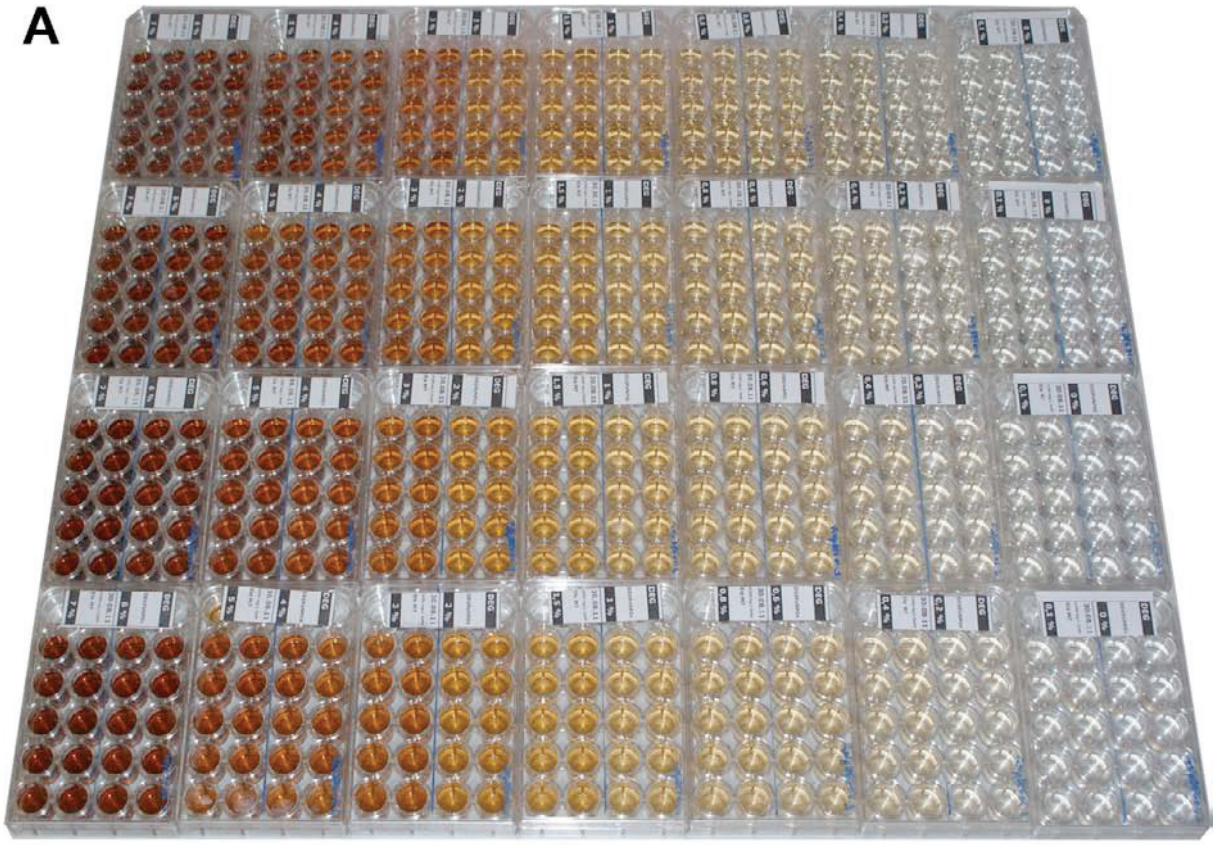

Fig. 6: Ecotoxicological testing of IONP with *D. magna*.
A: Acute test performed with the miniaturized test system in microtiter plates.
B: Chronic reproduction test performed according to OECD Guideline 211 (OECD 1998).

3 Publications and Manuscripts

3.1 Publication 1

Baumann J, Sakka Y, Bertrand C, Köser J, Filser J. (2014) **"Adaptation of the *Daphnia sp.* Acute Toxicity Test: Miniaturization and Prolongation for the Testing of Nanomaterials."** *Environmental Science and Pollution Research (international)* 21: 2201-2213.

The article can be downloaded from:
http://link.springer.com/article/10.1007%2Fs11356-013-2094-y

DOI: 10.1007/s11356-013-2094-y

Contributions of J. Baumann:

- Development of the miniaturized *D. magna* tests system
- Performance of experiments for Figure 1
- Support of experiments for Figure 2 and 3
- All statistical calculations and preparation of all figures and tables
- Preparation of the manuscript
- Finalization and submission of the manuscript

Y. Sakka performed all tests and analysis with silver nanoparticles (Fig. 2, 3; AgNP/$AgNO_3$ in Table 1). C. Bertrand supported several experiments with the daphnids and EC_{50} calculations with the statistics software R. J. Köser supported the AAS measurements of silver nanoparticles. J. Filser revised the manuscript.

3.2 Publication 2

Filser J, Arndt D, **Baumann J**, Geppert M, Hackmann S, Luther EM, Pade C, Prenzel K, Wigger H, Arning J, Hohnholt MC, Köser J, Kück A, Lesnikov E, Neumann J, Schutrumpf S, Warrelmann J, Bäumer M, Dringen R, von Gleich A, Swiderek P, Thöming J. (2013) **"Intrinsically Green Iron Oxide Nanoparticles? From Synthesis via (Eco-)toxicology to Scenario Modelling."** *Nanoscale* 5: 1034-1046.

The article can be downloaded from:
http://pubs.rsc.org/en/Content/ArticleLanding/2013/NR/c2nr31652h#!divAbstract

DOI: 10.1039/C2NR31652H

Contributions of J. Baumann:

- Performance of acute tests with *D. magna* and preparation of Figure 3
- Text parts concerning *Daphnia* tests in the Experimental section and Results and Discussions (Acute toxicity to *Daphnia magna*)

The rest of the manuscript was prepared by the co-authors.

3.3 Publication 3

Baumann J, Köser J, Arndt D, Filser J (2014) **"The Coating Makes the Difference: Acute Effects of Iron Oxide Nanoparticles on *Daphnia magna*."** *Science of the Total Environment,* 484: 176-184.

The article can be downloaded from:
http://www.sciencedirect.com/science/article/pii/S0048969714003532

DOI: 10.1016/j.scitotenv.2014.03.023

Contributions of J. Baumann:

- Performance of all *Daphnia* experiments and IONP analyses
- Statistical analyzes for Figures 3 and 5
- Preparation of all Figures and Tables
- Preparation of the manuscript
- Finalization and submission of the manuscript

J. Köser supported the AAS measurements of free iron in the dispersions. He did the theoretical assumptions for Figure 2 and parts of Table 1. Furthermore, he prepared text parts for the theoretical assumptions in the Methods part as well as in the Results and Discussions. D. Arndt synthesized all IONP for this study. J. Filser revised the manuscript.

3.4 Publication / Manuscript 4

Baumann J, Köser J, Bertrand C, Filser J (*under revision*) **"Acute Combinatory Effects of Iron Oxide Nanoparticles with Selected Contaminants on *Daphnia magna*."**

Contributions of J. Baumann:

- Performance of all *Daphnia* experiments
- All statistical analysis
- Preparation of Figure 1, Table 1 and 2, Table SI 1
- Preparation of the manuscript
- Finalization and submission of the manuscript

J. Köser did all theoretical calculations of binding capacities, provided text parts on binding kinetics and prepared the first draft of the supporting information. C. Bertrand supported the experiments. J. Filser revised the manuscript.

Acute Combinatory Effects of Iron Oxide Nanoparticles with Selected Contaminants on *Daphnia magna*

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Abstract

In the past decade iron-based nanoparticles (NP) have more and more come into the focus for remediation of contaminated groundwater and soil. Risks from this new technology to biota are unknown. In this account we conducted combinatory tests with iron oxide NP (IONP) and four contaminants – cadmium, copper, resorcin, and glyphosate. Acute toxicity over 96 h to *Daphnia magna* was investigated. Bioavailability of cadmium and copper was significantly reduced in the presence of IONP, which was supported by theoretical calculations of binding capacities. IONP did not affect the toxicity of resorcin. The toxicity of glyphosate was halved by IONP in the first 72 h, but reached values comparable to the single substance after 96 h. The toxicity of Cu remained constant between 48 h and 96 h, whereas it increased continuously in all other substances. The strongest increase was found for glyphosate + IONP (EC_{50} 48h: ~180 mg/L, 96 h: ~35 mg/L). This hints at a transporter effect, by which the substance is ingested bond to the IONP and then released during passage of the digestive tract.

Keywords: *Daphnia magna*, combinatory toxicity, mixtures, iron oxide nanoparticles, heavy metals, organic compounds

Introduction

Remediation of contaminated groundwater and soil with traditional techniques is very expensive [1]. With the advancing development of nanotechnology, iron and iron compound nanoparticles (INP) have come into the focus of cost-effective methods [2] for (1) *in-situ* injection [3] and (2) *ex-situ* treatment of contaminated (ground) water [4]. The advantage of the nano form is a significantly enlarged reactive surface compared to bulk materials, guaranteeing higher reaction rates [5]. Furthermore, iron is relatively non-toxic to organisms [6] compared to commonly applied injection substances [1]. Mainly consisting of zero valent iron (nZVI), INP have a high redox potential. While

nZVI is oxidized, organic compounds can be reduced to less toxic compounds. Furthermore, INP may bind heavy metal ions [1, 3, 7-10].

nZVI easily oxidizes in air and hydrolyzes in water [11] and is quickly transformed to iron oxide nanoparticles (IONP) when released to the environment. Usually they remain at the contaminated site since recuperation and recycling are cost-intensive. Depending on their colloidal stability – often enforced by surface functionalizations – they can keep their nano form for a long time [12]. Their potentially high mobility may turn into a disadvantage when INP might prevent pollutants from sorption to the solid matrix, thus increasing their bioavailability. Mobilized by INP, local hazards might be transported to uncontaminated sites, surface-waters or even enter drinking water resources [13, 14]. The risks for biota from the end-products of a nanoremediation have not been investigated so far.

Due to the fast oxidation of nZVI we focused on the testing of IONP consisting of magnetite (Fe_3O_4), expecting a higher environmental relevance. Furthermore, the handling of less reactive IONP is easier. The IONP were functionalized with polyvinyl pyrrolidone (PVP) against agglomeration. Since these IONP are relatively non-toxic [15] high concentrations can be applied to achieve high reaction rates. At the same time, the toxicity of IONP should not mask the toxicity of the contaminant or of reaction products.

The combinatory toxicity was investigated with the *Daphnia sp.* acute immobilization test according to OECD guideline 202 [16] over a prolonged test span of 96 h. The used IONP had already been tested with daphnids in maximum concentration of 100 mg Fe/L without inducing significant effects [15]. Due to filter-feeding, the main uptake route for NP in daphnids occurs via ingestion [17, 18].

The combination tests were performed with four substances: The heavy metals cadmium und copper of which their ions are known to bind to INP [3, 19-22]; the organic compound glyphosate which was tested since it is the active ingredient in the widely used herbicide RoundUp®; the aromatic compound resorcin – a dihydroxy benzene – which is mainly used in the production of diazo dyes and plasticizers and as a UV stabilizer in polyolefins [23].

The study aimed to investigate the possible use of PVP-coated INP for remediation by comparing the toxicity of the four substances with and without IONP. Furthermore, the toxicity tests should show whether the presence of IONP might increase toxicity, e.g. by increasing the uptake of sorbed compounds.

Materials & Methods

Culturing of Daphnids

The waterflea *Daphnia magna* was obtained from IBACON laboratories (Roßdorf, Germany) and cultured continuously in a climate controlled chamber at 20±1°C and a 16:8 h (light:dark) photoperiod. Animals were cultured in Elendt M7 medium (EM7; detailed composition in OECD guideline 211 [24]), which was renewed twice a week. They were fed with the green algae *Pseudokirchnerialla subcapitata* (#61.81, SAG, Göttingen, Germany) on a basis of 150 µg C per daphnid & day [24].

Synthesis and properties of IONP

IONP were synthesized and characterized in our laboratories. The synthesis of monodisperse and water-soluble magnetite IONP (Fe_3O_4) was based on the thermal decomposition of iron(III) acetylacetonate ($Fe(acac)_3$) in diethylene glycol (DEG). IONP were functionalized with polyvinyl pyrrolidone (PVP) during the formation process. The IONP had a primary particle diameter of 6.1 ± 0.6 nm (without coating) [25]. Suspended in EM7 medium, they were colloidal stable, their hydrodynamic diameter was around 135 nm and their zeta-potential was nearly neutral with slight negative charge (-1 mV) [26]. A detailed description of the synthesis and characteristics of the PVP-IONP can be obtained from Arndt et al. [25].

Preparation of test solutions

Stock dilutions of cadmium chloride ($CdCl_2$ • 2 H_2O; Fluka, purum, CAS# 10108-64-2), copper chloride ($CuCl_2$ • 2 H_2O; Merck, p.a., CAS# 10125-13-0), and resorcin (C_6H_4-1,3-$(OH)_2$; Sigma-Aldrich, ReagentPlus®, CAS# 108-46-3) were prepared by diluting substance powders in deionized water (see Table 1). From these stocks a second stock in EM7 medium was made by mixing the first stock with deionized water and double concentrated EM7 medium. The same method was used for transferring RoundUp® (commercial product RoundUp® UltraMax from Monsanto, water soluble concentrate with 450 g glyphosate/L) and IONP (900 mg Fe/L) to EM7 medium. Test concentrations were diluted directly from the EM7 stocks in medium. For the combinatory test, IONP were added from the EM7 IONP stock (250 mg Fe/L) to achieve a final concentration of 100 mg Fe/L. To achieve complete equilibrium, all test dilutions were aged between 3 and 8 days on a horizontal shaker at 60 rpm in dark at room temperature. The different aging intervals could not be avoided, since test starts had to be staggered over several days due to the large quantity of neonates needed and the time-consuming test preparations. At least one single substance test was always performed simultaneously to the corresponding mixture tests.

Test design & procedure

Tests were performed according to OECD guideline 202 [16], but with some adaptations. The test design was miniaturized and conducted in 24-well microtiter plates. Furthermore, the duration was prolonged from 48 h to 96 h. A detailed description of the changed test design and the test procedure can be obtained from Baumann et al. [27]. All tests were performed with a negative control and a minimum of 9 different substance concentrations. In the combinatory tests additional to the negative control an IONP control with 100 mg Fe/L was run to ensure no effect of IONP. Tests were only counted valid if the negative control or both controls, respectively, did not exceed a mortality of 10 % after 96 h. Concentration ranges are given in Table 1. Numbers of test repeats are shown in Table 2. Due to the heavy workload, combination tests were performed only three times, except for cadmium. Because in the first test series only one of the three cadmium tests was valid, the test series was repeated resulting in finally four valid tests. Single substance tests were performed five times. Different numbers of repeats are results of invalid tests. In the case of glyphosate additional tests were performed since there was a high variance of data after the first test series.

Table 1. Concentrations of stock dispersions and the range of tested concentrations

Substance	1. stock (water)	2. stock (EM7)	Tested concentration range [a,b]
IONP	900 mg Fe/L	250 mg Fe/L	100 mg Fe/L
Cadmium	2.3 g/L	50 mg/L	w/o NP: 100 – 5,000 µg/L
			with NP: 100 – 10,000 µg/L
Copper	1.9 g/L	10 mg/L	w/o NP: 10 – 400 µg/L
			with NP: 750 – 2750 µg/L
Resorcin	10.3 g/L	5 g/L	w/o NP: 0,1 – 500 mg/L
			with NP: 0,1 – 1,000 mg/L
RoundUp®	450 g/L	5 g/L	w/o NP: 10 – 200 mg/L
(Glyphosate)			with NP: 1 – 1,000 mg/L

[a] w/o NP = single substance, with NP = combinatory test with 100 mg Fe/L IONP

[b] Each test was performed with a control group and at least 9 different substance concentrations

Data analysis

All statistical calculations were made with GraphPad Prism 5.0 (GraphPad Software, San Diego, California, USA). EC_{50} values were calculated separately for each test repeat. For the EC_{50} calculations, concentrations were transformed to log scale. EC_{50} values were calculated using a nonlinear fit/dose-response equation (log(agonist) vs. response) with variable slope and an ordinary fit (least squares) with the top plateau set to 100%. Afterwards, EC_{50} values were re-transformed to linear scale and

mean EC_{50} were calculated. Statistical differences between the corresponding treatments were calculated with repeated measurements ANOVA.

Theoretical calculations of binding capacities

To support observed effects found for combinations of cadmium/copper and IONP, speciations were calculated using PHREEQCi (v.3.0.6-775, USGS, http://wwwbrr.cr.usgs.gov/projects/GWC_coupled/phreeqci) using the database file minteq.v4 (see detailed description in SI). The software allows the estimation of adsorbed species of cations like Cu^{2+} and Cd^{2+} on hydro-ferrous oxides (Hfo) with weak and strong binding sites (Hfo_wOH & Hfo_sOH, respectivily) [28]. The interaction of Cd^{2+} and Cu^{2+} with PVP was estimated by integrating equilibria data to the database file minteq.v4 generated from experimental results of Yildiz et al. [29].

Results & Discussion

For the comparison of combinatory toxicity of the four test substances an acute test span of 96 h was chosen. Previous studies have shown that the IONP used here exhibited low toxicity within this time span. Baumann et al. [26] and Filser et al. [15] found no or very low, but insignificant toxicity only at the highest test concentration of 100 mg Fe/L after 96 h. Therefore, in the present study an IONP concentration of 100 mg Fe/L was chosen. This is the highest concentration where no significant effect of the IONP was expected.

In this scenario we expected that effects were related to (1) the toxicity of the test substance, (2) the combination of the substance and the IONP (due to possible addition of sublethal toxicity, leading to lethal effects), (3) reaction between toxicant and IONP e.g. due to increased transport of toxicants bound to IONP into the daphnids via ingestions (transporter effect). The reaction between toxicant and IONP might also (4) decrease toxicity due to decreased bioavailability of processed or NP-bound substances.

The toxicity of cadmium (Fig. 1A) was about 7-times lower in the presence of IONP after 24 h. Over time the effect was slightly reduced, but toxicity was still about 3-times lower after 96 h. For copper (Fig. 1B) IONP decreased toxicity even between 20 and 15-times (24 h/96 h) compared to single copper. Test duration, IONP, and their interaction were highly significant for both combinations (Table 2). Obviously the toxicity of both heavy metals is strongly decreased by the IONP.

The decreased toxicity of both heavy metals should be due to decreased bioavailability. In principle, there are three mechanisms of metal detoxification by iron: a) reduction, b) complexation, c) sorption [21]. Referring to Merkel et al. [28], for both cadmium and copper only b) and c) should occur on magnetite NP. To clarify the observed effects, theoretical calculations of sorption kinetics and

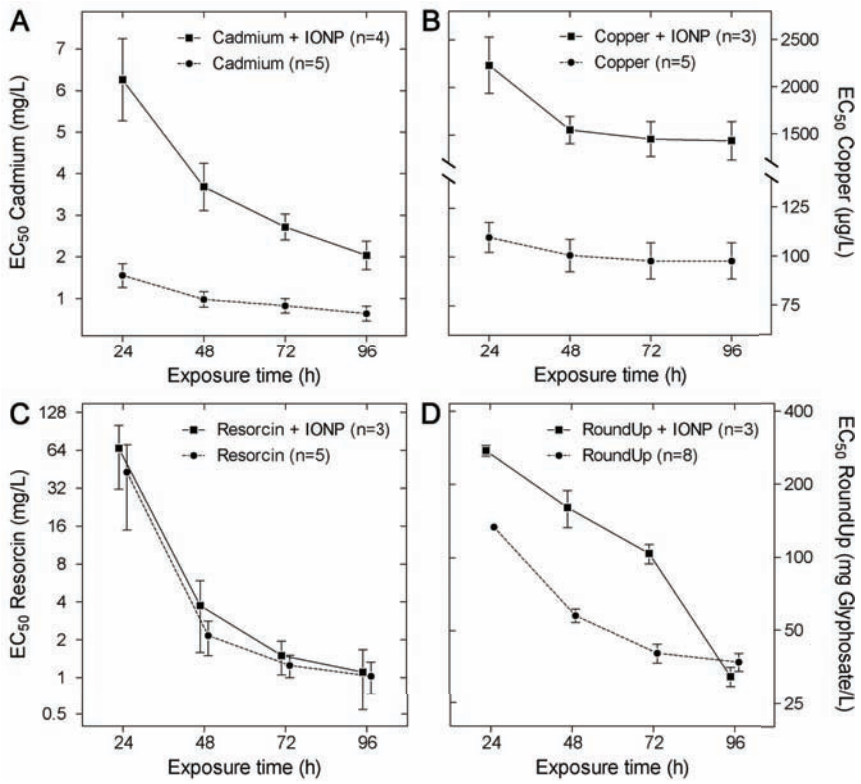

Fig. 1. *Daphnia magna* acute toxicity shown as the EC_{50} of the four tested substances over 96 h with (**A**) cadmium, (**B**) copper, (**C**) resorcin, and (**D**) RoundUp® related to glyphosate as the reactive compound. Graphs compare single substance toxicity and the mixture with IONP (100 mg Fe/L). Error bars = SE.

capacities were made (detailed description see SI). First, cadmium and copper ions should have got into contact with the PVP shell of the NP. PVP was already shown to have high binding capacities for heavy metal ions [29-31]. With 100 mg Fe/L (as IONP) about 3.75 mmol/L PVP (related to the monomer) was present [15]. Considering the average EC_{50} values for cadmium of 3677 µg/L (32.7 µmol/L Cd^{2+}) and copper of 1671 µg/L (26.3 µmol/L Cu^{2+}) observed with 100 mg Fe/L IONP, about 38.3 % of cadmium and 31.8 % of copper could have been bound to PVP (Table SI 1). The integration of speciation calculations using the hydro-ferrous oxide (Hfo) surface sorption model for the iron oxide cores suggested stronger binding of copper (78.8 %) than of cadmium (64.4 %) to IONP, leaving 1307.3 µg/L Cd^{2+} and 354.6 µg/L Cu^{2+} bioavailable to the daphnids (Table SI 1). These values do not correspond to the measured average EC_{50} of the pure heavy metals with 998.5 µg/L cadmium and 101.7 µg/L copper. Due to higher binding affinities to iron oxide, the integration of Hfo in the calculations led to highly reduced amounts of Cd^{2+} (15.1 %) and Cu^{2+} (0.07 %) bound to PVP. Unfortunately the core-shell structure of the NP cannot be integrated in the model. Therefore iron oxide and PVP are treated like competitive compounds in equilibrium for the binding of cadmium and copper, which should not be true *in situ*. In fact, the heavy metal ions were most likely bound to PVP

in the beginning and were then transferred to the iron oxide cores due to higher binding affinity until complete saturation of binding sites. Reduced bioavailability in the tests compared to the theoretical assumptions should therefore result from interaction of the heavy metal ions with the PVP shells. With the limited data on binding characteristics of heavy metals to PVP available in literature actual interactions were not completely reproducible with theoretical calculations. However, the calculations clearly show the higher binding potential of Cu^{2+} ions compared to Cd^{2+} ions to both the iron oxide cores and the PVP shell alike, which can be also deduced from the toxicity tests.

Interestingly, when considering the slightly lower pH inside the daphnid's anterior part of the midgut of pH 6 – 6.8 [32], the binding behavior of cadmium changes drastically compared to copper. Lowering the pH in the speciation calculations to this range for copper led to slightly stronger binding of Cu^{2+} to Hfo and PVP, leaving only 17.6 % (293.6 µg/L) bioavailable at pH 6.2. Doing the same for cadmium led to weakening of the binding of Cd^{2+} to Hfo and PVP, leaving 53.9 % (1979.6 µg/L) of the Cd^{2+} bioavailable at pH 6.6. This might be a further explanation for the generally higher binding affinity of copper to the IONP and the lower toxicity. Further, due to the probably weakened binding of cadmium during the passage of the digestive tract of the daphnids, cadmium may have been remobilized, which could explain the continuously increasing toxicity of cadmium especially with IONP over time (Fig. 1A), unlike the toxicity of copper (Fig. 1B).

As a representative aromatic organic compound, resorcin was tested (Fig. 1C). Results showed an increasing toxicity between 24 h and 48 h. Between 48 h and 96 h toxicity increased only slightly. The toxicity for pure resorcin and in combination with IONP remained equal over the whole test period with no statistical differences (Table 2). Apparently there was no interaction between resorcin and the IONP. This is straightforward: when considering the possible interaction in terms of the TSAR concept [33] of the components PVP, resorcin, and water, resorcin is more likely interacting with water.

Glyphosate was tested as a representative non-aromatic organic compound. In the presence of IONP it was significantly less toxic (Table 2). Between 24 h and 72 h toxicity decreased by about factor 2 compared to the pure formulation (Fig 1D). After 96 h this effect had disappeared. Whereas the toxicity of the pure formulation of RoundUp® remained nearly equal between 72 h and 96 h, the combinatory toxicity strongly increased during this time span with an even slightly higher toxicity in the combinatory test, supported by a highly significant interaction (Table 2). This might hint at a long-term combinatory effect. Effects should not result from reaction products of glyphosate and IONP, since both substances had already been mixed about one week before the tests and placed on a shaker to ensure complete reaction. If the effect was related to end or by-products of the reaction, stronger combination effects should have already been visible after 24 h. It seems likely that the effects resulted from a direct interaction between glyphosate and IONP. Using the TSAR approach [33] for glyphosate, PVP and water, due to the charges the glyphosate molecule at neutral pH is much more likely found in

Table 2. Significance tests of the EC_{50} via *repeated measurements* ANOVA.

Test [a]	*N*	Treatment [b]		Time [b]		Interaction [b]	
Cd^{2+} vs. Cd^{2+} + IONP	5 4	**	0.0014	***	<0.0001	***	<0.0001
Cu^{2+} vs. Cu^{2+} + IONP	5 3	***	<0.0001	***	<0.0001	***	<0.0001
Resorcin vs. Resorcin + IONP	5 3	ns	0.5866	**	0.0082	ns	0.8615
RoundUp® vs. RoundUp® + IONP	8 3	***	<0.0001	***	<0.0001	***	<0.0001

[a] pure substance toxicity versus the combination with IONP (100 mg Fe/L)

[b] significance level and *p*-value

ns = not significant

** p<0.01

*** p<0.001

the water phase. Borggaard et al. [34] report that glyphosate is supposed to behave similar to phosphates considering adsorption behavior to soil minerals like aluminium and iron oxides. Therefore, possible binding of glyphosate should appear directly to the iron oxide cores. Obviously, there was less glyphosate bioavailable in the first three days, but then its bioavailability seemed to be highly increased (Fig. 1D). This may hint to a transporter effect. In the beginning glyphosate may have been only transiently bound to the IONP, reducing its bioavailability. With continuous test span the IONP were ingested by the neonates and concentrated in the digestive tract. Due to digestion of the IONP surface, possibly bond glyphosate was released from the IONP. Normally, the passage of the digestive tract only takes some minutes up to a few hours [35, 36], depending on the amount of food (or particular matter) provided [37]. Food is expelled from the hindgut by peristaltic movement but also requires the pressure of more recently acquired food particles [32]. Since the juvenile daphnids were not fed during the tests, gut passage time should have been increased. However, this assumption cannot explain the late effects after 72 h. Under the given concentrations of 100 mg Fe/L IONP, pre-tests showed high accumulation into the gastrointestinal tract of neonates, but also excretion of feces within a few hours, and re-ingestion of excreted IONP (*personal observation*). It is more likely that the observed effects are a combination of (1) slow gut passage (with enforced digestion due to longer residence time), (2) re-ingestion of excreted IONP, and (3) several passages of the digestive tract, leading to enforced destabilization of the glyphosate-PVP-IONP complex after 72 h.

Conclusions

Our tests have shown very different results depending on the substance tested. The combination of IONP and the two heavy metals – cadmium and copper – led to decreased toxicity since their bioavailability was significantly reduced by the IONP. Theoretical assumptions revealed possible binding of heavy metal ions to both, the PVP shells and the iron oxide cores. Combined with their superparamagnetic characteristics, which allow their recuperation by magnetic treatment [4], these IONP might be a highly efficient tool for the cleaning of heavy metal contaminated waters [38, 39]. Effects of the two organic compounds were completely different. Toxicity of resorcin was not affected by the IONP. The toxicity of glyphosate was reduced in the first 72 h, but then substantially increased up to positive control (single substance) levels, which could hint at a transporter effect. This study has shown that a test duration of 48 h (of the *Daphnia* acute test [16]) may lead to wrong conclusions – not only for the toxic potential of NM (e.g. Dabrunz et al. [40]) – but also for their remediation potential, as exemplified by glyphosate.

Our results give a first idea of how remediation products can influence organisms. Certainly tests should also be conducted with potential target organisms living in soil and groundwater, since the way of life and further biotic and abiotic factors highly influence the exposure. Furthermore, tests should also be made with different target contaminants and mixtures, since contaminants might also compete e.g. for binding sites on the NP surface.

Acknowledgments

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Supporting information

The surface of the IONP in presence of water is most likely hydrated and should behave similar to hydro-ferrous oxide (Hfo) as described in Merkel & Planer-Friedrich [1]. The amount of binding sites was roughly estimated from the particle surface (707 nm^2/particle or 77.4 m^2/g IONP), calculated assuming spherical particles with diameter 15 nm [2]. The number of available binding sites should match the number of iron atoms on the particle surface. This number was estimated from the iron-iron distances, which were assumed to be the approximate twice the average atom distance (Fe-O) in hematite of 0.2 nm [3], which should be close to the value of magnetite, considering this rather rough approach. In this way the actual number of binding sites was estimated to amount to 4949 binding sites per particle. For 100 mg iron/L (1.823 mmol/L) the fraction of binding sites was estimated to 125

µmol/L (122 µmol/L weak and 3 µmol/L strong binding sites). The fractions of the weak binding sites Hfo_wOH (97.6 %) and of the strong binding sites Hfo_sOH (2.4 %) were assumed to be the same as given by Merkel & Planer-Friedrich [1]. However, our estimated fraction of binding sites was approximately factor 3 lower than the values given there of 0.2 mol weak binding sites and 0.005 mol strong binding sites per mol iron. This could be associated to higher active surface area assumed by Merkel & Planer-Friedrich [1] compared to our approach with a particle diameter of 15 nm.

Speciation calculations were done using the software PHREEQCi (v.3.0.6-775, USGS, http://wwwbrr.cr.usgs.gov/projects/GWC_coupled/phreeqci) with the database minteq.v4 including dissolved oxygen (0.26 mmol/L) and gas phase equilibrium with CO_2 containing atmosphere (390 ppm). The test medium Elendt M7 in itself contains Fe^{2+} 3.6 µmol/L which are then oxidized by the dissolved oxygene and are thermodynamically unstable and prone to precipitate completely as $Fe(OH)_{2.7}Cl_{0.3}$, in case strict thermodynamical equilibrium conditions are considered. However, no precipitation was observed, which is related to the stabilizing component EDTA in Elendt M7 medium. For Cu^{2+} concentrations up to 10 mg/L and for Cd^{2+} up to 50 mg/L in Elendt M7 medium likewise no precipitation occurred in the timeframe of the tests. Considering these facts and that all EC_{50} values for copper and cadmium were below these values, the speciation calculations were carried out not allowing for precipitation processes. The used equilibria data for the surface adsorption equlibria with Hfo as given in the database minteq.v4 is listed below:

$Hfo\_sOH + Cd^{2+} = Hfo\_sOCd^{+} + H^{+}$, log_k = 0.47 (K = 3.0)

$Hfo\_wOH + Cd^{2+} = Hfo\_wOCd^{+} + H^{+}$, log_k = -2.9 (K = 0.0013)

$Hfo\_sOH + Cu^{2+} = Hfo\_sOCu^{+} + H^{+}$, log_k = 2.89 (K = 776)

$Hfo\_wOH + Cu^{2+} = Hfo\_wOCu^{+} + H^{+}$, log_k = 0.6 (K = 4.0)

The database uses logarithms to the base 10 for the equilibrium constants. The data suggest that the interaction of copper with the hydro-ferrous oxide surface is about 1000 times stronger than for cadmium.

To account for the possible interaction with the PVP bound to the IONP first step calculations were conducted without the Hfo surface sorption model to estimate the binding of Cd^{2+} and Cu^{2+} to PVP alone. The results show weak binding to PVP (Table SI 1). The integration of PVP and the Hfo model resulted only in small changes on the final equilibrium compared to the Hfo model alone.

Table SI 1. Binding capacities of cadmium and copper to polyvinyl pyrrolidone (PVP) and hydro-ferrous oxide(Hfo). [a, b]

	mean EC_{50} w/o NP	mean EC_{50} with NP		PVP 3.75 mmol/L	Hfo sOH	Hfo wOH	Hfo + PVP sOH	Hfo + PVP wOH	Hfo + PVP PVP
Cadmium	998.5 µg/L	3677 µg/L	bond:	1333 µg/L	293.4 µg/L	1907.6 µg/L	283.3 µg/L	1532.2 µg/L	556.4 µg/L
	8.88 µmol/L	32.71 µmol/L		11.86 µmol/L	2.61 µmol/L	16.97 µmol/L	2.52 µmol/L	13.63 µmol/L	4.95 µmol/L
				36.3 %	8.0 %	51.9 %	7.7 %	41.7 %	15.1 %
			unbond:	2343 µg/L	1476 µg/l		1307.3 µg/L		
				20.84 µmol/L	13.13 µmol/L		11.63 µmol/L		
				63.7 %	40.1 %		35.6 %		
Copper	101.7 µg/L	1671 µg/L	bond:	531.9 µg/L	76.3 µg/L	1240.4	75.6 µg/L	1240.4 µg/L	1.1 µg/L
	1.6 µmol/L	26.33 µmol/L		8.37 µmol/L	1.20 µmol/L	19.52 µmol/L	1.19 µmol/L	19.52 µmol/L	0.0173 µmol/L
				31.8 %	4.6 %	74.2 %	4.5 %	74.2 %	0.07 %
			unbond:	1139.1 µg/L	354.3 µg/L		354.6 µg/L		
				17.95 µmol/L	5.62 µmol/L		5.58 µmol/L		
				68.2 %	21.4 %		21.2 %		

[a] in Elendt M7 medium at pH 7

[b] Association constants of Cd^{2+} and Cu^{2+} ions to PVP (containing approx. 5 % PEG) were calculated assuming sorption according Langmuir isotherm behavior with experimental data of Yildiz et al. [4].

Cd^{2+} + PVP ⇆ $PVPCd^{2+}$, K = 337.6 L/mol (at pH 8); Cu^{2+} + PVP ⇆ $PVPCu^{2+}$, K = 2438 L/mol (at pH 8).

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3.5 Publication / Manuscript 5

Baumann J, Bertrand C, Becker M., Filser J (*submitted manuscript*) **"Colloidal Properties of PVP-coated IONP affect their Bio-distribution and Life History Responses of *Daphnia manga*."**

Contributions of J. Baumann:

- Performance of all *Daphnia* experiments
- Analyses of IONP
- Statistical analyses
- Preparation of all Figures and Tables
- Preparation of the manuscript

C. Bertrand supported the experiments and statistical analyses for the comparison of life history responses (PCA). M. Becker supported the accumulation/depuration tests. J. Filser revised the manuscript.

Colloidal properties of PVP-coated IONP affect their bio-distribution and life history responses of *Daphnia magna*

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Abstract

We studied long-term effects of iron oxide nanoparticles (IONP) on Daphnia magna using two types of polymer (PVP)-coated IONP: Two batches were synthesized with identical methods. One of them remained colloidally stable over 30 days (s-IONP), whereas the second did not (i-IONP); this resulted in hydrodynamic diameters of 133 and 215 nm, respectively. IONP were tested in concentrations between 1 and 100 mg iron L^{-1}.

s- and i-IONP were both effectively ingested and eliminated by mature daphnids, with higher accumulation rates of i-IONP. Depending on the concentration (from 1 mg iron L^{-1} onwards), both IONP induced death or significantly reduced development and reproduction, with stronger inhibition by i-IONP. Our data suggests that effects were mainly related to disturbed nutrient assimilation in the daphnids' guts. Stronger effects of i-IONP were explained by the additional flocculation of algae and their adhesion to the filtering apparatuses and other exoskeleton parts of the daphnids. Increased specific weight and swimming resistance may have increased the daphnids' energy demands, which could not be compensated for because algae clogged the filtering apparatuses.

Our results show that even NP composed of less toxic or non-toxic materials can have significant impacts on filter-feeding organisms such as daphnids. Due to the long-term effects of such NP, acute tests might be inadequate for a reliable risk assessment.

Keywords: iron oxide nanoparticles, colloidal properties, agglomeration, *Daphnia magna*, accumulation, elimination, chronic test, reproduction, development.

Introduction

Nanomaterials (NM) and nanoparticles (NP) are increasingly used in a wide range of applications and products. Due to their small size and highly increased reactive surface, NM often provide new beneficial properties. However, with increasing application, NM will consequently be released to the environment and will end up in surface waters with unknown risks to human and environmental health [1-3].

Due to their small size, NP may enter non-target organisms, organs, the brain, or even penetrate membranes and cells [4]. The toxicity of NM, especially of metallic NP, can mainly be linked to the production of reactive oxygen species (ROS). ROS can be directly formed at the highly increased surface of the NP (primary toxicity). Furthermore, the release of toxic ions (secondary toxicity) is often described as the main source of toxicity [5]. This might be enforced by a "trojan horse" effect, by which NP are accumulated inside organisms or cells, with toxic ion release eventually exceeding their naturally occurring concentrations [6].

Most studies evaluating the risk of NM are based on *in vitro* assays [7]. They can provide initial data on comparative toxicity of NM, but can hardly estimate impacts on organisms or the environment [4]. To assess the environmental risk of NP, often the crustacean *Daphnia magna* is used for *in vivo* testing [8]. This filter feeder was already shown to actively ingest NP [8, 9]. Although it mainly feeds on suspended food particles, it may also browse over surface substrates to pick up small food particles, especially when food becomes scarce [10]. Thus, it might also be able to ingest NP which agglomerated and settled to the ground or which are loosely adsorbed to surfaces.

Most studies on the effects of inorganic NP on daphnids are based on short-term investigations. Toxicity is usually related to the direct or indirect (via ions) induction of oxidative stress [5]. A few studies give reason for concern since they have revealed unexpected side effects during NP exposure. These effects basically provoked physiological inhibitions. Studies showed disturbed ecdysis, NP adsorbed to the daphnids' exoskeleton, leading to increased swimming resistance and body weight, and presumably to increased energy demands [11, 12]. However, energy uptake may have been reduced due to NP which have disturbed natural nutrient exchange in the gut [5]. Additional effects such as these might have an especially strong influence on the life history of daphnids under NP exposure, but studies on long-term effects are rare [13].

In this account we investigated the chronic effects of iron oxide nanoparticles (IONP). Until now, IONP had not come into the focus of chronic NP investigations, since iron itself is less toxic to organisms than are other metals [14]. First studies on their acute toxicity revealed only moderate or no effects [12, 15]. Nevertheless, IONP have a huge environmental relevance. Apart from their use in medicine [16], they are tested for the remediation of contaminated (ground-) water and soil [17]. During such applications, tons of iron-based NM are released to the environment. However, there is

still a serious lack of knowledge about the efficiency, fate and persistence of iron-based NP released to the environment [18, 19].

Since the toxicity of metallic NP is often related to the increased release of toxic ions, direct effects of NP, e.g. due to their size, might be masked by the higher toxicity of the released ions. Apart from their environmental relevance, IONP gave us the opportunity to investigate long-term "nano-effects" on daphnids because of the low toxicity of the core material [14]. In an earlier study, the tested IONP were stabilized with a polymer coating (polyvinyl pyrrolidone, PVP) and had a high colloidal stability [20] with no significant release of iron ions and moderate toxicity to *D. magna* [12, 15].

However, in a second batch of the same synthesis route, agglomeration occurred. Thus, in this study we concentrated on colloidal stability as the influencing factor. We studied mortality, reproduction and a number of other life history parameters in chronic tests with *D. magna*. In addition, we measured IONP uptake and elimination. Our main hypothesis was that the measured parameters would be differentially affected by s- and i-IONP.

Material & Methods

Synthesis & Characterization of IONP

IONP were synthesized and characterized in our laboratories. The synthesis of monodisperse and water-soluble magnetite IONP (Fe_3O_4) was based on the thermal decomposition of iron (III) acetylacetonate ($Fe(acac)_3$) in diethylene glycol (DEG). IONP were functionalized with polyvinyl pyrrolidone (PVP) during the formation process. After synthesis, particle concentrations of the suspensions in deionized water were measured using atomic absorption spectroscopy (AAS). A mean primary particle diameter of 6.1±0.6 nm was determined via transmission electron microscopy (TEM). A detailed description of methods and characterization results is given in Arndt et al. [20]. The hydrodynamic diameter (HDD) was measured via dynamic light scattering (DLS; Delsa™Nano C, Beckman Coulter, Krefeld, Germany) in EM7 medium in 100 mg iron L^{-1} solutions. All stocks were stored in glass bottles (Duran® glass bottle with PP cap, Schott AG, Mainz, Germany) and placed in darkness at room temperature.

Preparation of IONP stocks

IONP were transferred to Elendt M7 medium (EM7; detailed composition see OECD [21]), one week before the tests. For this, one part of the 1 g L^{-1} water stock was mixed with one part of double-concentrated EM7 medium and two parts of normal EM7 medium to achieve a 250 mg iron L^{-1} EM7 stock. From these stocks, all test dilutions were freshly prepared during the tests. All 250 mg L^{-1} EM7

stocks were stored in glass bottles (Duran® glass bottle with PP cap, Schott AG, Mainz, Germany) and placed in darkness at room temperature.

Culturing of daphnids

The water flea *Daphnia magna* was obtained from IBACON laboratories (Roßdorf, Germany) and cultured continuously in a climate-controlled chamber at 20±1°C with a 16:8 h (light:dark) photoperiod. Four semi-static cultures each containing 30 animals were cultured in 1.5 L EM7 medium, which was renewed twice a week. Animals were fed with the green algae *Pseudokirchneriella subcapitata* (#61.81, SAG, Göttingen, Germany) on a basis of 150 µg C per daphnid and day [21].

Accumulation and depuration tests

Test procedure

Tests were performed in 2 L glass beakers (Boro-siliacte) with gentle aeration in a climate-controlled room at 20±1°C with a 16:8 h (light : dark) photoperiod. 100 mature daphnids (21 d) were put together in a volume of 1.8 L EM7 medium. During the whole test they were fed with living algae (*P. subcapitata*), providing 200 µg C daphnid^{-1} day^{-1}. Daphnids were exposed for 48 h to both s- and i-IONP at a concentration of 1 mg iron L^{-1} and additionally to i-IONP at a concentration of 10 mg iron L^{-1}. s-IONP were replicated twice, i-IONP five times at 1 mg L^{-1} and twice at 10 mg L^{-1}, and a negative control twice. After exposure, daphnids were transferred to pure medium for 144 h for the elimination of IONP. Medium was renewed after 12 and 24, 48, 72, 96 and 120 h. During the tests samples were constantly taken from each replicate. Therefore, at each time point 3 daphnids were caught and merged into a 1.5 mL Eppendorf tube (0030125.150; Eppendorf AG, Hamburg, Germany). Prior to this, each tube was weighed. Daphnids were instantly killed by shock freezing in liquid nitrogen. Samples were dried at 60 °C in a heating cabinet over night. After cooling to room temperature, samples (tube + daphnids) were weighed again for measuring the daphids' dry weight.

Iron measurement

The method for photometric iron content measurement was adopted from Riemer et al. [22]. For this reason, we will only be providing a short description of the crucial preparation steps or parts which differ from the original protocol.

Samples were re-suspended in 300 µL 50 mM NaOH (30620; for analysis; Riedel-deHaën, Seelze, Germany), and daphnids were milled with plastic pistils to lyse their tissue and release all iron (NP). After one hour, 300 µL 10 mM HCl (solvent of the Fe standard; 30720; puriss. p.a., Riedel-deHaën, Sigma-Aldrich, Steinheim, Germany) and 300 mL of freshly prepared iron-releasing agent were added. The releasing agent was composed of a 1:1 mixture of 1.4 M HCl and 4.5 % potassium permanganate

(60459; BioUltra ≥99.0 %; Fluka, Sigma-Aldrich, Steinheim, Germany) solutions. NP digestion was achieved at 60 °C in a fume hood for 16 h.

Fe standards ($FeCl_3$: 44944; puriss.p.a. 99.0-102 %; Fluka, Sigma-Aldrich, Steinheim, Germany) were prepared in accordance to Riemer et al. [22]. For measuring the iron content, the detection reagent composed of 2.5 M ammonium acetate (A7330; BioXtra ≥98.0; Sigma-Aldrich, Steinheim, Germany) 1 M ascorbate (3525.1; ≥99.0 % p.a.; Carl Roth GmbH+Co, Karlsruhe, Germany), 6.5 mM ferrozin (82950; for spectrophotometric det. of Fe ≥97.0 %; Sigma-Aldrich, Steinheim, Germany) and 6.5 mM neocuproin (N1626; BioReagent, crystalline; Sigma-Aldrich, Steinheim, Germany) was freshly prepared. 90 µL of detection reagent was added to each sample. After 30 min of incubation samples were pipetted onto a 96 h well microtiter plate (82.1581.500; Sarstedt AG, Nümbrecht, Germany). 2 repeats of each standard and 3 repeats of each sample in a volume of 280 µL were measured using a photometric micro plate reader (MRX; 992-8031-13; Dynatech Laboratories, Denkendorf, Germany) at a wavelength of 550 nm.

Chronic test procedure

IONP were tested in concentrations of 0, 1, 2.5, 5, 10, 25, 50, and 100 mg iron L^{-1}. Each test concentration was replicated 10 times. HDD was measured in the highest test concentration of 100 mg iron L^{-1} one day before the tests started, once during the tests (day 14) and one day after the tests had been completed.

The tests were performed in small glass beakers (100 mL, Boro-silicate) covered with a glass lid against evaporation, but leaving an opening at the spout for aeration. Test dilutions were changed every 3-4 days. Due to the time-consuming synthesis of IONP, the provided test volume per daphnid was reduced to 25 mL in the beginning under the assumption that young daphnids require less space. As the development of the daphnids progressed, the volume was increased to 35 mL on day 4, to 45 mL on day 8 and to the final volume of 50 mL on day 12. The OECD guideline 211 [21] actually suggests a minimum volume of 50 mL per daphnid. In accordance with the OECD guideline, the daphnids were fed with living algae (*P. subcapitata*), providing between 100 and 200 µg C $daphnid^{-1}$ day^{-1}. Algae were separated from their culture medium by centrifugation (2200 *g*, 10 min), re-suspended in EM7 medium, counted (Neubauer cell counting chamber), and then added during the renewal of test dilutions.

24 h before the test start, the mother daphnids were transferred to freshly prepared culture vessels to separate them from their offspring. 16-18 h later the newly-born offspring were caught with a glass pipette. 22 - 24 h after separating the mothers, the neonates were put into the prepared test vessels. One daphnid was exposed per replicate.

Tests were performed in a climate controlled room at 20±1°C with a 16:8 h (light : dark) photoperiod. Test vessels were randomly distributed and their position was changed daily. For the periodic renewal of the test dilutions, new test vessels with fresh dilutions were prepared and daphnids were carefully transferred with a glass pipette in the smallest possible volume of old test dilution. Every replicate was checked daily for mortality and reproduction. Offspring were caught with a Pasteur-pipette and counted. Abiotic parameters (pH, oxygen) were recorded during renewal and temperature was measured daily.

After the tests, daphnids were conserved in 5% formol for further investigations. Lateral pictures of daphnids were taken to measure their body size. The dorsal length from the rostrum to the basis of the distal spine was measured with the free software tool ImageJ 1.47 (http://rsbweb.nih.gov/ij/).

Data analysis

All chronic test data was checked for significant outliers using the Gubbs' test with a significance level of 0.05 (http://graphpad.com/quickcalcs/grubbs1/). If the test found a significant outlier for a measured endpoint, the data of this replicate was completely excluded from all calculations. The validation criteria of the OECD guideline were then checked (mean control reproduction >60 and variance of reproduction <25 %). All test data presented fulfilled these criteria; if not, tests would have been repeated.

The plotting of graphs and significance tests were made with GraphPad Prism 5.0 (GraphPad Software, San Diego, California, USA). For the calculation of HDD, intensity data of three independent measurements with 2 - 5 repeats (depending on the validity of the measurement) was exported from the measurement software (Delsa^{TM}Nano Version 2.31, Beckman Coulter, Krefeld, Germany) to the statistics software. HDD ±SD were calculated with a Gaussian distribution.

For plotting mortality against time, a non-linear regression (dose-response curve; normalized response/variable slope) was used. All life history data was tested on normal distribution. Significant differences to the control of parametric data were compared with a students t test, and of non-parametric data with a Mann-Whitney U test.

The overall effect of s- and i-IONP on *D. magna* was compared with a principal component analysis (PCA) using the statistics software R (version 3.0.3, R Core Team 2014). All life history data was pooled and tested independently of the concentration to analyze the effect of the two treatments (s-/i-IONP). For the PCA, a *within class component analysis* (library ade4; http://cran.r-project.org/web/packages/ade4/index.html) was used. Although the PCA data was normally distributed, a Bartlett test revealed non-homogeneous variances of the PCA results. Therefore, the PCA data of the two treatments was tested for significant differences using a Mann-Whitney U test.

Results & Discussions

IONP properties

Dispersed nanoparticles often tend to agglomerate in aqueous solutions. For this reason, nanoparticles are usually functionalized with a stabilizing surface coating. Our IONP were stabilized with a polymer coating (PVP). PVP prevents agglomeration via steric stabilization. The IONP were thus only slightly charged with zeta potentials between 0 and -5 mV (in both water and EM7; *data not shown*). The applied synthesis of PVP-IONP led to monodisperse nanoparticles with HDD of 22.6 nm in deionized water (see Arndt et al. [20]). Transferred to the *Daphnia* culture medium EM7, the HDD slowly increased within a couple of days. This is a common phenomenon of PVP, which tends to swell in the presence of high salt concentrations such as those in EM7 medium [20, 23].

In this account, colloidally stable and instable PVP-IONP (s- and i-IONP) were tested and compared. The size distribution was continuously investigated in parallel to the reproduction tests. Figure 1a shows the mean HDD of s-IONP (132.9 ±31.24 nm; ±SD) before the tests. A second DLS measurement after the test showed nearly unchanged HDD (138.9 ±30.35 nm). An additional measurement of s-IONP two months after they were dispersed in EM7 showed equal HDD (*data not shown*), indicating high long-term colloidal stability. For another test series, a second batch of IONP was synthesized with the same method. These i-IONP did not have colloidal properties equal to s-

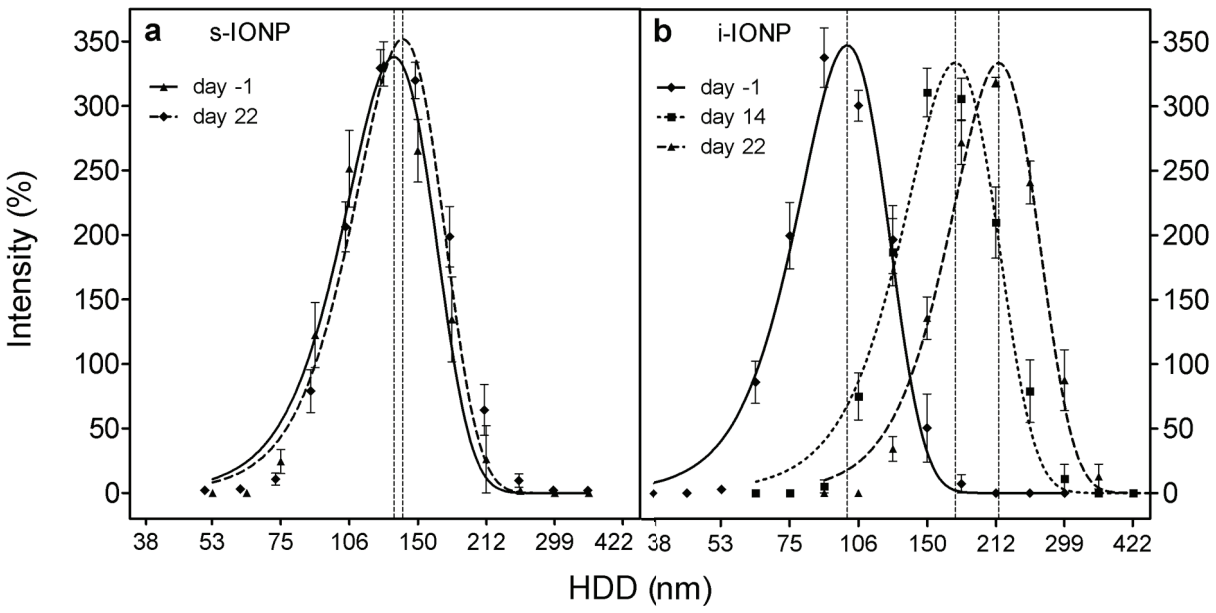

Fig. 1. Size-distribution of (a) s-IONP and (b) i-IONP during the chronic tests. The hydrodynamic diameters (HDD; mean values ±SE) were measured via dynamic light scattering (DLS) in three samples at a IONP concentration 100 mg iron L^{-1} one day before the chronic tests started (solid lines), at day 14 of the tests (dotted lines), and one day after the tests had been finished (dashed lines).

IONP (Fig. 1b). 7 days after transferring to EM7, the mean HDD was 100.2 ±22.7 nm, only indicating common swelling, and the *Daphnia* test was started. 14 days later (day 12 of the test), the HDD had already increased to 172.8 ±40.62 nm and continued to increase to 214.8 ±47.40 nm until the end of the test. The i-IONP were not colloidally stable and slowly agglomerated.

Although the IONP from both batches were synthesized using the same procedure, the s-IONP were colloidally stable, whereas the i-IONP were colloidally instable. In the synthesis of i-IONP, PVP obviously did not equally bind to the iron cores like in s-IONP. PVP was most probably released from i-IONP over time, consequently leading to their agglomeration. Bare IONP have a high tendency to agglomerate, which we already proved in a previous study with the DLVO theory [12].

Daphnia tests

Visible effects

Both IONP were visibly accumulated in the digestive tract (Fig. 2a/b; [12]) during both the accumulation and chronic exposure tests. For i-IONP, an additional effect occurred: Algae provided as food flocculated in the presence of i-IONP. The algae agglomerates stick to the filtering apparatuses and the swimming antenna. This effect occurred in both the accumulation and chronic tests, but was stronger in the latter and increased with increasing i-IONP concentration. There, especially the younger daphnids suffered from the algae agglomerates during the first two weeks, which partly completely inhibited their movements (Fig. 2c). The mature daphnids used in the accumulation test were less affected. On the one hand, they were stronger, had more energy reserves, and were able to strip off the agglomerates during ecdysis (Fig. 2d). On the other hand, they were only exposed for 48h, which was possibly too short to induce significant effects in mature daphnids.

At this point, we cannot completely explain this effect, but the agglomerates were obviously directly formed in the daphnids' filtering apparatuses (Fig. 2c). We hypothesize that turbulences caused by the swimming and filtering movements of the daphnids might have accelerated the colloidal destabilization of the i-IONP. We had already demonstrated accelerated agglomeration of IONP due to the water-movements of daphnids through the *Daphnia*-induced agglomeration of charge-stabilized IONP [12].

Since i-IONP had a tendency to swell and agglomerate (Fig. 1), the PVP-coating was most likely only loosely bonded to the i-IONP. Due to the *Daphnia* movements, PVP was probably increasingly detached from the i-IONP. The colloidally destabilized i-IONP, the released PVP, or a mixture of both then interacted with the algae. PVP is known for swelling in the presence of salt ions, cross-linking, and hydrogelization [23-25]. PVP – released or still loosely bonded to the i-IONP – might have interacted with the daphnids' filtering apparatuses and the swimming antenna as well as with the algae

passing the filtering stream. This induced the strong flocculation of algae which clogged the thoracic legs and other fine structures of the exoskeleton.

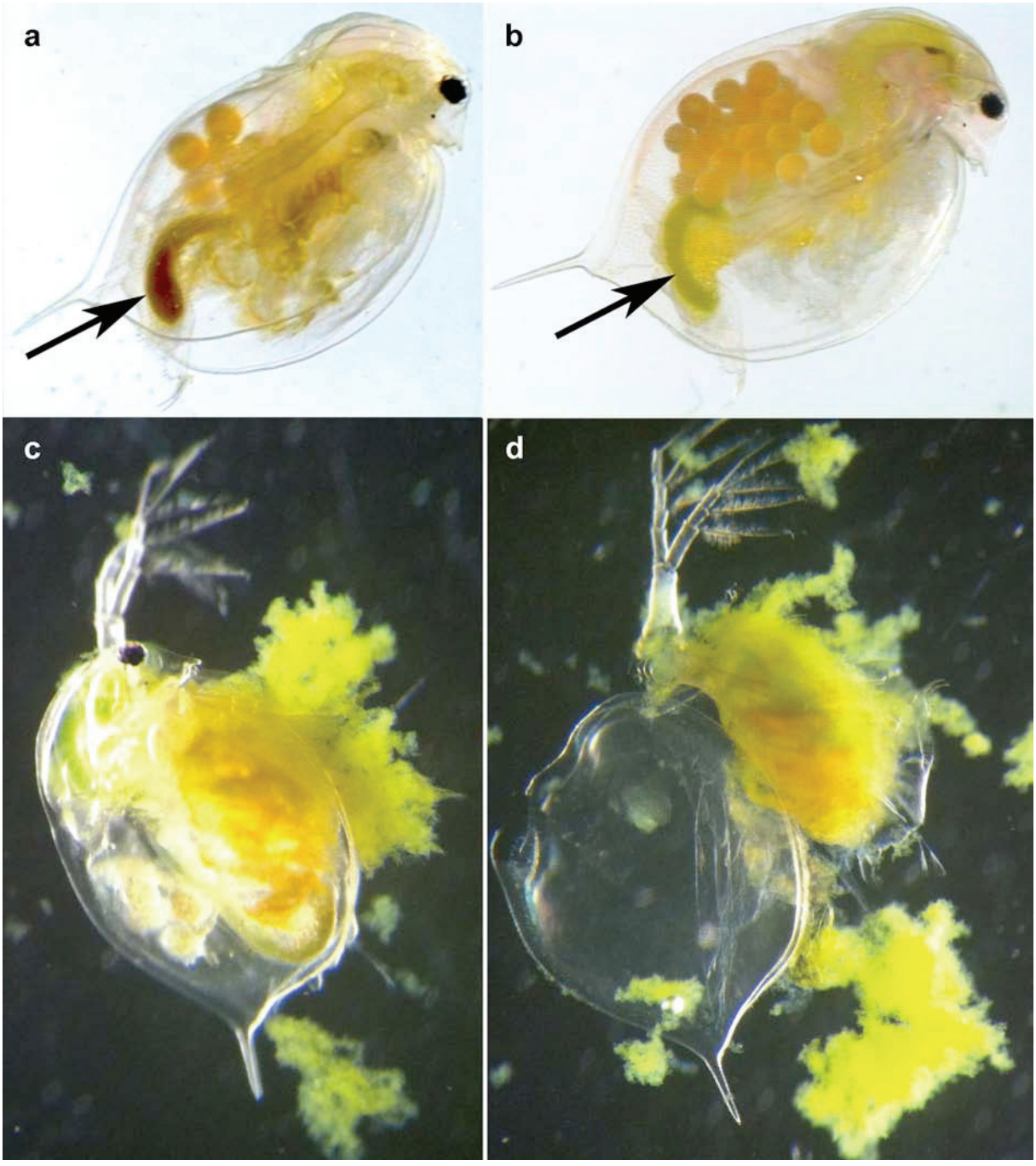

Fig. 2. Visible effects of 5 mg iron L^{-1} i-IONP exposure on *Daphnia magna*: (a/b) Daphnids at the end of the reproduction test; (a) i-IONP were concentrated in the digestive tract and stained the hind gut dark brown (arrow); (b) in comparison the hind gut of control animals with green staining due to the algae diet (arrow); (c) i-IONP induced agglomeration of algae in the filtering apparatuses of daphnids; (d) at concentrations of ≤ 10 mg iron L^{-1} daphnids were able to shed off the IONP-algae agglomerates during ecdysis.

Accumulation and depuration

In general, daphnids filter particles mainly in the range of 1 to 50 µm without any selective mechanism [26, 27]. With their thoracic appendages, daphnids produce a constant water current along the opening of the carapace. Fine setulae located on the thoracic legs filter the food particles from the feeding current and move them to the mouth [26, 28]. Daphnids are thought to actively filter ultra-fine particles as small as 200 nm [29]. However, due to their size, the ingestion of NP should be a passive rather than an active process. NP are intercepted and transported into the esophagus with the feeding current or are ingested by drinking the surrounding media [28].

Since ingestion is the most common uptake route for NP in invertebrates, their digestive system probably becomes the vulnerable target [30]. Different NP such as iron oxide NP [31], cerium dioxide NP [32], zinc oxide NP [33], copper NP [34], silver NP [35], gold NP [36], titanium dioxide NP [9], quantum dots [37, 38], polystyrene beads [28], diamond NP [39], and other carbon NM [40, 41] were already shown to be accumulated in the digestive tract of daphnids [42]. Most studies reported quick uptake of NP into the intestine within a few hours. In most cases, the depuration was very effective within the first 12 h after exposure and was stronger when daphnids were fed. In all reported cases, daphnids were not able to eliminate all NP within 24 to 96 h. Although authors often speak of the bio-accumulation or bio-concentration of NP when NP are ingested. But this is a wrong use of the terminology. Only when the net transfer of a substance from the external environment to the systemic circulation of the organism (influx) exceeds the efflux, accumulation occurs. (NP) accumulation thus only refers to the internal compartments of an organism[43]. At the moment, only a few studies have reported interactions of NP with gut epithelial cells or the penetration into these structures [33, 34, 36].

In a previous study we showed the strong enrichment of PVP-IONP in the digestive tract of *D. magna* [12]. Now, we aimed to investigate the bioaccumulation potential of IONP by measuring the total iron content of daphnids over an accumulation period of 48 h and elimination period of 144 h. IONP had been prepared as in the chronic test. Random measurements showed equal HDD as measured at the beginning of the chronic tests (*data not shown; see above*). Tests were performed with both s- and i-IONP at a concentration of 1 mg iron L^{-1}.

Figure 3a shows the results of the accumulation and depuration experiments. At the beginning of the test (t0), mature daphnids had a basal iron content of 0.13 µg iron mg^{-1} (*Daphnia* dry weight [dw]). In the first 4 h, the iron content equally increased in s- and i-IONP to 0.184 µg mg^{-1} (dw). Then the uptake of i-IONP increased compared to s-IONP. It is very likely that at that point the above-described destabilization of i-IONP set in. As a consequence, (1) more i-IONP bound to algae were passively ingested, (2) due to agglomeration i-IONP were large enough to be actively intercepted, and (3) by losing their colloidal stability, i-IONP adsorbed to the daphnids' carapace, filtering apparatuses and swimming antennae (Fig. 2c). After 48 h, iron concentration in daphnids exposed to i-IONP (2.0 µg

mg^{-1} dw) was about five times higher than in s-IONP (0.42 µg mg^{-1} tw). However, the concentration in s-IONP was also three times higher than the basal iron content in unexposed daphnids (0.14 µg mg^{-1} tw).

For the following depuration period, daphnids were transferred to fresh medium. Both IONP were quickly eliminated by the daphnids within the first 2-4 h, illustrated by the quick decrease in iron content (Fig. 3a). Then, the reduction rate decreased for both IONP between 2-8 h. s-IONP were completely eliminated from the daphnids within 12 and 24 h, reaching control levels. In contrast, the

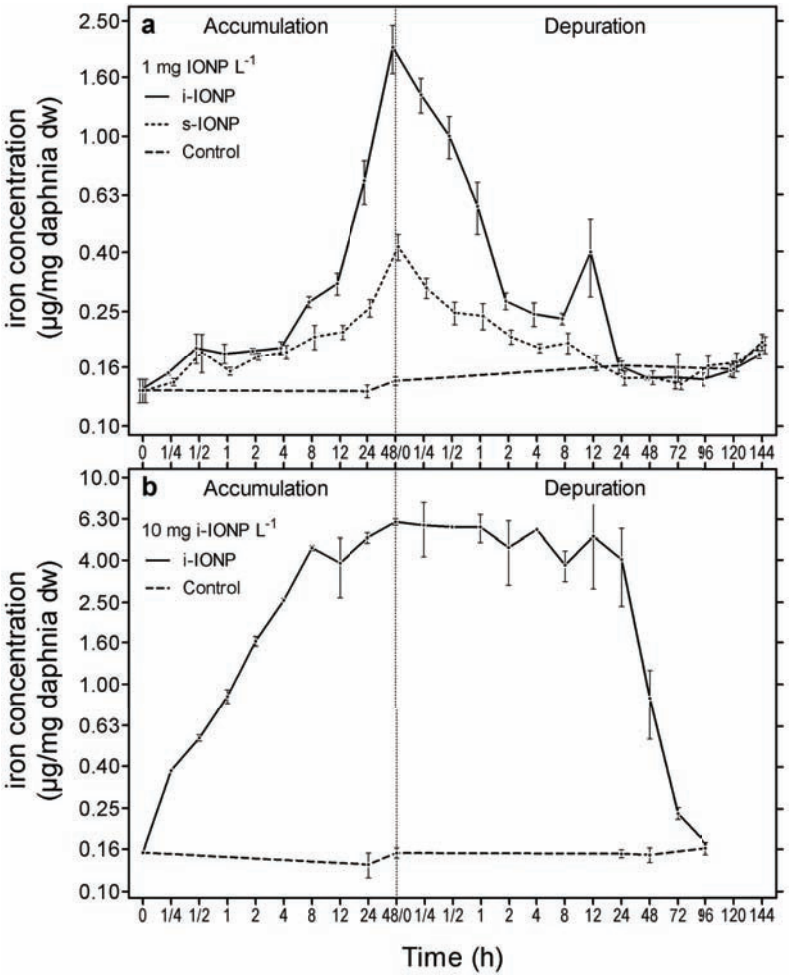

Fig. 3. Accumulation of IONP in *D. magna* over a period of 48 h and their following depuration. The vertical dotted line indicates the time when daphnids were transferred from the exposure medium to clean medium for depuration. Iron contents were measured using a photometric ferrozine staining by which the iron concentration in µg per mg *daphnia* tissue (dw = dry weight) was calculated; error bars denote the SE. (a) 1 mg iron L^{-1} comparison of s-IONP (dotted line; *N*=5), i-IONP (solid line; *N*=5) and the unexposed control (dashed line; *N*=5); (b) To evaluate the maximum possible IONP burden a test with 10 mg iron L^{-1} i-IONP (solid line; *N*=2) was performed. (Control: dashed line; *N*=2).

iron content in i-IONP increased between 8 and 12 h, but then also decreased to the control level between 12 and 24 h. In the following 5 days, the iron content was equal in both treatments and the control. This shows that no iron or IONP were actually bio-accumulated by the daphnids within the 48 h exposure. The plateau phase between 4 and 8 h in both IONP and the following increase in i-IONP occurred due to the water change rhythm. The medium was refreshed for the first time after 12 h. In the hours before, daphnids most likely re-ingested already excreted IONP.

Interestingly, this is the first study to show the complete elimination of NP by daphnids. None of the previous studies concerning the depuration after exposure to NP ever found the complete elimination of the NP from the daphnids within the chosen time frames (max. 96 h) [9, 28, 31, 37, 38].

In order to determine the maximum uptake of IONP, a second test with 10 mg iron L^{-1} i-IONP (expecting higher accumulation rates) was performed (Fig. 3b). This test was only performed with two replicates due to the higher concentration and therefore to the huge amounts of IONP needed. In the first 8 h, the iron content quickly increased from 0.15 µg mg^{-1} (dw) to around 4.59 µg mg^{-1} (dw). Thereafter, the influx rate decreased and reached an iron content of 6.1 µg mg^{-1} after 48 h. Although the influx still showed a slightly increasing trend at 48 h, the approximate maximum IONP accumulation burden should have been reached. The iron content was three times higher than in the 1 mg L^{-1} treatment (Fig. 3a) and 20 times higher than in the control (0.15 µg mg^{-1} dw). During the first 24 h of the excretion experiment, the iron content fluctuated but did not significantly decrease as in the 1 mg L^{-1} test although the medium was renewed after 12 h. After 24 h, a strong elimination of iron set in and after 96 h, the control level was reached.

The slower excretion of i-IONP at 10 mg L^{-1} might also be related to the re-ingestion of already excreted IONP, which also explains the fluctuating iron content between 1 and 24 h. However, the daphnids were not able to eliminate significant amounts of IONP within the first 24 h as in the case of 1 mg L^{-1}. Most likely, this phenomenon had two reasons: (1) the algae-IONP agglomerates clogged the digestive tract, disturbing an effective elimination; and (2) a significant amount of i-IONP was attached to the daphnids' exoskeleton or was incorporated in algae agglomerates, which were attached to the filtering and swimming apparatuses. Jacobasch et al. [44] found that titanium dioxide NP adsorbed to the filtering apparatuses and covered the setae. For *Daphnia pulex,* Auffan et al. [32] showed that during the depuration cerium dioxide NP were not efficiently removed by newly acquired food (algae). The authors identified ecdysis as the main physiological mechanism of NP depuration. Thus, i-IONP attached to the exoskeleton and clogging the digestive tract were most likely only effectively eliminated by the molting of the daphnids. Mature daphnids usually molt every 48 to 72 h (59±21 h [32]), which corresponds very closely to the elimination period with a 90 % decreased iron burden after 72 h (compared to the climax). After 96 h, all IONP were eliminated from the daphnids' bodies and the iron content control reached the control level.

The results indicate that the applied IONP are ingested by the daphnids or at least adsorb to their exoskeleton. The internal iron content thus increases many times within a few hours. However, the IONP used here were not bio-accumulated, since all IONP were effectively eliminated after 96 h at the latest. We hypothesize that the depuration of IONP is a combination of direct excretion and a shedding of the chitinous exoskeleton.

Chronic exposure

Chronic and reproduction tests originally aim to investigate sub-lethal effects on organisms such as to their development and reproduction; wherever possible, they should not induce the death of test organisms. Lethal concentrations should be determined with acute toxicity tests. In previous acute tests – already prolonged to 96 h – the PVP-coated s-IONP had no [12] or only slight, but insignificant harmful effects on daphnids at concentrations between 1 and 100 mg iron L^{-1} [15]. For this reason, the same concentration range was chosen for the chronic reproduction test.

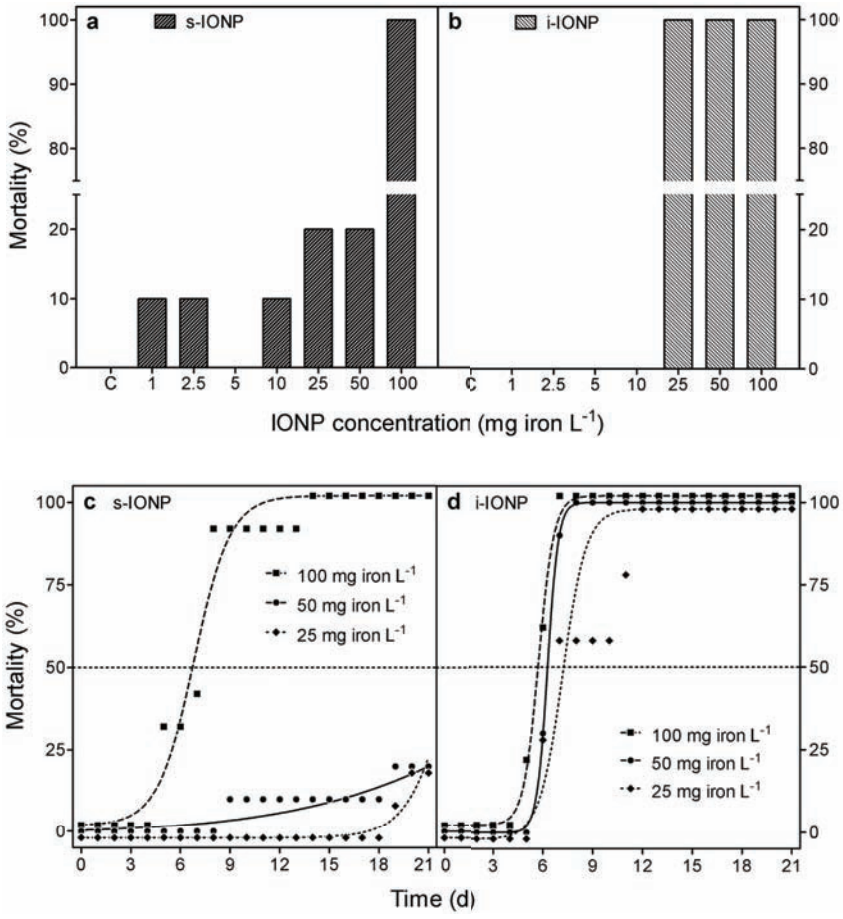

Fig. 4. Concentration dependent mortality (% dead after 21 days) of *Daphnia magna* due to the exposure to (a) s-IONP and (b) i-IONP. (c/d) The mortality of *D. magna* over time at the three highest test concentration of 25 (dotted lines), 50 (solid lines), and 100 mg iron L^{-1} (dashed lines) for s- and i-IONP.

Due to the prolonged exposure, mortal concentrations were now reached. s-IONP induced slight mortality in all tested concentrations, except for the 5 mg L^{-1} treatment (Fig. 4a). However, it was only at the highest test concentration of 100 mg L^{-1} that all daphnids died. In contrast, i-IONP did not kill any daphnids at low concentrations between 1 and 10 mg L^{-1}, but at the higher concentrations of 25, 50 and 100 mg L^{-1} all daphnids died (Fig. 4b). Fig. 4c/d illustrate the point in time of the daphnids' deaths. With the exception of the highest concentration of 100 mg L^{-1} (Fig. 4c), s-IONP mostly killed the daphnids during the last days of exposure.. In the former case, about 50 % of daphnids had died after 6.5 days. In contrast, i-IONP killed most daphnids in all three concentrations after 5 to 7 days (Fig. 4d).

Life history responses could only be determined for test concentration with mortalities < 100 %. We therefore cannot provide data for s-IONP at 100 mg L^{-1} and i-IONP ≥ 25 mg L^{-1}. We monitored the development of the daphnids by measuring their body size at the end of the tests. Their reproduction parameters were determined by the total mean production of progeny per mother, the mean number of days until they released their first brood and the mean quantity of neonates of the first brood.

s-IONP did not significantly reduce the growth of the daphnids at concentrations between 1 and 25 mg L^{-1} as compared to the control with a mean dorsal length of 5.4 mm (Fig. 5a). Growth was only significantly reduced to 4.8 mm at 50 mg L^{-1}. Daphnids were about 21.3 % smaller than in the control. In contrast, i-IONP had a much stronger influence on the daphnids' development. Their growth was already significantly reduced from 5.76 mm in the control to 5.42 mm at the lowest test concentration

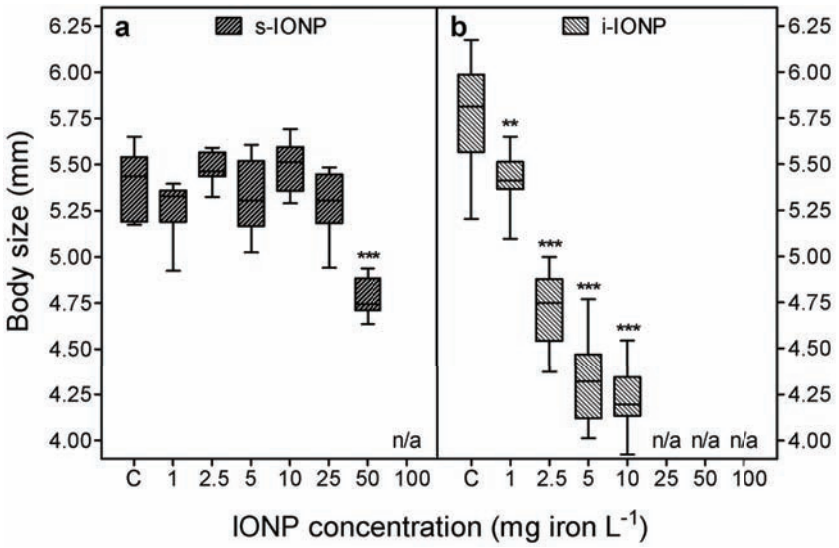

Fig. 5. Effects of s-IONP (a) and i-IONP (b) on the growth of *D. magna* measured at the end of the reproduction test (day 21). Shown are box plots; whiskers indicate min/max values. Significant differences to the control were calculated using a *students* t test. ** $p < 0.01$, *** $p < 0.001$; n/a = data not available due to the death of all daphnids.

of 1 mg L^{-1} (Fig. 5b). The effect increased with increasing concentration. At 10 mg L^{-1} daphnids had a dorsal length of only 4.23 mm and were about 26.6 % smaller than in the control.

Figure 6 shows the effects of IONP on reproduction parameters. s-IONP only significantly reduced the overall reproduction at 50 mg L^{-1} from 65.6 in the control to 34.3 (Fig. 6a). i-IONP induced a much stronger inhibition. In the control, daphnids produced 66.2 neonates. The lowest test concentration of 1 mg L^{-1} the reproduction was already significantly reduced to 43.2 neonates (Fig. 6b). With increasing i-IONP concentration, the reproduction continued to decrease to 11.7 neonates at 10 mg L^{-1}. This is a significant reproduction inhibition of 82.3 %. Fig. 6 c-f show the IONP effect on the first brood. We measured the day when the mothers released their first progeny (Fig. 6c/d) and counted the amount of neonates in this first brood (Fig. e/f). For s-IONP, the day of first offspring was slightly, but not significantly, delayed at 25 and 50 mg L^{-1} (Fig. 6c). In contrast, i-IONP significantly delayed the production of first offspring between 1.5 to 3.5 days in all tested concentrations (Fig. 6d). The quantity of neonates in the first brood was not significantly affected by s-IONP at any concentration (Fig. 6e). i-IONP significantly reduced the quantities of neonates at concentrations from 2.5 to 10 mg L^{-1} (Fig. 6f). However, the effect was less strong compared to the other life history responses.

In vitro assays already showed the cytotoxicity of IONP [45] and their ability to induce oxidative stress [46]. Both IONP might therefore also have induced oxidative stress after ingestion inside the intestine. The effect of i-IONP was more pronounced due to the higher influx rates (Fig. 3a). However, the creation of reactive oxygen species (ROS) as a result of the Fenton reaction [20] is often connected to the release of free iron ions from the IONP [47]. In an earlier study, we showed that even after one year no iron was released from the PVP-IONP [12]. A non-quantitative test as described there confirmed that no diluted iron was present in either IONP dispersions (*qualitative data, not shown*). Klein et al. [47] also described that ROS might be directly produced on the reactive surface of IONP. However, we hypothesized that the thick PVP shell might have suppressed possible ROS production [12]. This was supported by the research of Arndt et al. [20], who compared different IONP and found the lowest production of ROS for PVP-IONP. Thus, it is to be expected that toxicity did not significantly originate from ROS.

Some authors hypothesized that inhibiting effects of ingested NP were related to the mechanical clogging of the digestive tract and diminished energy accumulation [5, 48]. Both IONP were shown to be ingested by the daphnids (Fig. 2 and 3). Even at the lowest test concentration of the reproduction test (1 mg L^{-1}) mature daphnids very effectively concentrated the IONP in their intestine, as demonstrated by the accumulation experiments (Fig. 3a). It is thus very likely that, at the highest test concentrations of the reproduction tests, high amounts of IONP were concentrated in the daphnids' guts and disturbed nutrient uptake or the production of energy. Different possible modes of action of IONP or NP which might inhibit digestion in general are: (1) NP filling the lumen of the gut. In consequence, fewer food particles are ingested; (2) a paratrophic membrane (PTM) regulates the

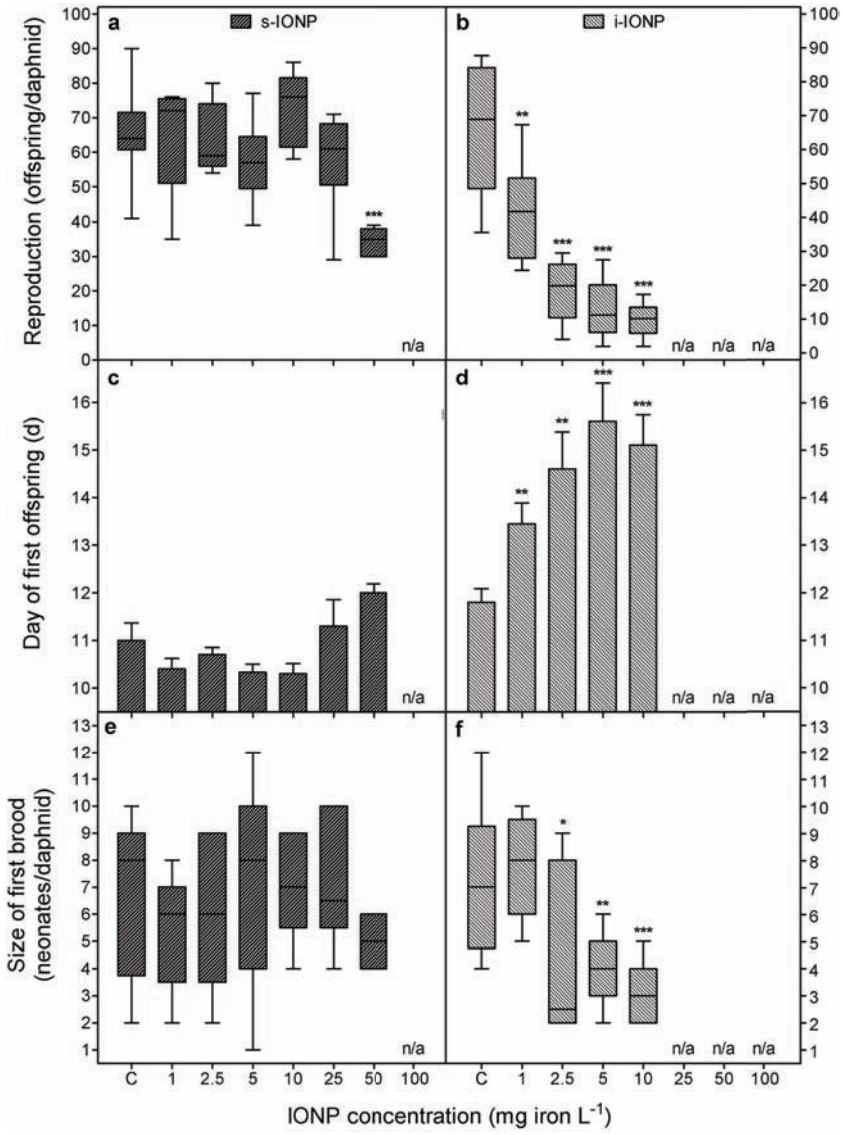

Fig. 6. Influence of s- and i-IONP on reproductive parameters of *D. magna*: (a/b) amount of progeny produced per mother; (c/d) day when the daphnids released their first offspring; (e/f) amount of neonates (size) in the first brood. Shown are box plots; whiskers indicate min/max values. Significant differences where calculated using a *students* t test. ** $p < 0.01$, *** $p < 0.001$; n/a = data not available due to the death of all daphnids.

exchange of nutrients inside the gut, protects the epithelial cells [49, 50], and prevents the settling of harmful microorganisms [51, 52]. The PTM has a mesh-like structure. Its pores have diameters of about 130 nm. NP might block the PTM pores, suppressing nutrient exchange; (3) NP, which are able to pass the PTM, may get stuck between the microvilli, inhibiting nutrient uptake there; and (4) NP destroying the function of gut epithelial structures. This can be mechanical destruction of the PTM or other gut structures during passage or destruction due to direct toxicity of the NPs' material.

The effects of s-IONP on *D. magna* were less strong than those of i-IONP, because, as shown above, fewer s-IONP were ingested. It was only at 100 mg L^{-1} that all daphnids died within a few days. We hypothesize that at that concentration virtually no nutrient exchange was possible due to the effects described above; consequently, the young daphnids starved to death. Baumann et al. [53] showed that neonates can survive four days without feeding. After this period, the energy reserves are depleted and external food uptake is needed for further development. At 100 mg $L^{-1,}$ s-IONP suppressed the efficient use of energy and the daphnids died at the age of five to eight days (Fig. 4c). Some daphnids also died at 50 mg L^{-1}, but much later. This implies that most daphnids were able to ingest enough food to develop. However, reproduction was still significantly reduced (Fig. 6). At concentrations between 1 and 25 mg L^{-1}, the presence of s-IONP did not significantly affect the life history responses of *D. magna*.

The inhibition of the daphnids' life history parameters by i-IONP was amplified by a second effect, the flocculation of algae (Fig. 2c/d), which did not occur for s-IONP. The algae stick to the filtering apparatuses and the swimming antenna, partly completely inhibiting the movements of the daphnids (Fig. 2c). The adsorption of algae to the daphnids exoskeleton (1) increased the resistance during swimming, leading to higher energy demands [11], which could not be compensated since (2) the filtering apparatuses were blocked by the algae, inhibiting normal food uptake. Furthermore, (3) respiration might have also been affected since the filtering current which serves to transport oxygen-rich water to the gills was most likely reduced by the algae clogging the filtering-apparatuses. Additionally, the gills might have also been covered by the agglomerates, which would have disturbed efficient oxygen exchange. The phenomenon of algae agglomeration already occurred at the lowest test concentration of 1 mg L^{-1} and increased with increasing i-IONP concentration. In concentrations between 1 and 10 mg L^{-1}, the daphnids survived due to lower effect size, but also due to the possibility of eliminating the algae agglomerates from their bodies during ecdysis since algae kept sticking to the old *exuvia* (Fig. 2d). At concentrations of 25 mg L^{-1} and higher, the inhibition was too strong and all daphnids died after a few days (Fig. 3d).

The results showed the different effect size of s- and i-IONP. The effect size strongly depended on the exposure concentration in each treatment with stronger effects of i-IONP at lower concentrations. To compare the effects of both IONP on the life history parameters (size, reproduction, day of first offspring and size of the first brood), we performed a within-class Principal Component Analysis (PCA). This analysis allowed us to eliminate the concentration effect on the investigated responses and to compare them. The PCA, followed by a Mann-Whitney U test, revealed no significant differences between the responses to s- or i-IONP exposure ($W = 5124$, $p = 0.38$). This result indicates that both particles had the same effect on the daphnids. From this, we can hypothesize that the mode of action for both IONP was the same. Life history parameters were most likely affected by the insufficient nutrient assimilation. The effect of i-IONP was stronger due to the agglomeration of algae

clogging the filtering apparatuses and additionally preventing the daphnids from sufficient nutrient uptake.

Conclusions

Daphnids are able to effectively filter NP and concentrate them in their digestive tract. The main exposure route of NP in daphnids therefore appears via ingestion. When NP are ingested, they disturb nutrient uptake. NP can inhibit the function of filtering setae and clog the digestive tract or disturb the function of the gut epithelium. Ingested NP have no alimentary value, but when they are ingested they displace food. Even if NP are composed of non-toxic materials, they have strong harmful effects on filter-feeders, since they consequently lead to starvation. On the other hand, we demonstrated efficient and complete excretion of accumulated IONP after medium exchange. Filter feeders such as daphnids are primary consumers and play an important role in the food chain. As they are able to concentrate NP during permanent exposure, trophic transfer of NP to higher tier organisms such as fish is possible [54].

The tested PVP-IONP were effectively ingested by *D. magna* and highly concentrated in its intestine. Due to their low toxicity, we were able to conduct a good investigation of their physiological effects on daphnids. By chance, one of our IONP batches exhibited decreased colloidal stability, which gave us the opportunity to compare equal NP with different colloidal properties. In previous investigations, PVP-IONP did not induce significant acute immobilization within 96 h [12, 15]. Under chronic exposure, both IONP significantly affected the daphnids and even induced their death, with stronger effects of colloidally instable i-IONP. We were able to demonstrate the stronger physiological inhibition by i-IONP due to the additional clogging of the filtering apparatuses by agglomerating algae.

Our results show that even NP composed of uncritical materials with low or no toxicity can significantly affect filter-feeding organisms. Since their effects were closely related to physiological inhibitions, effects were often pronounced only after long-term exposure. For the hazard and risk assessment of NM in general, acute tests might not be appropriate and toxicity cannot be exclusively extrapolated from the toxicity of the core material. Physicochemical properties also play an important role in how NM may affect (aquatic) organisms. As we showed, a little change in the colloidal properties can have significant influence on the impacts of NM.

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4 Summarizing Discussion and Conclusions

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In the year 2009, at the beginning of this thesis, knowledge about NP and their possible impacts was limited, and the available data was very diverse and partly contradictory. From the outset, the focus of this thesis was placed on the effects of iron oxide NP (IONP) in the aquatic environment, but also investigated AgNP as a reference NM. As the research discussed here progressed, new goals and hypotheses were developed, changed and adopted. Due to the manifold different aspects influencing the mode of action of NP in the aquatic environment, it was decided to focus on *Daphnia magna* as the only exemplary test organism.

The following section summarizes and discusses the key findings of this thesis and aims to make recommendations for future investigations of NM in aquatic, ecotoxicological tests for a reliable hazard assessment.

4.1 Accumulation and Depuration of IONP in Daphnia

Daphnids are small crustacean filter feeders. On their thoracic legs they carry small setae to filter food particles. Due to the size of their setae daphnids should be able to actively filter particles as small as 200 nm from the surrounding water (Hartmann & Kunkel 1991). With their appendages they create a water current along the opening of the carapace that funnels food towards their mouth and oxygen-rich water into the carapace to facilitate respiration (Mendonca et al. 2011, Pirow et al. 1999, Porter et al. 1982, Rosenkranz et al. 2009). Since the filter feeding of daphnids is not very selective, NP are ingested with the feeding current (Ebert 2005, Hund-Rinke & Simon 2006).

NP including iron oxide NP (Hu et al. 2012), cerium dioxide NP (Auffan et al. 2013), zinc oxide NP (Santo et al. 2014), copper NP (Heinlaan et al. 2011), silver NP (Zhao & Wang 2010), gold NP (Lovern et al. 2008), titanium dioxide NP (Zhu et al. 2010), quantum dots (Feswick et al. 2013, Lewinski et al. 2010), polystyrene beads (Rosenkranz et al. 2009), diamond NP (Mendonca et al. 2011), and other carbon NM (Petersen et al. 2009, Tervonen et al. 2010) were already shown to be accumulated by daphnids. Therefore, the main exposure route of NP in daphnids – and most likely in all filter feeding species – should be via ingestion (Feswick et al. 2013).

Most studies reported quick accumulation of NP in the daphnids' digestive tract and slower depuration rates. Some showed interactions between the NP and the gut epithelium such as

penetration into the first epithelium layers or destruction of gut epithelium structures (Heinlaan et al. 2011, Lovern et al. 2008, Santo et al. 2014). Due to these findings, NP bear a high potential to disturb natural nutrient uptake.

It was already found during the first investigation of acute effects that different IONP were evidently ingested by neonate daphnids (Baumann et al. 2014, Publication 3). In order to determine accumulation and depuration rates of IONP, tests with mature daphnids were performed. The iron content of daphnids was measured with an adopted, photometric ferrozine-based assay (Riemer et al. 2004). The accumulation of colloidally stable and instable PVP-IONP was investigated over 48 h and the depuration over a period of 144 h and compared to the iron content of unexposed daphnids.

Both IONP were ingested by the daphnids or adsorbed to their exoskeletons, with higher rates of instable IONP. During depuration daphnids excreted the whole IONP burden within 96 h. The IONP were expelled by the pressure of more recently acquired food, but also ecdysis was hypothesized to play an important role in eliminating IONP from the digestive tract (Auffan et al. 2013). The results indicated that IONP were not bio-accumulated by the daphnids.

Given the limited retrieved data, only postulates on the modes of action of IONP accumulation and depuration were possible. More research is needed to fully understand uptake and elimination mechanisms. Furthermore, the exposure of 48 h was very short. At a concentration of 1 mg iron/L, the internal iron concentration was still increasing at the end of the exposure period. Additional tests monitoring the accumulation of IONP over longer periods of several days, weeks or even generations might deliver new insights into the bio-accumulation potential of (IO)NP in daphnids.

4.2 The Role of Colloidal Properties of IONP on their Effects

Colloidal characteristics of NP have the most important influence on their distribution in the (aquatic) environment (Wiesner et al. 2009). These properties determine whether NP build stable dispersions, whether they agglomerate or adsorb to surfaces and other particles, or whether they tend to decompose and release potentially critical substances such as toxic ions (Kahru et al. 2008, Navarro et al. 2008a). The colloidal stability therefore determines exposure routes and interactions at the bio-nano interface (Rivera-Gil et al. 2013).

The colloidal properties of NP highly depend on their reactivity, their surface chemistry and the composition of the dispersion medium (Handy et al. 2008a). Usually NP are supplied with

a surface coating which serves to increase the colloidal stability to maintain their nano-specific characteristics in dispersion (Batley et al. 2013, Nowack & Bucheli 2007). NP can be (colloidally) stabilized by increasing their charge to achieve electrostatic repulsion, or they can be surrounded by a sterically stabilizing shell, which suppresses direct contact between the NP (Segets et al. 2011, Studart et al. 2007).

IONP with different coatings were synthesized by Darius Arndt and tested on their colloidal properties in different media including the *Daphnia* medium Elendt M7. The four colloidally most stable IONP were chosen for ecotoxicological investigations with *D. magna*. These IONP were present in the water column at least for several days or even months, guaranteeing high exposure potential to the pelagic filter feeding daphnids. One set of IONP was stabilized via electrostatic repulsion with ascorbate (ASC) and citrate (CIT). Sterically stabilized IONP were functionalized with dextrane (DEX) and polyvinylpyrrolidone (PVP).

First, the acute toxicity of the four different IONP was investigated over a period of 96 h. Since (negatively) charged NP were already shown to be more (cyto-) toxic than less or neutrally charged NP due to increased reactivity and the potential to form more reactive oxygen species (ROS) (Lee et al. 2013a, Park et al. 2013, Schaeublin et al. 2011), ASC- and CIT-IONP were expected to be more toxic than DEX- and PVP-IONP. However, the greatest immobilization and toxicity was found for ASC- and DEX-IONP which was attributed to an increased agglomeration in the tests. Although ASC/DEX-IONP were tested to be colloidally stable, they quickly agglomerated in the presence of daphnids. It was hypothesized that the colloidal destabilization was related to turbulences induced by the filtering and swimming movement of the daphnids. The agglomeration and adsorption of IONP disturbed the molting of the neonates. The incomplete ecdysis often immobilized the neonates, but usually did not cause their death. Comparable agglomeration of both IONP in the Elendt M7 stocks was only observed after one year. Most frequent cases of incomplete ecdysis were induced by the colloidally instable ASC- and DEX-IONP. IONP possibly adsorbing to the carapace were obviously able to disturb the molting process. This effect was also less frequently observed for CIT-IONP and did not occur for PVP-IONP.

In CIT-IONP additional release of iron ions was measured. However, toxicity could not exclusively be related to free iron since release rates were too low. Toxicity was hypothesized to also originate from the possible formation of ROS. For ASC- and CIT-IONP Arndt et al. (2012) had shown increased production of ROS.

PVP-IONP did not have any significant harmful effects within 96 h at concentrations of up to 100 mg iron L^{-1}. PVP-IONP had the highest long-term stability without any agglomeration or decomposition even after one year in Elendt M7 stocks. The thick PVP-shell effectively

prevented the IONP from agglomeration (Studart et al. 2007). These IONP are therefore a very promising tool to investigate long-term NP effects without the influence of effects from released (toxic) ions or decreasing colloidal stability.

Consequently, PVP-IONP were chosen for the investigation of the long-term effects of IONP on *D. magna*. The chronic test aimed to investigate sub-lethal endpoints such as inhibition of reproduction and growth over 21 days. By chance, one batch of PVP-IONP synthesized for the tests showed decreased colloidal stability with slow agglomeration. Although this effect was not desired, it opened up the opportunity to compare long-term effects of equal IONP with different colloidal properties.

Both IONP induced increased mortality at concentrations ≥ 25 mg iron/L, but with stronger effects of instable IONP. Many or even all daphnids died after 5 to 8 days, which indicates that acute tests might not be sufficient for the testing of NM since effects are often delayed compared to those of diluted chemicals. Also the reproduction as well as the development (growth) was significantly reduced under exposure to both IONP. However, stable IONP only induced significant effects at 100 mg iron/L, instable IONP already affected the life history responses of daphnids significantly at 1 mg iron/L.

When NP are present, they are very effectively concentrated by daphnids in their digestive tracts (*see above*). NP composed of non-toxic materials (such as IONP) might also significantly decrease nutrient uptake (Mendonca et al. 2011) by physically clogging the digestive tract and slowing down the gut passage (Campos et al. 2013). In consequence, many life history effects and even increased mortality observed under chronic IONP exposure should be related to the starving of daphnids as a consequence of insufficient nutrient uptake. For a complete understanding of effects and to confirm the developed hypotheses the accumulation of (IO)NP in daphnids has to be investigated in more detail. Investigations of the intestines of exposed daphnids with histological methods might help to prove the assumptions of disturbed nutrient exchange in the daphnids' gut.

The increased effects of colloidally instable IONP were explained by the additional flocculation of algae, which were administered as food. The algae flocs were concentrated between the filtering legs of the daphnids, partly completely inhibiting their locomotion. This greatly increased physiological stress, which may have even been amplified by the reduced food acquisition. Furthermore, respiration might also have been affected sicne the filtering current also serves respiration. It was hypothesized that the filtering movements and the associated water current accelerated the release of PVP from the IONP. Released PVP, colloidally instable PVP-IONP or the mixture of both interacted with the algae forming the visible algae agglomerates. However, since the observed effects might exclusively be related

to PVP, more research on the interaction of algae and i-IONP would allow a better understanding of this phenomenon.

4.3 Combinatory Toxicity and Application of IONP for Remediation

Iron-based NM are tested for remediation applications as a cost effective method due to their high efficiency in processing many different contaminants (Brame et al. 2011, Crane & Scott 2012, Karn et al. 2009, Li et al. 2006a, Li et al. 2006b, Sanchez et al. 2011, Zhang & Elliott 2006). Risks for the environment are largely ignored, although there is only limited data available concerning the fate, transport and the effects of nano-remediation products on biota (Grieger et al. 2010, Noubactep et al. 2012, Yin et al. 2012).

Since iron is easily hydrolyzed or oxidized to iron oxides under environmental conditions (Kharisov et al. 2012, Khin et al. 2012), studies presented within this thesis focused on more environmentally relevant IONP. In order to identify potential benefits and risks from nano-remediation products, combinatory tests with PVP-IONP and four exemplary contaminants, the heavy metals cadmium and copper and the organic compounds glyphosate and resorcin (aromatic compound), were performed.

The addition of PVP-IONP reduced the toxicity of both heavy metals many times over. Cadmium and copper can be sorbed or complexed by IONP (Merkel & Planer-Friedrich 2009), but also interactions with the PVP shell were demonstrated by theoretical assumptions (Publication/Manuscript 4). The results indicated that the bioavailability of heavy metals can be significantly reduced by IONP. In the case of glyphosate, the toxicity was reduced by the IONP within the first 72 h, but after 96 h toxicity reached the level of the single substance. It was hypothesized that – at first – glyphosate was incorporated within the PVP-IONP complex. Over time glyphosate was released from the IONP, e.g. due to digestion processes during passage of the daphnids' guts. The results hint at a possible carrier effect where IONP serve as a transport vehicle (into biota). The toxicity of resorcin was not affected by the addition of IONP.

Within the test duration of 96 h, no negative effects of the IONP-contaminant mixtures were observed. At least in the case of heavy metals their toxicity was even reduced. However, complexed or adsorbed contaminates are just immobilized at the surface of IONP, which temporarily reduces their bioavailability. At the moment there is only limited data available on the long-term remobilization of such contaminants from NP (Yin et al. 2012). NP may act as a carrier by which a local contamination might be induced, drift with the (ground-) water

stream, and be spread to uncontaminated areas (Grieger et al. 2010, Noubactep et al. 2012). By ingesting NP-bound substances, the NP might act as a transport vehicle for immobile contaminants into organisms, possibly increasing the uptake of critical substances compared to their natural accumulation rates. Baun et al. (2008b) found faster uptake and highly increased toxicity of phenanthrene in *D. magna* and algae (*Pseudokirchneriella subcapitata*) as a result of the presence of C_{60} fullerenes. C_{60} fullerenes also increased the toxicity of copper in *D. magna* by increasing its uptake (Tao et al. 2013). TiO_2 NP increased the toxicity of arsenic in *Ceriodaphnia dubia* (Wang et al. 2011). Zhang et al. (2007) showed increased cadmium accumulation in carp and Hu et al. (2011) in zebra fish when TiO_2 NP were present.

However, first results on the transport of iron NP in artificial soil and groundwater systems indicate that their transport under natural conditions is limited to a few centimeters (Emerson et al. 2014, Gomes et al. 2013, Lin et al. 2010). To increase their efficiency, the mobility of future NP for remediation will most likely be increased (Phenrat et al. 2007), but their transport might still be limited. Therefore, the application of iron NP on highly contaminated sites is a promising future remediation tool (Karn et al. 2009), especially when taking into account that soil and groundwater organisms are usually no longer present in contaminated areas.

Nevertheless, the unknown side effects and the behavior of iron NP under environmental conditions need further investigation before they can be commercially applied. Although the high efficiency of iron NP in decontamination was already proved in laboratory studies (Huang & Chen 2009), more research on long-term effects such as transport and release of contaminants from NP or the risks from degradation products is needed (Yin et al. 2012).

Although *Daphnia* tests provided new insights into the combinatory toxicity of IONP, future tests should also be conducted with potential target organisms living in soil and groundwater, since the way of life and further biotic and abiotic factors highly influence exposure. Furthermore, tests should also be carried out under more environmentally relevant conditions and with different target contaminants and mixtures, since contaminants might also compete e.g. for binding sites at the NP surface. In order to determine the complete hazard potential of iron NP, combinatory tests would have to be performed with each iron NP and each target-contaminant in environmentally relevant mixtures.

4.4 Daphnia OECD Standard Tests and their Suitability for the Testing of NM

In order to gain reproducible data, it is very important to rely on standardized test protocols. All *Daphnia* tests of this thesis were performed according to OECD standards. It was a major objective to prove the suitability of both tests, namely the acute immobilization (OECD 2004) and the chronic reproduction test (OECD 1998), for testing NP.

The OECD suggests using artificial media such as Elendt M4 and M7 (OECD 2004). Elendt is a very complex medium containing over 20 different substances, resulting in an ionic strength of 8.3 mM. High (salt) concentrations, especially those of calcium and magnesium present a problem for the colloidal stability of dispersed NP (Filser et al. 2013, Jin et al. 2010). Furthermore, the contained EDTA can complex metal ions and affect their bioavailability (OECD 1998). Hence, some ecotoxicologists suggest less complex media with reduced salt concentrations for the testing of NP (Handy et al. 2012b, Tejamaya et al. 2012).

Chronic reproduction tests performed with different media revealed astonishing results (**Fig. 7**). The best reproduction was found in both Elendt media as well as in the UFT tap water. In the alternative EPA hard water medium (US EPA 2002) and ISO medium (ISO 1996), the reproduction of *Daphnia* was significantly reduced, showing that the transfer of daphnids to other media can significantly affect life history parameters. Due to these findings and the reduced concentration of microelements, Elendt M7 was the preferred medium for the culturing of *D. magna* and the testing of IONP.

Test guidelines recommend accompanying analyses of the tested substances during the tests to determine the bioavailable concentrations. Furthermore, abiotic factors such as temperature, pH, salinity, conductivity, and hardness of the medium should also be recorded (e.g. OECD 1998). However, for NP, additional analyses have to be recommended to understand the effects of NP in biotests (Crane et al. 2008). The most important information for ecotoxicological tests is: (1) size: the primary particle diameter usually measured via TEM, and the mean hydrodynamic diameter in the test medium measured via DLS; (2) size distribution: exact size partition determination to assess whether NP dispersions have a narrow or broad distribution and the tendency of NP to form agglomerates; (3) coating materials and (4) surface charge: the mode of action on how NP are stabilized plays an important role for their colloidal properties and their interactions at the bio-nano interface (Cunningham et al. 2013, Kim et al. 2013, Rivera-Gil et al. 2013, Zhu et al. 2013). The surface charge is usually measured via the zeta potential; (5) concentration: the overall mass concentration, e.g. measured via AAS, to assess the bioavailable concentrations as well as

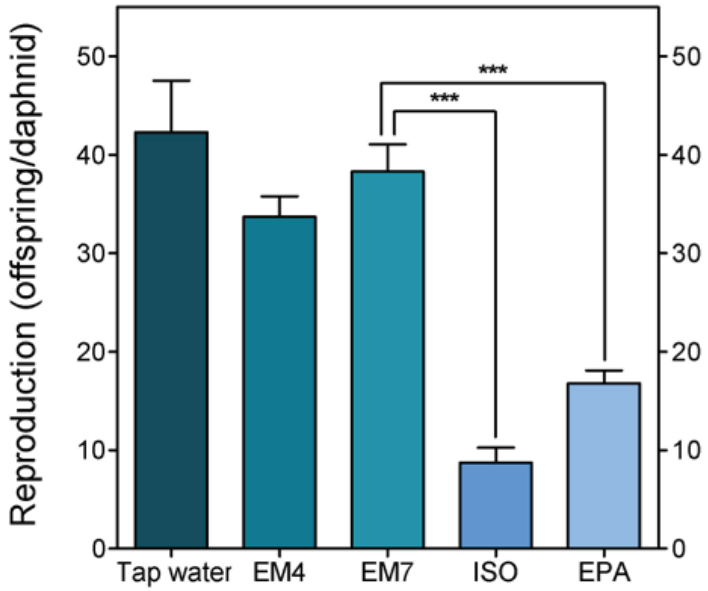

Fig. 7: Mean reproduction (±SE) of *Daphnia magna* in a 21-day reproduction test with different (artificial) test media. Daphnids produced the most offspring in the UFT tap water, Elendt M4 (EM4) and Elendt M7 (EM7) media. Reproduction was significantly reduced in the ISO medium (ISO 1996) and the EPA hard water medium (US EPA 2002). Statistics: 1-way ANOVA with Dunnetts' multiple comparison test, *** $p < 0.001$ (*unpublished data by kind permission of Yvonne Sakka; the experiment was designed by J.B. + Y.S. and conducted by Y.S.*)

the tendency for adsorption, but also differentiation between nanoparticular matter and material released from the NP such as metallic ions, e.g. achieved by a pretreatment of samples with ultra-filtration or centrifugation. These mentioned analytical endpoints were assessed in most investigations of this thesis. However, additional measurands may deliver important information such as the particle concentration, the overall reactive surface (Handy et al. 2008b) and other material-specific properties such as the photocatalytic (Hund-Rinke & Simon 2006) or redox reactivity and the ability to build ROS, respectively (Xia et al. 2006).

The tests with IONP and daphnids have shown that new, unknown biological effects can appear under NP exposure. Test organisms and their reactions during NP exposure have to be carefully monitored. In daphnids more research must be done on the phenomenon of incomplete ecdysis and the mechanism disturbing the molting process. The effects of NP clogging the filtering apparatuses and the digestive tract should be investigated in more detail.

These findings reveal that “nano-effects” might often be connected to modes of action other than those of dissolved chemicals. Basically, the physiological fitness of the daphnids was affected. Since these are indirect effects, they should harm organisms much more slowly than the direct toxicity of dissolved substances. In order to determine “long-term acute effects” in daphnids, the acute toxicity test (OECD 2004) was prolonged from 48 to 96 h. This was the longest duration the neonates survived without changing the standard test procedure, for example by adding food. For both NP tested, IONP and the exemplary tested AgNP, additional information was gathered during the prolonged test span. These findings are supported by the research of Dabrunz et al. (2011) who found increasing toxicity of TiO_2 NP and incomplete ecdysis of *Daphnia* during the prolonged test span. A prolongation of acute tests seems especially worthwhile for NP consisting of uncritical material(s).

It appears that in most cases *Daphnia* standard tests (and also other ecotoxicological standard tests) should be appropriate for the testing of NM (Handy et al. 2012a). However, additional test endpoints such as incomplete ecdysis of daphnids might have to be introduced. Furthermore, additional physicochemical analyses have to be performed to understand the reactions at the bio-nano interface. Many analytical methods to characterize NM were launched in the market over the past decade, but we are still lacking guidelines prescribing standardized analytical methods which should accompany ecotoxicological tests (Lopez-Serrano et al. 2014).

4.5 Miniaturization of the Daphnia Acute Toxicity Test

Due to the time-consuming synthesis of IONP, a miniaturized test design of the *Daphnia* acute test (OECD 2004) was developed at the beginning of this thesis. For the miniaturization, 24-well microtiter plates were used, which are cheap, disposable test vessels made of polystyrene. This can be particularly important for the testing of NM. Cleaning reusable (glass) test vessels often demands a time-consuming treatment with dangerous chemicals such as strong acids and the additional disposal of contaminated cleaning liquids.

With the developed miniaturized test design, not only 50 % of test substances and test animals were saved, but the time to setup and check the tests was also significantly reduced. Thus allowed for more tests to be performed simultaneously. The miniaturized test was compared to the classic standard test performed in glass beakers and in 6-well microtiter plates (in order to evaluate possible influences of the plate material). Potassium dichromate was used as the reference substance. The comparison tests revealed no significant differences between the miniaturized and the two standard tests designs. It was concluded

that the miniaturized test system is an appropriate alternative to the standard test system, at least for soluble and non-adsorbing substances.

Since the reduction of the test volume automatically increases the surface-to-volume ratio, the miniaturization may influence the bioavailability of potentially adsorbing substances. Most AgNP and released Ag^+ ions are known for their strong adsorption to surfaces (Lau et al. 2013, Welz & Sperling 1997). For this reason, additional tests with NM-300K AgNP were conducted.

Contrary to expectations, the toxicity of AgNP increased in the miniaturized test although the overall Ag content was measured to be lower than in the standard tests. It was hypothesized that the effects were related to increasing decomposition of AgNP when adsorbing to the test vessels' surface, thus releasing more toxic Ag^+ ions (**Fig. 8**). In order to confirm this assumption, it would have been necessary to differentiate between the partitions of AgNP and Ag^+ ions e.g. by ultra-filtrating the samples before further processing for the AAS measurement. However, this requires a very sophisticated preparation of samples due to the extreme adsorption of AgNP/Ag^+ ions. Furthermore, the available AAS measurement was just sensitive enough to measure the overall Ag content at concentrations corresponding to the EC_{50} values. Even with a reliable separation method, exact concentration measurements would have been nearly impossible. Measuring Ag at biologically and environmentally relevant concentrations might be improved by concentrating the samples after the drying process. Nevertheless, a reliable separation of AgNP and Ag^+ ions and concentration measurement methods must be improved and further developed to understand the effects observed. For example Mwilu et al. (2014) have recently demonstrated a method using magnetic NP to capture the Ag^+ ions fraction and concentrating it up to 250 times. After magnetic separation, Ag bound to the magnetic NP was measured via ICP-MS.

The results showed that a miniaturization of the *Daphnia* acute toxicity test is possible. However, due to the increased surface-volume ratio the bioavailability of substances may be significantly affected, especially of those with a strong adsorption potential. The increasing toxicity of AgNP was surprising and contrary to expectations. It could only be hypothesized that decomposition processes might have played a role (**Fig. 8**).

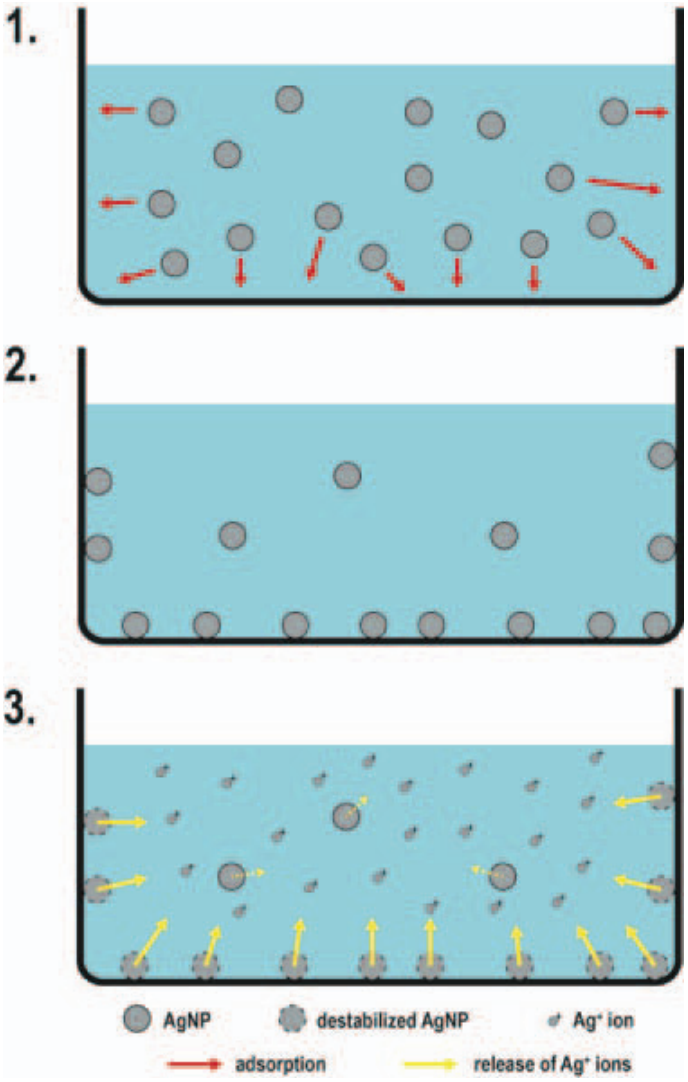

Fig. 8: Scheme of the hypothetical processes causing higher toxicity of AgNP in the miniaturized test design. (**1**) AgNP adsorbed to the test vessels' walls (**2**) building a monolayer. (**3**) Adsorbed AgNP increasingly released toxic Ag^+ ions. Due to the increased surface-volume ratio in the miniaturized test, more AgNP adsorbed in less time and more Ag^+ was released. Although the overall Ag content was lower compared to the standard test, more toxic Ag^+ ions were present, explaining the increased toxicity.

4.6 Future Perspectives and Recommendations

When iron NP enter the environment, they are quickly covered by an oxidized shell or are even completely oxidized or hydrolyzed. Hence, this thesis focused on the investigation of iron oxide NP. The tests proved that IONP had relatively low toxicity in daphnids compared to other metallic NP such as AgNP. They effectively reduced the bioavailability of at least two heavy metals. For environmental remediation, iron NP appear as a promising future tool. The risks to the environment are low compared to the benefits from iron NP remediation. Due to their moderate toxicity, IONP are also very well suited for the investigation of "nano-effects" without the influence of other effects such as that of toxic ions released from the NP.

Daphnids such as *Daphnia magna* are one of the preferred test organisms in aquatic ecotoxicology. They are easy to culture, have short reproductive cycles, high reproduction rates, and are sensitive to most toxic substances (Griffitt et al. 2008, Kahru et al. 2008, Zitova et al. 2009). Daphnids are pelagic filter-feeders which feed on suspended microparticles such as algae. Daphnids are predestined for the testing of NM (Baun et al. 2008a) since they are able to ingest and concentrate suspended NP in their digestive tract, greatly increasing internal or intestinal exposure and hence potential hazards from NP.

The investigations have proven that both OECD *Daphnia* standard tests, the acute immobilization test (OECD 2004) and the chronic reproduction test (OECD 1998), are suitable for the ecotoxicological investigation of NM. However, some adaptations for future D*aphnia* tests should be made:

1. **Prolonged duration:**

   The acute immobilization test is limited to 48 h. Within this period, diluted chemicals often induce significant effects and mortality only moderately increases thereafter. The effects of NM are often delayed since other mechanisms of action might take place. The possibility to prolong the *Daphnia* acute test to 96 h without changing the test procedure was proven. Tests have shown that within the prolonged test span, effects of NP can significantly increase (e.g. AgNP, DEX-IONP). Since a few NP such as PVP-IONP may not induce any harmful effects even within the prolonged test, the effectiveness of acute tests for the determination of hazard potentials of NM needs further discussions.

2. **More standardized analyses:**

   The complete characterization of physicochemical properties of NP is important to understand their effects on biota (Crane et al. 2008). Test guidelines should be extended with suggestions for additional analytical endpoints and appropriate measuring methods

of NP. Provided that material compositions of NP are known, NP analyses should at least cover: (1) concentration measurement with exact determination of NP fractions; (2) information on the primary particle size and morphology and the hydrodynamic diameters in the test media as well as a time-dependent detection of size distributions since colloidal properties can significantly influence the effects of NP on daphnids; and (3) exact knowledge of the surface chemistry such as surface-coatings and surface-charge which might be extended by NP specific measurements such as the potential to build ROS.

3. New endpoints:

This thesis provides a number of new insights on how NP can affect the physiology of daphnids. First, disturbed molting or incomplete ecdysis is to be mentioned. IONP were also found to disturb the ecdysis, which was first described by Dabrunz et al. (2011) for TiO_2. Although more research has to be performed to explain the mechanisms behind this phenomenon, researchers should already draw attention to this “nano-effect”. Another effect was described as the “clogging” of the filtering apparatuses and the digestive tract, which consequently reduced effective nutrient accumulation. This effect also needs further investigation, since intestinal interactions might be one of the driving forces of the reduced development of daphnids under chronic (sub-lethal) exposure to NM.

Laboratory data on the effects of NM on biota is important to estimate their hazard potential. Nevertheless artificial laboratory single species tests can hardly simulate the complex processes in nature. In order to better understand how NP may actually behave in the environment, much more work has to be done. This begins in the laboratory, where new and standardized test strategies and instructions have to be developed. More research on the bioaccumulation potential of NM and their entrance and transport in food webs needs to be conducted. Environmentally relevant mixtures of NP and natural substances (e.g. NOM) or contaminants will need to be investigated further. At last, we need reliable, standardized measurement methods for NP/NM in the environment and the differentiation between natural and engineered NM to understand the behavior of and to predict the risks from NM to human and nature health.

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Annex

VI Acknowledgements

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VII Curriculum vitae

Personal Data

Name	Jonas Baumann
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Education

01/2009 - 08/2014	PhD thesis *The Ecotoxicity of Iron Oxide Nanoparticles: Acute, Chronic and Mixture Effects on Daphnia magna* Department of General and Theoretical Ecology, Center for Environmental Research and Sustainable Technology (UFT), University of Bremen, Bremen, Germany
06/2008 - 09/2008	Subsequent work on open questions of the Diploma thesis
03/2007 - 04/2008	Diploma thesis *Effects of Nanoparticles on Aquatic Invertebrates* Department of Aquatic Ecotoxicology, Institute of Ecology, Evolution and Diversity, Faculty of Biosciences, Goethe-University, Frankfurt/Main, Germany
09/2006 - 02/2007	Preliminary work for the diploma thesis
10/2004	Intermediate diploma Faculty of Biosciences, Goethe-University Frankfurt/Main, Germany

10/2001 - 08/2006	Full time study General Biology with later specialization on Aquatic Ecotoxicology
	Faculty of Biosciences, Goethe-University Frankfurt/Main, Germany
10/2000 - 09/2001	Full time study Engineering
	Technical University Darmstadt, Germany
08/1999 - 07/2000	Military/social service
	Reha-Werkstatt Rödelheim, Frankfurt/Main, Germany
09/1996 - 07/1999	Upper/Grammar school
	Heinrich-Böll-Schule, Hattersheim, Germany

VIII Awards

09/2013	1st price, young scientists award, best poster presentation, 18th Annual SETAC GLB Conference 2013, Essen, Germany
09/2008	1st price, young scientists award, two best poster presentations, 3rd Annual Joint Conference of SETAC GLB and GDCh 2008, Frankfurt/Main, Germany

IX Publications

International peer-reviewed Articles

2014

Baumann J, Bertrand C, Becker M, Filser J (2014, *submitted manuscript*). "Colloidal properties of PVP-coated IONP affect their bio-distribution and life history responses of *Daphnia magna*."

Baumann J, Köser J, Bertrand C, Filser J (2014, *under revision*). "Acute combinatory effects of iron oxide nanoparticles with selected contaminants on *Daphnia magna."*

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2013

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2007

Vogt C, Pupp A, Nowak C, Jagodzinski LS, Baumann J, Jost D, Oetken M, Oehlmann J (2007). "Interaction between genetic diversity and temperature stress on life-cycle parameters and genetic variability of *Chironomus riparius* populations." Climate Research 33: 207-214.

Presentations at scientific meetings

Platform Presentations

2012

Baumann J (2012). “Ecotoxicological investigations with iron (oxide) nanoparticles.” (in German language). Final symposium of the graduate school nanoToxCom, Haus der Wissenschaften, Bremen, Germany.

Baumann J (2012). “Ecotoxicological investigations with iron oxide nanoparticles (IONP): Acute and chronic effects and bioaccumulation in daphnids.” (in German language). 5th Joint Annual Conference of SETAC GLB and GDCh, UFZ Leipzig, Germany.

2011

Baumann J (2011). “Iron (oxide) nanoparticles for environmental remediation? A case study on single and combinatory effects in *Daphnia magna*.” (in German language). 16th Annual SETAC GLB conference, Landau, Germany.

2007

Baumann J, Nowak C, Vogt C (2007). “ ‘Real-world’ relevance of laboratory data – the example of TBT induced genetic erosion in *Chironomus riparius*.” (in German language). S*potlight*-presentation of the results of the BW-Plus project ‘Genetische Verarmung in Folge von Schadstoffstress’. 12th Annual SETAC GLB conference, UFZ Leipzig, Germany.

Posters Presentations

2013

Baumann J, Sakka Y, Bertrand C, Filser J (2013). “Miniaturization and economization of the *Daphnia* acute toxicity test (according to OECD 202) with microtiter plates.” (in German language). 18th Annual SETAC GLB Conference 2013, Essen, Germany.

Sakka Y, Baumann J, Bertrand C, Köser J, Filser J (2013). “Effects of miniaturizing the *Daphnia* acute test on the toxicity of silver nitrate and silver nanoparticles (NM-300K).” (in German language). 18th Annual SETAC GLB Conference 2013, Essen, Germany.

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2012

Sakka, Y., Baumann J, Siol A, Filser J (2012). “Pollutants in culturing medium: active carbon filters as an alternative.” (in German language). 5th Joint Annual Conference of SETAC GLB and GDCh, UFZ Leipzig, Germany.

Baumann J, Bertrand C, Arndt, D., Filser J (2012). “Is there a risk from iron (oxide) nanoparticles used for remediation? A case study on combinatory effects in *Daphnia magna*.” 6th SETAC World Congress / SETAC Europe 22nd Annual Meeting, Berlin, Germany.

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2011

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2010

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Baumann J, Bertrand C, Arndt D, Filser J (2010). "Ecotoxicologival investigatins with iron oxide nanoparticles: toxicity, combinatory effects and bioaccumulation in *Daphnia magna*." (in German language). 4th Joint Annual Conference of SETAC GLB and GDCh, UBA, Dessau, Germany.

2009

Baumann J, Oehlmann J (2009). "Golden particles – The uptake of nanogold in the Zebra mussel *Dreissena polymorpha*." 1st Young Environmental Scientists Meeting (SETAC-YES), Landau, Germany.

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2008

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2007

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2006

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Changes made to the original version

Page III: The “date of defense” was added

Publication 1 (pages 33-46), publication 2 (pages 49-62) and publication 3 (pages 65-74) were removed from this dissertation due to copyright reasons. Download links of the publications are given on the front pages of the corresponding publication chapter.