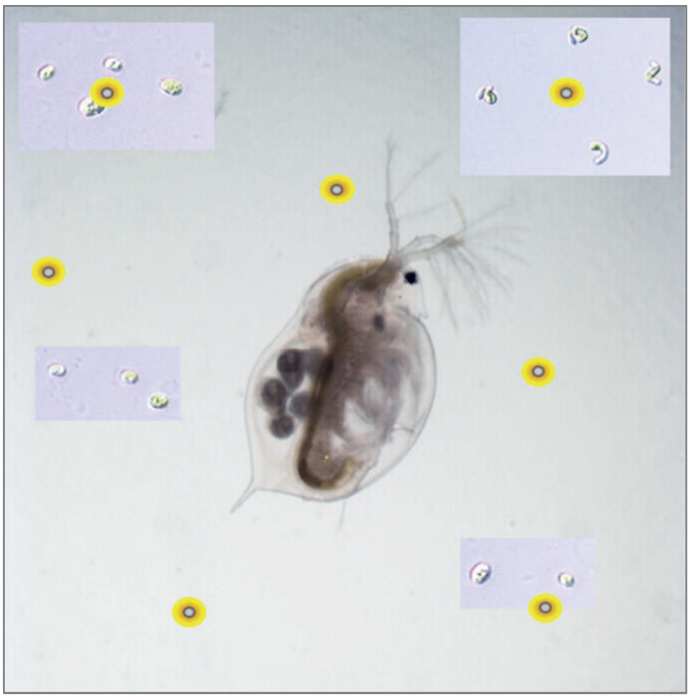
Universität Bremen*

Abiotic and biotic influences on silver nanoparticle fate and effects in aquatic model ecosystems



PhD thesis for the attainment of the scientific doctoral degree

Dr. rer. nat

submitted to the Department of Biology (Dept. 2)
at the
University of Bremen
by
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August 2016

„When we try to pick out anything by itself, we find it hitched to everything else in the Universe.“

John Muir

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Date and place of the colloquium:
23rd of September, 2016
Center for Environmental Research and Sustainable Technology (UFT), Bremen

Universität Bremen

Name: _____ Ort, Datum: _____

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ERKLÄRUNG

Hiermit erkläre ich, dass ich die Doktorarbeit mit dem Titel:

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selbstständig verfasst und geschrieben habe und außer den angegebenen Quellen keine weiteren Hilfsmittel verwendet habe.

Ebenfalls erkläre ich hiermit, dass es sich bei den von mir abgegebenen Arbeiten um drei identische Exemplare handelt.

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(Unterschrift)

Table of contents

1 List of abbreviations

AAS	atomic adsorption spectrometry
Ag	silver
$Ag_{i/m/s}$	ionic silver/silver in the medium/silver sorbed to the surface
AgCl	silver chloride
$AgNO_3$	silver nitrate
Ag_2O	silver oxide
Ag^+	silver ions
Ag^+-MT	miniaturised test design with silver nitate as test substance
Ag^+-ST	standard test design with silver nitate as test substance
AgNP	silver nanoparticles
AgNP-MT	miniaturised test design with silver nanoparticles as test substance
AgNP-ST	standard test design with silver nanoparticles as test substance
AgNP-ST-P	standard test design in plastic beakers with silver nanoparticles as test substance
ANOVA	analysis of variance
bPEI	branched polyethyleneimine
citAgNP	citrate coated AgNP by Cline Scientific AB (Gothenburg, Sweden)
D. subspicatus	*Desmodesmus subspicatus*
detAgNP	detergent stabilized AgNP, NM-300K by ras materials GmbH (Regensburg, Germany)
D. magna	*Daphnia magna*
DLS	dynamic light scattering
DLVO	Derjaguin-Landau-Verwey-Overbeek (theory)
DTU	Technical University of Denmark
DTU M7	Elendt M7 medium prepared at DTU
DW	dry weight
EC_{10}	10 % effect concentration
EC_{50}	50 % effect concentration
EPA	Environmental Protection Agency (USA)
GF-AAS	graphite-furnace atomic adsorption spectrometry
glm	generalised linear model
HDD	hydrodynamic diameter

HCl	chloric acid
HNO_3	salpetric acid
ICP-MS	inductively coupled plasma mass spectrometry
ISO	International Organisation for Standardisation
$K_2Cr_2O_7$	potassium bichromate
lm	linear model
LOEC	lowest observed effect concentration
MT	miniaturised test design
MT-F	miniaturised test design with food
$NaHCO_3$	sodium bicarbonate
NaOH	sodium hydroxide
NM	nanomaterials
NNLS	non negative least square
NOEC	no-observed effect concentration
NP	nanoparticles
OECD	Organisation for Economic Cooperation and Development
PDI	polydispersity index
PEG	polyethylene gylcol
PEN	The Project on Emerging Nanomaterials
PS	polystyrene
PTM	peritrophic membrane
PVP	polyvinylpyrrilidone
R. subcapitata	*Raphidocelis subcapitata*
REACH	European chemical legislation
se	standard error
SEM	scanning electron microscopy
ST	standard test design
ST-G	standard test design in glass beakers
SVR	surface:volume ratio
TAGAT® TO	polyoxyethylene glycerol trioleate
TEM	transmission electron microscopy
TWEEN 20	polyoxyethylene (20) sorbitan monolaurate
TWEEN 80	polyoxyethylene (80) sorbitan monolaurate
T-SAR	Thinking in terms of Structure-Activity-Relationships

UB M7	Elendt M7 medium prepared at the university of Bremen
UB	University of Bremen
UFZ	Center for Environmental Research/ Zentrum für Umweltforschung
UV	ultra-violet (light)
UV/VIS	ultra-violet/visible (light)
v/v	volume/volume (dilution)
½ N	½ of nitrogen content
¼ N	¼ of nitrogen content
½ P	½ of phosphorous content
¼ P	¼ of phosphorous content
% wt	% of weight

2 Abstract

Silver nanoparticles (AgNP) are used dominantly for disinfection purposes due to their high toxicity against bacteria. Even though this property is desired during use, it may become unwanted, if AgNP are released into the environment. As the toxicity of AgNP is not limited to bacteria, also other taxonomic groups may suffer from release of AgNP into the environment. Release of silver ions from the AgNP represents an important source for toxicity, however, also AgNP themselves can act toxic. In aquatic systems, toxicity of AgNP is highly affected by their colloidal stability (dissolution and agglomeration) in the medium, which is in turn closely related to the coating of the used AgNP. As also the test conditions, such as pH, medium composition, light intensity, or test duration interact with the coating, generalizations on the mode of action or toxicity of AgNP to aquatic organisms can only be made for a single coating or a certain test design. Still, the high number of studies using AgNP provide a good reference for investigating the relation between colloidal stability in the test medium and toxicity in more detail.

For this dissertation, two rarely considered aspects of this relation were chosen: 1) the influence of surface area and surface properties on colloidal stability and actual concentrations of AgNP in the test medium, and 2) the effect of resource reduction on AgNP toxicity. By using these two topics, biological as well as chemical influences on AgNP toxicity could be investigated. For this purpose, an aquatic model system was used including two differently coated AgNP, two green algae, *Raphidocelis subcapitata* and *Desmodesmus subspicatus*, and the big water flea, *Daphnia magna*, in varying compositions.

The investigation of surface-related effects revealed that differences in surface area were of minor importance for AgNP colloidal stability and fate compared to surface properties. Higher hydrophobicity increased the attachment of detergent stabilized AgNP to the test vessel's surface, thus reducing the actual exposure concentrations and causing lower levels of toxicity in this test vessel. In case of variation of the AgNP surface itself, the coating, higher degrees of attachment of the AgNP to each other, so a higher degree of agglomeration, increased uptake of the corresponding AgNP and caused higher levels of toxicity. These results support the importance of surface properties for the fate of AgNP in a given test system and identified hydrophobicity as well as surface charge as most important properties for the attachment of AgNP to surfaces. In addition, the interactions between biological surfaces and citrate coated AgNP were identified as most probable link between colloidal stability and observed toxicity, suggesting further investigations on this topic.

The reduction of resources had a close connection to the other topic of research, as changes in media composition, as required for changes in nutrient supply for algae, highly affected colloidal stability of the AgNP. By the use of intensive analytics, however, effects resulting from changes in colloidal stability could be separated from changes caused by differences nutrient reduction. Resource reduction caused an increase in AgNP toxicity in both trophic levels with the response also differing between the two algae species. Consequently, AgNP toxicity can be expected to be higher for various taxa when resource provision is low, but the intensity of this change is likely to vary between species.

3 Zusammenfassung

Silber Nanopartikel (AgNP) werden aufgrund ihrer hohen Toxizität gegenüber Bakterien hauptsächlich zur Desinfektion verwendet. Obwohl diese Eigenschaft während der Anwendung erwünscht ist, kann sie unerwünscht werden, wenn AgNP in die Umwelt gelangen. Da die Toxizität der AgNP nicht auf Bakterien beschränkt ist, können auch andere Organismengruppen unter der AgNP Freisetzung leiden. Freisetzung von Silberionen von AgNP stellt eine wichtige Quelle der Toxizität dar, allerdings können auch die AgNP selbst toxisch wirken. Die Toxizität der AgNP in aquatischen Systemen ist stark von der kolloidalen Stabilität (Lösung und Agglomeration) im Medium abhängig, welche wiederum eng mit der Beschichtung der AgNP verknüpft ist. Da auch die Testbedingungen, wie pH, Medienzusammensetzung, Lichtintensität oder Testdauer die Beschichtung beeinflussen, können Verallgemeinerungen über den Wirkmechanismus oder die Toxizität von AgNP nur für eine einzelne Beschichtung oder ein einzelnes Testdesign gemacht werden. Dennoch bietet die große Anzahl an Studien mit AgNP eine gute Vergleichsbasis, um das Verhältnis von kolloidaler Stabilität im Testmedium und Toxizität im Detail zu erforschen.

Für diese Dissertation wurden zwei selten beachtete Aspekte dieses Verhältnisses ausgewählt: 1) der Einfluss von verfügbarer Oberflächengröße und Oberflächeneingeschaften auf die kolloidale Stabilität und realer Konzentration von AgNP im Testmedium, und 2) der Einfluss von Ressourcenreduzierung auf die Toxizität von AgNP. Mit der Wahl dieser beiden Themen konnten sowohl biologische als auch chemische Einflüsse auf die Toxizität von AgNP untersucht werden. Zu diesem Zweck wurde ein aquatisches Modellsystem verwendet, welches aus zwei verschieden beschichteten AgNP, zwei Grünalgenarten, *Raphidocelis subcapitata* und *Desmodesmus subspicatus*, und dem Großen Wasserfloh, *Daphnia magna*, in wechselnder Zusammensetzung, bestand.

Die Erforschung der Oberflächen-bezogenen Effekte zeigte, dass Unterschiede in der verfügbaren Fläche weniger Einfluss auf die kolloidale Stabilität und den Verbleib der AgNP hatten als die Oberflächeneigenschaften. Höhere Hydrophobizität steigerte die Anlagerung von Tensid-stabilisierten AgNP an die Oberfläche des Testgefäßes, wodurch die realen Expositionskonzentrationen reduziert wurden und eine geringere Toxizität in diesen Testgefäßen beobachtet werden konnte. Bei einer Veränderung der Oberfläche der AgNP selbst, der Beschichtung, führte eine höhere Anlagerung der AgNP aneinander, also eine höhere Agglomeration, zu einer erhöhten Aufnahme dieser AgNP und bewirkte eine höhere Toxizität. Diese Ergebnisse unterstützen die Wichtigkeit des Einflusses von Oberflächeneigenschaften auf den Verbleib von AgNP in einem gegebenen Testsystem und identifizierten Hydrophobizität und Oberflächenladung als wichtigste Eigenschaften im Rahmen der Anlagerung von AgNP an Oberflächen. Außerdem wurden die Interaktionen zwischen biologischen Oberflächen und Citrat-beschichteten AgNP als wahrscheinlichste Verbindung zwischen kolloidaler Stabilität und beobachteter Toxizität identifiziert, was weitere Forschung zu diesem Thema nötig macht.

Die Ressourcenreduzierung hatte einen engen Bezug zum anderen Forschungsthema, da Änderungen der Medienzusammensetzung, wie sie zur Anpassung der Nährstoffzufuhr für Algen nötig war, sich stark auf die kolloidale Stabilität der AgNP auswirkten. Dennoch konnten mit Hilfe von intensiver Analytik, die Effekte von kolloidaler Stabilität von denen von Nährstoffverfügbarkeit getrennt werden. Ressourcenreduzierung erhöhte die Toxizität von AgNP für beide trophische Ebenen mit Unterschieden des Effekts zwischen den beiden Algenarten. Daher kann eine höhere Toxizität von AgNP für verschiedene Taxa bei geringer Ressourcenverfügbarkeit erwartet werden, obwohl die Intensität dieses Effekts wahrscheinlich mit der Art schwankt.

Introduction

4 Introduction

4.1 Motivation

„When enough is enough“ titled a review on silver nanoparticles (Hansen and Baun, 2012) – so why yet another thesis on this topic?

Even though many reviews on silver nanoparticles (AgNP) cover the same studies (Hansen and Baun, 2012), the search terms “silver”, “toxic*”, and “nano*” return about 21,000 hits (ScienceDirect, May 2016). By adding the term “aquatic”, this number is strongly reduced to slightly more than 2,600 containing mainly studies with algae (about 2,100). The rest addresses AgNP effects on daphnids. However, it is clear from these results that the present thesis can only add one small piece to this huge amount of information – so again, why did I choose AgNP and *Daphnia magna*?
This is my personal answer: Because this system is a good model to investigate rather basic research questions of nanoecotoxicology, like the effect of test conditions on the result, the link between AgNP behaviour and toxicity, or potential influences of environmental variation or conditions. Due to the large amount of data available on AgNP toxicity to algae and daphnids, we can compare results obtained in a broad range of settings, and thus design our experiments accordingly and improve the interpretation of our results.
Consequently, this thesis aims at understanding the patterns of AgNP toxicity in an aquatic model ecosystem rather than assessing their environmental risk.

4.2 Structure of the thesis

In the context of understanding the patterns of AgNP toxicity in a given test system, the exposure of the test organism(s) is of high importance, especially the actual concentration and the colloidal stability of the used AgNP in the test medium (see 4.3.1 and 4.3.2). In the present work, effects of surface interactions on fate and behaviour of AgNP were in focus in the first three parts of the results section (5-7).
In ecotoxicology, optimal test conditions are preferred to exclude confounding effects on the observed toxicity. Consequently, nutrient concentrations in ecotoxicological algae media or food levels for daphnids are comparatively high compared to environmental nutrient levels or algae abundances (see 4.3.4). The last two parts (8 + 9) investigate the effect of more natural conditions on AgNP toxicity and also address the question whether this additional (natural) stress can increase AgNP toxicity to predicted environmental concentrations (see 4.3.1).
In addition, this thesis aimed to stepwise increase the complexity starting with “simple” acute toxicity and abiotic influences (Results 5 + 6), followed by abiotic influences on chronic toxicity (Part 7) and finally the influence of biotic variation on acute and chronic toxicity (Part 8 + 9).

Summarized, the questions in focus of this thesis were:

1) How do surface interactions affect the exposure conditions of AgNP?
2) How do test organisms respond to AgNP exposure, if they experience limiting conditions similar to their natural environment?

A third question rose while conducting the experiments:

3) How do biological traits like feeding behaviour or nutrient requirements affect the toxicity of AgNP?

This topic was also included as it serves as connection between the two main questions of the present work. Table 4.1 provides an overview on the topics covered by single results sections and the tests and organisms used to investigate the corresponding research question.

4.3 Background information

4.3.1 Silver nanoparticles

AgNP and also engineered AgNP in general are nothing new to science or the environment: natural or UV light can reduce silver ions (Ag^+) present in watery solution to elemental silver of nanosize (Zhang et al., 2015), and chemical papers describe the production, analysis, and use of silver colloids already early in the last century (Gutbier et al., 1922). However, the production of AgNP increased since the beginning of this century and environmental exposure to engineered AgNP came into focus of ecotoxicology (Handy et al., 2008).
One main problem in determining the environmental risk of AgNP is the missing regulation: in the EU, manufacturers do not need to label their products whether they contain nanoparticles, with the exception of cosmetics (Hansen et al., 2016). Still, neither concentration nor coating of these nanoparticles need to be declared. In addition, the number of products claiming to contain AgNP varies depending on the database used for search (Hansen et al., 2016). According to the Danish database "The Nanodatabase", 340 products available to European consumers claim to contain nanosilver in May 2016 (The Nanodatabase, 2016). Globally, slightly more products are listed at the same time: 442 (Consumer Products Inventory; PEN, 2016), but this source was last updated in October 2011, so this number can be considered less reliable. Due to the lack of guidelines for product labelling, these numbers can only be considered as rough estimates of actual exposure pathways for humans and the environment. The release of AgNP into the environment also depends on the use of the product (Wigger et al., 2015), e.g. washing procedures for an AgNP treated textile (Geranio et al., 2009), as well as its production process (Benn and Westerhoff, 2008). Despite these large uncertainties, the estimates of environmental concentrations for waste-water effluents or surface waters are rather constant and span the large range from < 1 to about 100 ng Ag L^{-1} (3 to 32 ng L^{-1} reviewed by Batley et al., 2013; < 1 ng L^{-1} to about 1 µg Ag L^{-1} in surface waters, and about 1 to 100 ng L^{-1} in waste water effluents modelled by Gottschalk et al., 2013). The most recent values for predicted environmental concentrations range between 100 – 150 ng L^{-1} (Wigger et al., 2105). Still, increasing numbers of consumer products are listed in the Danish nanomaterial inventory (Figure 4.1) during the last 4 years, so that an increase in environmental concentrations can be expected, if these products are sold and used.
Waste water effluent can be considered to be the main exposure route for aquatic environments in the European region (Hartemann et al., 2015; Wigger et al., 2015). Most of the products listed to contain AgNP are used for health and fitness applications and/or linked to dermal exposure as most possible exposure scenario for humans (Figure 4.2; The Nanodatabase, 2016), indicating a close link to washing of textiles or showering

Table 4.1: Overview on the structure of the thesis. Main research questions are related to the focus of this thesis. In addition, information on test species, test duration, and tested AgNP are provided.

Part of this thesis	Research questions	Research Focus	Test species	Test duration	AgNP
5	• Influence of test vessel size	1		96 h (acute)	
6	• Influence of test vessel size • Influence of test vessel surface properties	1 1		96 h (acute)	
7	• Influence of AgNP surface properties • Influence of uptake by *D. magna*	1 3		21 d (chronic)	
8	• Influence of limiting nutrient supply on AgNP toxicity • Influence of differences in sensitivity between two green algae	2 3		72 h (acute)	
9	• Influence of reduced food quantity on AgNP toxicity pattern • Influence of uptake by *D. magna*	2 3		21 d (chronic)	

 detergent stabilized AgNP  citrate coated AgNP

 *D. magna*  *R. subcapitata*  *D. subspicatus*

1: effect of surface interactions on exposure conditions of AgNP

2: response of test organisms to AgNP exposure under limiting conditions

3: biological influences on AgNP toxicity

Acute and chronic toxicity of AgNP to aquatic organisms shows a very broad range of toxicity values ranking from ng L^{-1} to several mg L^{-1} (Fabrega et al., 2011; Table 4.2). High toxicity in the range of ng L^{-1} was reported dominantly for bacteria, but in case of release of silver ions (Ag^{+}), also toxicity to algae was in a similar range (Fabrega et al., 2011). A summary on reviewed EC_{50} values in relation to organism group extracted from the review of Fabrega et al. (2011) and additional literature on the test organisms (*Daphnia* and related species, and algae) is summarized in Table 4.2. The results show that bacteria are the most sensitive species to AgNP while *Daphnia* and *Ceriodaphnia* are the most sensitive eukaryotic species (Garner et al., 2015). AgNP had toxic effects to Daphniidae at the ng Ag L^{-1} level in some studies (Angel et al., 2013; Becaro et al., 2015; Sakamoto et al., 2015; Silva et al., 2014; Völker et al., 2013). In two studies (Angel et al., 2013; Sakamoto et al., 2015) this could be linked to a release of silver ions, while no dissolution occurred during the other studies. However, in one study, the test species was *D. similis* and the higher toxicity was explained by the overall higher sensitivity of this species compared to *D. magna* (Becaro et al., 2015).

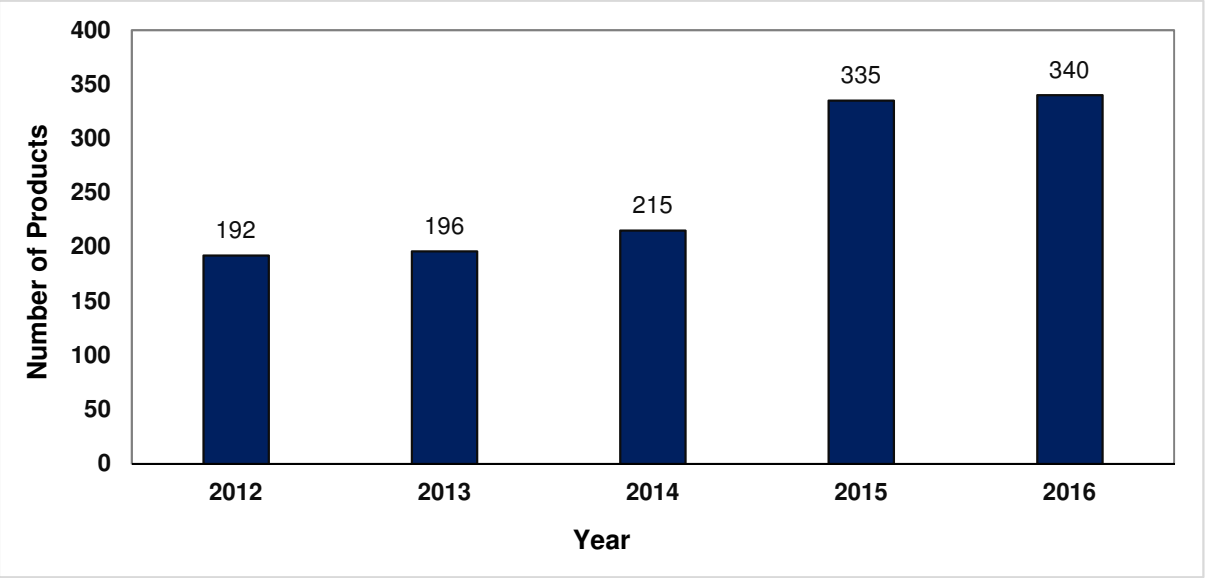

Figure 4.1: Number of products that claim to contain AgNP and are available to European consumers between 2012 and 2016 (The Nanodatabase, 2016).

Release of Ag^{+} as source of toxicity has been reported in most of the studies investigating AgNP toxicity to algae (e.g Angel et al., 2013; Miao et al., 2009; Navarro et al., 2015), and only in some cases, dissolved silver concentrations could not explain the observed toxicity. However, under more natural conditions (=using natural waters), dissolution was clearly reduced in some cases (Norman et al., 2015; das et al., 2014), but detected in others (Gil-Allué et al., 2015), and in one case differing with the coating (Tuominen et al., 2013). However, when expressed as total silver, toxicity of AgNP to algae spans a comparable broad range as in daphnids (minimal and maximal toxicity in Table 4.2).

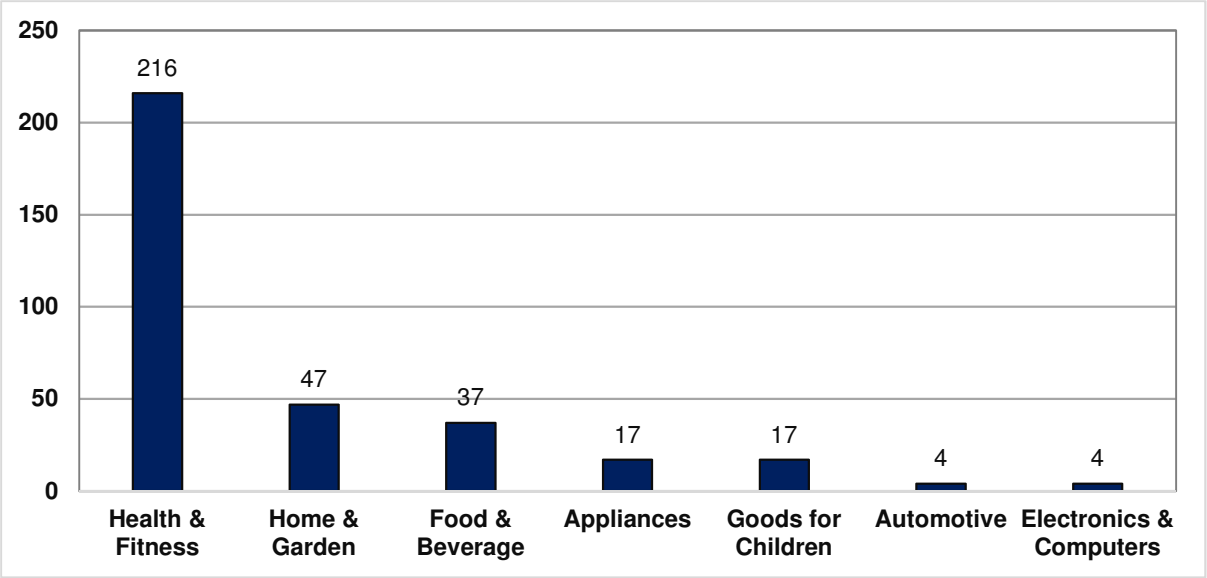

Figure 4.2: Number of products available to European consumers that claim to contain AgNP in relation to their application (source: The Nanodatabase, May 2016).

Aside from bacteria, the reported values are dominantly above the predicted environmental concentrations (Fabrega et al., 2011) suggesting no actual risk to the environment. For bacteria, results from microbial communities may also serve as an estimate for environmental risk due to the high numbers of bacteria found in any environment. The effects of AgNP on microbial communities have been discussed elsewhere in more detail (e.g. for soil: McKee and Filser, 2016), but community responses were highly variable in time and varied in their sensitivity towards AgNP similar to the results observed in single-species tests, so that a risk of AgNP to microbial communities at current predicted environmental concentrations (ng Ag L^{-1}) cannot be excluded. However, the conclusion that there is no actual risk by AgNP to the environment, is supported by the observation that AgNP were sulfidized during the waste water treatment: AgNP were transformed into Ag_2S NP probably with a silver core and an outer layer of Ag_2S during this process (Kaegi et al., 2011). Sulfidation was also observed when AgNP were added to a wetland mesocosm (Lowry et al., 2012). Sulfidation or sewage sludge treatments clearly affected colloidal stability and fate in a given test system (Baalousha et al., 2015; Whitley et al., 2013) and sulfide addition reduced acute and chronic silver toxicity to *D. magna*(Naddy et al., 2007). The same has been shown for the effect of sulfidized AgNP on nitrifying bacteria (Choi et al., 2009). Still, sulfidation increased the mobility of AgNP in partially saturated sand columns (Yecheskel et al., 2016) and silver body burdens of fish and chironomids were clearly higher than concentrations in the water column in the mesocosm study where sulfidation of AgNP was observed (Lowry et al., 2012), indicating remaining and also potentially increased bioavailability as well as possible bioaccumulation along food chains. Thus, overall environmental risk of AgNP cannot be determined yet, but for the chosen test organisms, algae and *Daphnia*, critical levels seem to be in the range of a few µg Ag L^{-1}.

Table 4.2: Overview on acute and chronic effects of AgNP in relation to taxonomic group based on Fabrega et al. (2011) and more recent studies addressing AgNP toxicity to daphnids and/or algae using standard test procedures.
Sources other than Fabrega et al. (2011) are indicated by numbers and listed below. Letters indicate the measure of toxicity: a: highest nominal concentration, b: EC_{50}, c: lowest observed effect concentration (LOEC), d: EC_{10}. For daphnids, chronic results are indicated by asterisks.

Organism group	**Min. value** [µg Ag L^{-1}]	**Test species**	**Max. value** [µg Ag L^{-1}]	**Test species**
Bacteria	0.086 [a]	*Escherichia coli*	1,000 [b]	*nitrifying bacteria community*
Algae	3 [1,b]	*Rapidocelis subcapitata*	355 [b]	*Chlamydomonas reinhardtii*
	89 [b]	*Chlamydomonas reinhardtii*	1,630 [2,b]	*Raphidocelis subcapitata*
Daphnia	0.15 [1,b]	*Ceriodaphnia dubia*	6,180 [b]	*Ceriodaphnia dubia*
	0.9 [3,d]*	*Daphnia magna*	148 [4,c]*	*Daphnia magna*
Fish	4,000 [a]	*Danio rerio*	7,000 [b]	*Danio rerio*
All	0.086 [a]	*Escherichia coli*	7,000 [b]	*Danio rerio*

[1] Angel et al. (2012) [2] Ksyazik et al. (2015) [3] Völker et al. (2013) [4] Blinova et al. (2015)
Stars * indicate results of chronic tests with daphnids.

The AgNP used in the present work were either citrate coated or stabilized by two detergents. The citrate coating caused a stabilization by negative surface charge and to some extent also by sterical hindrance of direct contact between single particles (Figure 2a, El Badawy et al., 2010). Citrate coated AgNP (citAgNP) were bought from Cline Scientific AB (Gothenburg, Sweden) and detergent stabilized AgNP (detAgNP) from ras materials GmbH (Regensburg, Germany). The detergent stabilized AgNP were NM-300K, one of the standard materials of the OECD for the testing of nanomaterials. They were chosen due to their status as standard material that guaranteed a high similarity of production charges. The disadvantage of these AgNP is the missing information on the bound between AgNP surface and stabilizer. They are delivered as uncoated AgNP in a very high concentration of detergents (4 % TWEEN 20 [polyoxyethylene sorbitan monolaurate] and TAGAT® TO [polyoxyethylene glycerol trioleate] each). However, they are comparatively colloidally stable in the *Daphnia magna* test medium (Elendt M7, Part 6, 7, and 9) and in seawater (Sakka, 2011). In combination with the high concentration of the detergents in dispersion, it is very likely that the uncoated AgNP cores are covered by these molecules. Kvitek et al (2008) showed that TWEEN 80 successfully covered AgNP during the production process. The result of Part 5 enabled us to develop a model of the detergent covering (Figure 4.2b) that is in line with all other results obtained in the presented work.

4.3.2 Surface Interactions

In accordance with increasing complexity, the basis for the presented work is the surface interaction between single particles in dispersion, so their colloidal stability. Besides, all other surfaces present during the test are considered, starting with the test vessel surface (Parts 5 + 6) and continuing with the test organisms (7 - 9) using varying test conditions (8 + 9). As surface interactions play a key role in determining the exposure scenario and thus for the toxic effect (Levard et al., 2012; Sharma et al., 2014), surface interactions can also be considered as the connecting topic of this work.
Nanoparticle behaviour in aquatic systems is mainly governed by their colloidal stability: if colloidal stability is high, particles remain monodispersed and in suspension. If colloidal stability decreases, three processes can take place: dissolution, agglomeration or aggregation (Mudunkotuwa and Grassian, 2011; Sigg et al., 2014). Agglomeration and aggregation are separated by the strength of the bound between the single particles (Sigg et al., 2014) which is hard to measure under ecotoxicological conditions, so the term agglomeration is used in this thesis.

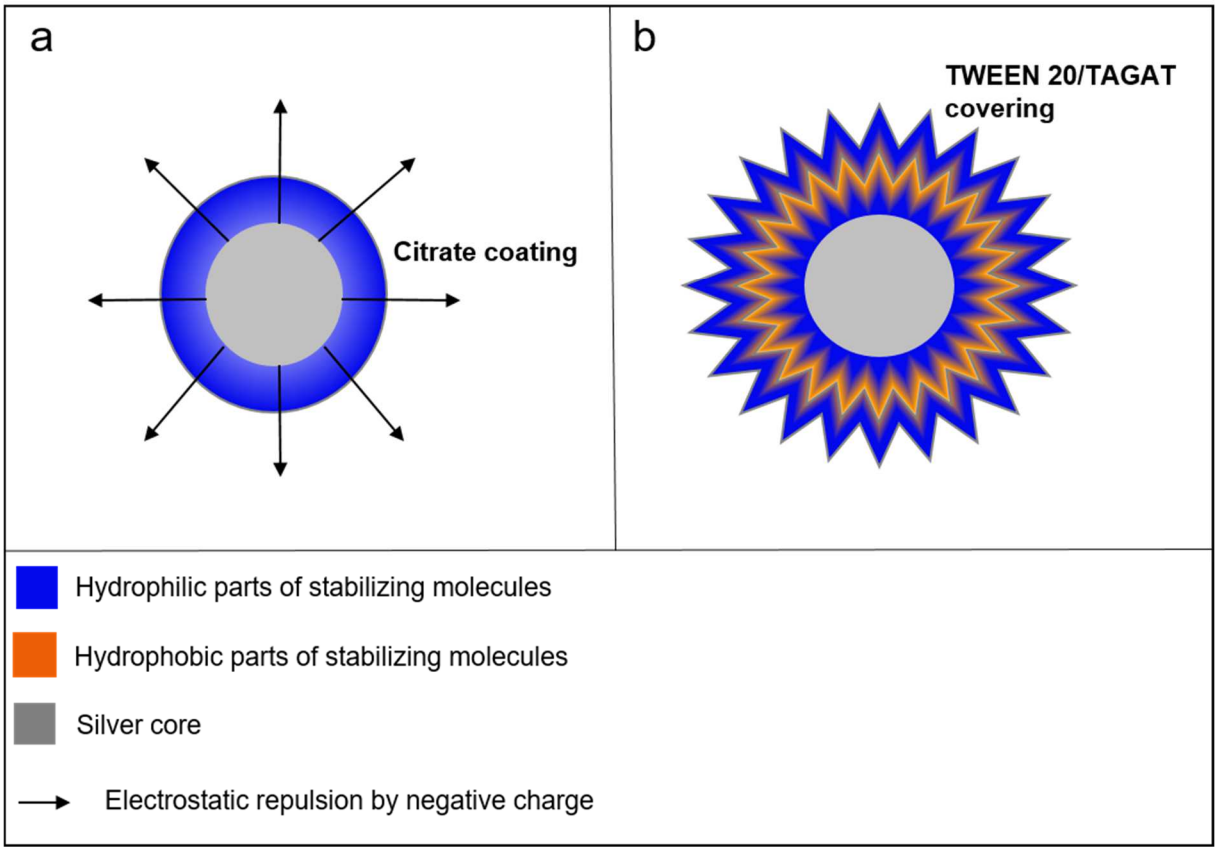

Figure 4.2: Schematic illustration of the two AgNP used in this thesis based on manufacturer information, chemical structures of the stabilizing molecules, and in case of the detergent stabilized AgNP, NM300-K, also on literature (Kvitek et al., 2008) and experimental results (Part 6). The core size of the spherical shaped AgNP was about 20 nm (Part 7, Figure 7.1). a) citrate coated AgNP, b) detergent stabilized AgNP.

Coilloidal stability is highly affected by numerous external factors such as pH, ionic strength and ion composition of the test medium, and is further modified by the “internal” factor of surface covering of the (Ag)NP (reviewed by Sharma et al., 2014; Levard et al., 2012). To understand the effects of external factors, the double layer theory (also: Derjaguin-Landau-Verwey-Overbeek, DLVO theory) can serve as basis; assuming that each single particle is surrounded by an electrostatic layer depending on van der Waals forces and electrostatic repulsion (Mudunkutova and Grassian, 2011). The thickness of this layer determines whether or not particles attach to each other. It strongly depends on the concentration and charge of ions in solution: with more ions present, the shell gets thinner and more interactions are possible, so that colloidal stability is reduced (Mudunkutova and Grassian, 2011; Sharma et al., 2014). Beside the ionic strength, also ion composition is crucial for colloidal stability (Levard et al., 2012). Especially divalent ions have been shown to cause agglomeration in various media (e.g. Baalousha et al., 2013; Jin et al., 2010; Zhang et al., 2012). This relation is strongly modified by an “internal” factor, the coating (Levard et al., 2012). The coating determines whether and which intensity of ionic strength is needed to cause agglomeration or which pH is changing colloidal stability (Levard et al., 2012; Sharma et al., 2014). Consequently, it is impossible to generalize AgNP behaviour without separating single coatings. For example, pH effects differed between citrate, starch and bovine albumin serum coated AgNP, despite all of them using electrosteric stabilization (Sharma et al., 2014). In addition, larger molecules like humic acids or proteins have been shown to modify the initial coating (Fabrega et al., 2009; Nasser and Lynch, 2016). Two examples for the influence of the coating on stability and subsequent on toxicity are described in the following lines to illustrate the extent of variation. In the first case, coating by citrate caused higher dissolution rates than coating by polyvinylpyrrolidone (PVP), and thus had higher toxicity (Angel et al., 2013). Here, toxicity was not caused by the AgNP anymore, but by released ions. In the second example, dissolution was negligible and even AgNP sizes in the test medium were not very different from each other. Higher toxicity could be explained by an increase in surface charge difference between the test organisms (*E. coli* and *D. magna*) and the differently coated AgNP, most likely causing higher levels of attachment (Silva et al., 2014).
As shown in the first example, dissolution may mitigate AgNP toxicity due to the much higher toxic effect of the released ions. Also agglomeration may change AgNP toxicity: agglomerated AgNP were less toxic to *Daphnia* in one study (Römer et al., 2011) while they were more toxic to *E. coli* in another (Reinsch et al., 2012) indicating differences in bioavailability or surface interactions in the two studies. The second example shows that colloidal stability alone is not sufficient to explain AgNP toxicity. Instead, interactions between surfaces play an important role in mediating toxicity.

Beside the surface of the test organism, also the surface of the test vessel, a surface often not considered, but yet always present in a test, may change AgNP behaviour: if AgNP strongly attach to the test vessel surface, medium concentrations are reduced and exposure conditions are changed. For dissolved silver, losses from the medium due to adsorption, formation of colloids or insoluble substances depend on several parameters such as pH, light exposure regime, ion composition, and container surface (Welz and Sperling, 1997). As long as a coating is lacking, surface interactions between AgNP and test vessel surfaces are likely to be similar. However, interactions between surfaces and AgNP are also highly influenced by the coating (Malysheva et al., 2016; Sekine et al., 2015; Song et al., 2011). Similar to dissolved silver, also the properties of the test vessel affect the interactions between the beaker and AgNP (Sekine et al., 2015, Song et al., 2011).

In the present work, this topic was investigated using detAgNP and differently sized test vessels of different materials (Table 4.1, Part 5 +6).

Beside test vessel surfaces, also the test organisms themselves have different external and internal surfaces that may interact with the surface of AgNP. As mentioned above, differences in charge cause attraction of AgNP and subsequent toxicity as has been shown for bacteria and *D. magna* (El Badawy et al., 2010; Silva et al., 2014). Most studies investigating the role of surface functionalization on toxicity use bacteria or cells as test systems (Bhatt and Tripathi, 2011; Levard et al., 2012). Kim et al. (2013) summarized the studies on cytotoxicity and showed that surface charge, hydrophobicity, and specific molecular interactions are crucial for surface interaction between cells and AgNP as well as for AgNP toxicity. For algae, attachment as well as internalization of various nanoparticles have been reported (Perreault et al., 2012; Sadiq et al., 2011; Van Hoecke et al., 2008), including AgNP (Das et al., 2014; Dash et al., 2012). However, the mechanisms driving this attachment are not clear in most cases but the cell wall seems to be an important factor in the related processes (Oukarroum et al., 2012; Perreault et al., 2012). In addition, small nanoparticles (5-20 nm) may enter algal cells via pores in the cell wall and subsequently interact with the plasma membrane similar to the processes suggested for animal cells (Bhatt and Tripathi, 2011). AgNP can not only attach to algae, but also interact with surfaces provided by larger organisms such as *D. magna*. Attachment to the carapax has been reported in some investigations on AgNP toxicity (Asghari et al., 2012; Zhao and Wang, 2010). Aside from outer surfaces, also internal surfaces may play an important role for nanoparticle attachment and several nanomaterials have been shown to attach and/or interact with the midgut epithelium (Dominguez et al., 2015; Heinlaan et al., 2011; Khan et al., 2014; Kwon et al., 2014; Lovern et al., 2008).

Even though attachment of AgNP to biological surfaces was not directly investigated in this work due to the lack of time for the required analytics, possible interactions between the used AgNP and algae and/or daphnids were an important influence on the establishment of silver body burden in *Daphnia* (Part 7 + 9) and possible reasons for the difference in sensitivity between the two algae species (Part 8).

4.3.3 Biological Influences

Even though this question rose during the conduction of this work, the influence of the biology of the test organism on the observed result as well as on the exposure scenario is an important one. *Daphnia*, for example, were chosen in the present work due to their filter-feeding behaviour that represents at the one hand a potential route for uptake and at the other ensures a permanent contact with the test medium (see 1.4). An influence of the filter-feeding on exposure is thus possible and needs to be considered in interpreting the obtained results.
Not much is known about the influence of algal species-specific biology on AgNP toxicity. Only few studies compared effects of AgNP to different species (Dash et al., 2012; Oukarroum et al., 2012). In these studies, algae responded similar to AgNP aside from small differences in sensitivity (Oukarroum et al., 2012) or attachment (Dash et al., 2012). These results indicate high similarity in algal response, yet only green algae were compared and also surface morphology was comparable. Citrate coated AgNP changed enzyme activities of a periphyton community (Gil-Allué et al., 2015), which is indicating a more biological response to AgNP exposure, most likely related to differences in AgNP sensitivity. Up to now, it remains unclear

whether diatoms with a completely different cell wall structure will respond similar to AgNP exposure or whether algae that form colonies under certain circumstances have increased or decreased sensitivity to AgNP exposure.
Even though additional experiments about attachment to algae surfaces are required to link AgNP attachment to biological traits, such as surface morphology or shape, the comparison of the toxicity of AgNP to two differently shaped green algae species (Part 8) supports the need for further studies on this topic.

As already mentioned above, *Daphnia* may directly or indirectly ingest nanoparticles and nanoparticle agglomerates. Uptake via direct filtration has been reported for citrate and tannic acid coated AgNP (Zhao and Wang, 2012), while dietary uptake was the main pathway for carbonate coated AgNP (Zhao and Wang, 2011). However, no food was added in the citrate and tannic acid coated AgNP experiment. For citrate coated gold nanoparticles, addition of food did not affect uptake (Skjolding et al., 2014b), indicating that uptake routes may vary between coatings and/or core materials. The amount of nanoparticles that is taken up by daphnids, irrespective of the uptake route, however, has been shown to highly depend on colloidal stability, and particle core size (Asghari et al., 2012; Feswick et al., 2013; Skjolding et al., 2014a, 2014b; Zhao and Wang, 2012).
A close relation between colloidal stability, body burden and toxicity was also observed in the present work (Part 7), but the link between body burden and toxicity was also shown to be less pronounced when additional stressors act on the test organism (Part 9).

4.3.4 Influence of resource limitation

Different from conditions during standard ecotoxicological tests, environmental conditions for algae as well as daphnids vary with year, season and daytime. Even though being fixed, light conditions during tests are adapted to conditions in nature concerning duration and intensity, reflecting optimal conditions (guideline No. 201, OECD 2006, 211; OECD 1998). The most prominent seasonal variations are temperature and nutrient quantities for both algae and daphnids (Müller-Navarra and Lampert, 1996; Sommer, 1989). Algae are also an important vector for the uptake of AgNP by *D. magna* (Zhao and Wang, 2010). Consequently, variation in resources in terms of macronutrients (Part 8) and algae concentration (Part 9) were chosen as limiting factors.
Other studies investigating the effect of nutrients on AgNP toxicity used phosphorous enrichment of natural instead of nutrient reduction of a defined growth medium (Das et al., 2014; Norman et al., 2015). Even though this clearly increases the environmental relevance, it reduces the repeatability of the experiment by the use of natural water samples. An additional effect rises from potential differences in natural water ion composition or natural organic matter content: both strongly changed the toxicity of PVP coated AgNP and also slightly changed silver nitrate, but not starch coated AgNP toxicity, making interpretation of the obtained results difficult (Tuominen et al., 2013). However, both studies investigating the effect of phosphorous enrichment on AgNP toxicity to algae show clear interactions between nutrient level and AgNP concentration. Phosphate enrichment reduced AgNP toxicity to planktonic as well as periphyton algae communities (Das et al., 2014; Norman et al., 2015), most likely due to increased algal growth (Das et al., 2014). In addition, both studies observed effects of AgNP on algal stoichiometry of carbon, nitrogen and phosphorous, but effects were contradictory and more research is required to identify possible reasons for this effect of AgNP.

Whether similar effects can be observed for a defined medium and also for the reduction of nitrogen aside from phosphorous was investigated in Part 8.

The effect of food reduction on *Daphnia* growth and reproduction has been well investigated for various species (Boersma and Vijverberg, 1994; Giebelhausen and Lampert, 2001; McCauley et al., 1990; Müller-Navarra and Lampert, 1996; Vigano, L., 1993; Vijverberg, 1976), providing a good basis for evaluating and interpreting the obtained results. One main result of these investigations is the development of food quantity based reaction norms for *Daphnia* population development (provided by e.g. Giebelhausen and Lampert, 2001; Rinke and Vijverberg, 2005; Rinke and Petzoldt, 2003) that allow extrapolation and modelling of energy allocation at low food conditions: first, investment in reproduction is shortened and less, but larger neonates are produced (e.g. Enserink et al., 1993; Guisande and Gliwicz, 1992). In addition, time to first brood increases, mainly as body growth is slowed down (e.g. Boersma and Vijverberg, 1994). However, this relation is further modified by other factors, so that first reproduction also occurred at smaller sizes at low food levels in some studies (Giebelhausen and Lampert, 2001). Mortality due to food limitation has only been observed in some studies and at very low food concentrations (Boersma and Vijverberg, 1994; McCauley et al., 1990), in one case this was clearly related to higher temperatures and in the same case, mortality also occurred at high food levels (Giebelhausen & Lampert, 2001).

Studies using other chemicals aside nanoparticles show varying results for the relation between food quantity and toxicity ranging from no change in relation to food quantity (Pavlaki et al., 2014; Pereira et al., 2007) to decreases or increases in toxicity (Antunes et al., 2004; Pavlaki et al., 2014). Pieters et al. (2006) showed that the effect of food quantity variation can be explained by differences in toxicokinetics and energy allocation rather than differences in sensitivity of the test organisms. The effect of food reduction on AgNP toxicity has been investigated in one other study using increased amounts of food, so providing additional energy for detoxification of AgNP (Mackevica et al., 2015). As was expected, AgNP toxicity was reduced when more food was present during the exposure, but it remained unclear whether this was related to a reduced uptake of AgNP (numbers of algae without AgNP on surface increased at high food quantities), or to additional energy available for stress responses.

The body burdens measured at low food levels support the first hypothesis, but their difference is rather small compared to the degree of variation, while the difference in toxicity (less food = higher AgNP toxicity) remained clearly visible (Part 9), supporting an interaction of both qualities of the feeding algae: vector of AgNP into the *Daphnia* and energy supply.

4.3.5 Test organisms

The organisms used to investigate the relation between AgNP behaviour and ecotoxicity were the Large Waterflea, *Daphnia magna*, and two green algae, *Raphidocelis subcapitata* (formerly: *Pseduokirchneriella subcapitata)* and *Desmodesmus subspicatus*. The choice of the test organisms mainly considered their use as model ecosystem in nanoecotoxicology to ensure a large database for comparison of the obtained results. In addition, some biological traits were taken into account: for *D. magna*, it was the filter-feeding behaviour that ensures a comparatively high exposure to dispersed nanoparticles and allows active and passive uptake of particles smaller than 650 nm (Geller and Müller, 1981; Heinz Brendelberger, 1991), as well as their translucent carapax (Anderson and Jenkins, 1942) and the possibility to detect moults

by eye that allow a visible control of nanoparticle attachment and uptake in the digestive tract. The two species of green algae were chosen due to their difference in shape (Figure 4.3) and their rather similar nutrient requirements (guideline No. 201, OECD, 2006; Sommer, 1989).

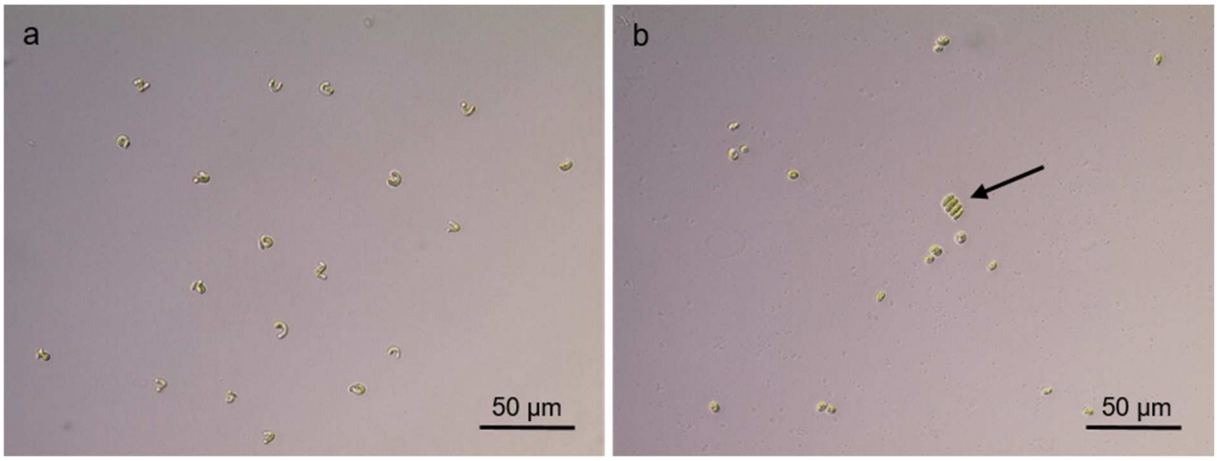

Figure 4.3: The green algae used as test species. a) R.*subcapitata*, b) *D. subspicatus*. A *D. subspicatus* colony is marked by an arrow.

Green algae are an important part of phytoplankton in most freshwater habitats with their abundance depending on nutrient availability and absence/presence of grazers (Sommer, 1989). They include a large number of species that vary in shape, flagellation or in complexity ranging from single celled to multicellular species with the latter being no part of the phytoplankton community (Reynolds, 2007). The most distinct difference to other algal groups are their photosynthetic pigments (chlorophyll a and b) causing the typical green colour (Reynolds, 2006). Green algae possess a cell wall of cellulose and hemicellulose, even though calcium carbonate is also incorporated in some species (Reynolds, 2007).

Green algae require rather high levels of phosphorous, but they were also found to be slightly nitrogen limited under natural conditions (Sommer, 1989). This difference can be explained by their competitive strength for these nutrients: overall, green algae are poor competitors for nitrogen compared to cyanobacteria and diatoms, but intermediate competitors for phosphorous (Sommer, 1989), resulting in nitrogen limitation in coexistence with the two other algal groups.

Algae are also included in the present work due to their role as food source for *D. magna* and other zooplankters. However, predation pressure highly depends on the food selectivity and predation efficiency, so differences between algal taxa or species cannot be linked to their predation risk in general (Schwoerbel, 1999). However, increasing population sizes of *Daphnia* increased evenness in phytoplankton (Sarnelle, 2005), illustrating their importance for algal community structure. Here, vulnerability to predation was most closely linked to the formation of colonies that decreased edibility for daphnids. Also gelatinous capsules reduce predation of green algae (Schwoerbel, 1999).

R. subcapitata is a standard food organism for *D. magna* and considered as optimal food source (OECD, 1998). Also *D. subspicatus* may be grazed by *D. magna*, but it is able to form colonies that cannot be filtered and ingested (Verschoor et al., 2004). *R. subcapitata* is a thread-shaped algae growing in spirals, so that most of them have a half-moon-shaped appearance under the microscope (Figure 1a). *D. subspicatus* is rather spherical (Figure 1b),

so that separation of both algal species under the microscope is easily possible.

Daphnids, especially *D. magna*, have a long tradition as test organism in limnology (e.g. Anderson and Jenkins, 1942) and ecotoxicology (Mark and Solbé, 1997). One main reason is their world-wide distribution and easy culturing (Mark & Solbe, 1997), but also their role as important phytoplankton grazers (Sarnelle, 2005; Steiner, 2004). Their use for ecotoxicological tests has been evaluated several times (e.g. Enserink et al., 1993; Martins et al., 2007). The results summarize the reasons for their use and confirm their high sensitivity to a large number of chemicals.
Daphnia (Figure 4.4) are Crustaceans (subclass Cladocera) and as such moult in regular intervals to grow and – after maturation – release offspring from the brood pouch (Baumann, 2014). Time to maturation varies between 6-8 instars (=inter-moulting stage), depending on the culture conditions (Anderson, 1932) and reproduction strongly decreased at about 30 d for the clones cultured in Bremen (personal observation). *Daphnia* can reproduce either sexually or parthenogenetically. Parthenogenetic reproduction takes place under optimal culture conditions, while sexual reproduction indicates the need of improving the actual culture conditions (Baumann, 2014; OECD, 1998).

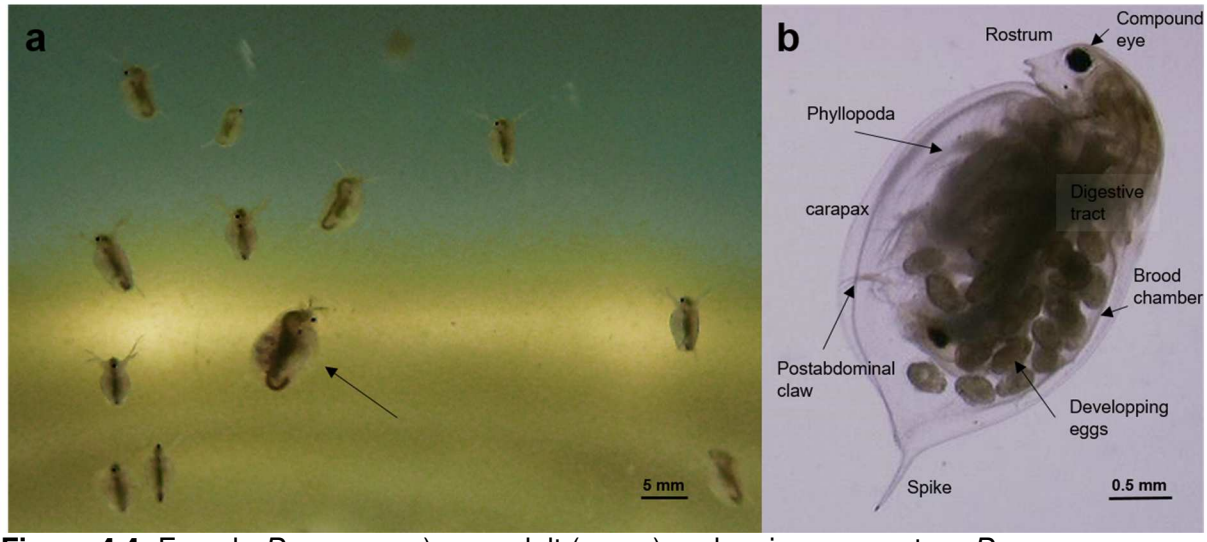

Figure 4.4: Female *D. magna*. a) one adult (arrow) and various premature *D. magna* swimming in the culture medium with feeding algae, b) morphological overview of an adult *D. magna* female.

Daphnia prey on algae by filtering their surrounding medium. Which algae and particles can be taken up via filtration is determined by the size limits for filtered particles – a range that has been reported to be between 150 nm and 50,000 nm (Geller and Müller, 1981; Heinz Brendelberger, 1991) with the minimum limit being independent of animal size (Geller and Müller, 1981; Lampert and Brendelberger, 1996). Filtered, and probably also grazed, particles are transported to the food groove where they are transported to the mouth (Baumann, 2014). After ingestion, food enters the digestive tract that is divided into three subsections (Quaglia et al., 1976). The midsection, the midgut, is of high interest to ecotoxicological studies, as it is not covered by chitin, but by an adsorptive epithelium (Avtsyn and Petrova, 1986; Quaglia et al., 1976). This epithelium is expected to be the most sensitive part of the digestive tract.

However, it is separated from the gut lumen by the peritrophic membrane (PTM), that hinders all particles larger than 130 nm to get into direct contact with the epithelium (Hansen and Peters, 1997). The comparison of this size limit and the minimal filtration size shows that actively filtered and ingested particles cannot interact directly with the adsorptive epithelium, but need to be digested for nutrition. In accordance, some digestive enzymes could be shown to exist in the digestive tract of *D. magna* with their optimal substrate turnover being in the range of the midgut pH (Hasler, 1935; von Elert et al., 2004)

For ecotoxicological research, parthenogenetic reproduction of daphnids is favoured by maintaining optimal culture conditions (Baumann, 2014; OECD, 1998). The resulting cultures are genetically identical and called clones. For the present work, three different clones were used: two clones from University of Bremen and one clone from the Technical University of Denmark (DTU). The Danish clone was used for the experiments described in Part 7, which took place during a collaboration with the department of Environmental Engineering (DTU Miljø). It originates from a pond in Birkedammen, Denmark, and has been cultured in the laboratory since 1978. One clone at University of Bremen was originally from IBACON GmbH (brought to Bremen from the Goethe University at Frankfurt) and the other from the Helmholtz Center for Environmental Research (UFZ), in Leipzig. Both clones were used for the work presented in Parts 5, 6, and 9, due to the differences in fitness of the cultures during this dissertation. Only healthy and well-reproducing cultures were chosen for experiments according to the criteria summarized in the test guideline (No. 211, OECD, 1998).

The algae cultures for all experiments conducted in Bremen were obtained from the culture collection of algae at the University of Göttingen (strain no. 61.81 for *R. subcapitata* and no. 54.80 for *D. subspicatus*) and kept as permanent cultures at the University of Bremen. *R. subcapitata* used as food in Part 7 were cultured at the department of Environmental Engineering of the Technical University of Denmark (DTU Miljø).

More details on culturing, the test protocols and analytics used for this thesis can be found in the materials & methods section of each part of the results and in the Appendix.

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Results

5 Adaptation of the *Daphnia sp.* Acute Toxicity Test: Miniaturization and Prolongation for the Testing of Nanomaterials

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Published in: Environmental Science and Pollution Research 21, p. 2201–2213, 2014
doi: 0.1007/s11356-013-2094-y

Contributions of Yvonne Sakka:

- Performance of the experiments with silver (AgNP and $AgNO_3$)
- Quantification of silver in the different test designs
- Writing of the silver-related parts of the materials & methods section
- Support of manuscript finalization

The test designs compared in this study were chosen and developed by Jonas Baumann, who also conducted the experiments with potassium bichromate, analysed the data and wrote the main part of the mauscript. Carole Bertrand supported the experiments as well as the data analysis. Jan Köser supported the silver measurements and Juliane Filser revised this manuscript**.**

Adaptation of the *Daphnia sp.* Acute Toxicity Test: Miniaturization and Prolongation for the Testing of Nanomaterials

Abstract

Manufacturing of nanomaterials (NM) is often complex and expensive, and their environmental risks are poorly understood or even unknown. An economization of testing NM is therefore desirable, which can be achieved by miniaturizing test systems. However, the downsizing of test vessels and volumes can enlarge the surface:volume ratio (SVR) which in turn can affect the bioavailable concentration of adsorbing substances like NM.

The present study focused on the miniaturization of the acute toxicity test with *Daphnia magna*. The adaptations were verified with three reference substances, the non-adsorbing potassium dichromate ($K_2Cr_2O_7$) and as potentially highly adsorbing substances silver nanoparticles (AgNP) and silver nitrate ($AgNO_3$). The miniaturized test was conducted in 24-well microtiter plates (MT) and simultaneously compared to the OECD standard test (ST). Furthermore, the test duration was prolonged from 48 to 96 h since NMs tend to show effects only after extended exposure.

The toxicity of $K_2Cr_2O_7$ and AgNP continued to increase within the prolonged test span. The test comparisons with $K_2Cr_2O_7$ did not reveal any significant differences between ST and MT. $AgNO_3$ toxicity was significantly decreased in MT compared to ST due to enlarged SVR. The toxicity of AgNP in MT after 24 h was equal to ST. Contrary to our expectations an exposure longer than 24 h resulted in an increase of AgNP toxicity in MT, possibly due to enhanced dissolution of silver.

Microtiter plates are appropriate alternative test vessels for the *Daphnia sp.* acute toxicity test; thus its miniaturization is possible. The enlarged SVR has to be taken into account since it can affect the toxicity of potentially adsorbing substances. Furthermore, the standard test duration of 48 h might underestimate the toxicity of many substances, especially of NM.

Keywords

miniaturization, prolongation, nanoparticles and nanomaterials, *Daphnia magna*, acute toxicity test, microtiter plate, potassium dichromate, silver nanoparticles

1. Introduction

For many years chemicals were put on the European market with a serious lack of knowledge about potential risks for human health and the environment. Therefore the European commission decided to launch the new European chemical legislation for registration, evaluation, authorization, and restriction of chemicals (REACH). Since gathering information on all these chemicals would dramatically increase the use of laboratory animals, REACH suggests to increasingly use in vitro testing methods. However, relying solely on such methods can underestimate the potentially hazardous properties of chemicals that could harm humans and the environment (European Commision 2012). Therefore, other tests have to be adopted for better economization. This can be achieved by miniaturizing the test systems, resulting in a reduced amount of organisms and chemicals, which also increases the cost efficiency. Furthermore, test systems must be simplified for a higher throughput, for example by using mircotiter plates in static test systems. They are comparably cheap and can be handled easily, which is ideal for single-use. Organisms can be easily placed, detected and observed in the wells. Furthermore, evaporation is reduced by the lid. The plates can be also closed with a sealing foil to prevent high loss of volatile substances from the test medium (Schreiber et al. 2008). Often they are used for photometric measurements since they can be directly placed in a photometer. Microtiter plates are mainly used for tests with bacteria or algae (Araujo et al. 2010; Blaha et al. 2010; Paixao et al. 2008; Pinto et al. 2012; Rojickova et al. 1998; Neumann-Hensel and Melbye 2006; Kamber et al. 2009; ISO 2004), but also for nematodes, other invertebrates (Nalecz-Jawecki and Sawicki 1998; Schmitz et al. 1999) and the fish embryo test (Embry et al. 2010). Only few investigations have used microtiter plates for tests with arthropods (Brown et al. 2005; Ghekiere et al. 2007; Perez and Beiras 2010; Zitova et al. 2009b).

Daphnia magna, the big water flea, is one of the most widely used organisms in standardized aquatic ecotoxicology tests (OECD 1998, 2004; Zitova et al. 2009a; ISO 1996); most often for testing acute effects of substances. The OECD-Guideline 202 (OECD 2004) suggests a test period of 24-48 h, the use of small beakers with a minimum volume of 10 ml, 5 neonates per replicate and a minimum number of 4 replicates per concentration. Only little information is available on the use of microtiter plates for daphnid tests (e.g. Daniel et al. 2004). Powell et al. (1996) conducted the test in 48-well microtiter plates with one neonate in a volume of 1 ml per replicate. The validation was performed with four organic reference substances. The results in the miniaturized test system were very similar to those using the EPA standard test procedure (U.S.EPA 1991). They also found that substances which might leach from the microtiter plate material (normally polystyrene) had no effect on the daphnids within the test duration.

Nanomaterials (NM) have increasingly come into the focus of ecotoxicological testing. In the nanometer range (1-100 nm at least in one dimension) a substance can have completely different characteristics and therefore also different toxic modes of action compared to bulk or dissolved material. In addition, size, structure, and surface charge of a nanoparticle (NP) play an important role. Reactivity and toxicity of NP often depend on the relative surface area (Van Hoecke et al. 2008), showing that smaller NP can have higher toxic potentials (Passagne et al. 2012). In eukaryotic cells the uptake of NP strongly depends on the NPs' size, shape, and surface charge (Canton and Battaglia 2012; Rancan et al. 2012; dos Santos et al. 2011). Furthermore, the surface of NM is often functionalized with a stabilizing capping agent to prevent agglomeration or dissolution. The stabilizer is covalently bound to the NP surface (chemisorption) or adsorbed to the surface (physisorption). Since size and surface chemistry significantly influence their effects on organisms, NM have to be tested with respect to all these influencing factors.

Starting tests with NM, we decided to transfer the standard test design of the *Daphnia* acute toxicity test to 24-well microtiter plates. Differences between the standard design according to OECD-guideline 202 (OECD 2004) and the miniaturized microtiter plate test were tested with the reference substance potassium bichromate ($K_2Cr_2O_7$).

To validate the miniaturized test with a NM, silver nanoparticles (AgNP) were chosen since some results on their toxicity to daphnids are already available (Allen et al. 2010; Zhao and Wang 2011; Bowman et al. 2012; Jo et al. 2012). Due to their antimicrobial activity AgNP are used in many products ranging from medical applications to textiles (Allen et al. 2010; Marambio-Jones and Hoek 2010). In general silver toxicity is mainly linked to silver ions (Ag^+) (Bowman et al. 2012; Radniecki et al. 2011), which may also be released from AgNP. Silver ions (Welz and Sperling 1997) and AgNP (Lau et al. 2012) often show a high adsorption to surfaces, which also affects the bioavailability and therefore toxicity. This plays an even more important role when the surface:volume ratio (SVR) differs between the test designs.

Another adaptation concerned the test duration, which was prolonged to 96 h. Especially NM tend to show increased long-term effects. The standard test duration of 48 h can underestimate the toxic effects of NM. Dabrunz et al. (2011) have already shown increased toxicity and changed modes of action of TiO_2 nanoparticles in a prolonged acute test with daphnids.

The present study aims to evaluate the use of microtiter plates to simplify and miniaturize (economize) the *Daphnia* acute toxicity test without influencing the results compared to the guideline 202 (OECD 2004). So far, there were no adaptation or miniaturization proposals based on this new guideline. In contrast to Powell et al. (1996), we tested with larger volumes to keep the same volume per neonate ratio as in the OECD test design and to provide enough volume to the neonates to swim freely, which is important for respiration. Furthermore, changes in the test system should be as small as possible compared to the OECD design. Comparative tests with two different microtiter plates – 24-well and 6-well plates – with different SVR were performed. We chose AgNP as a potentially adsorbing, toxic material. Sorption of Ag^+ ions as well as of AgNP was possible. Furthermore, we investigated if a test prolongation to 96 h delivers new information. Since daphnids are not fed during the test, starving might affect the results in the prolonged time span. Therefore, comparative tests with algae supplied as food source were conducted.

The present study focused on five main questions:

1. Can microtiter plates be used for the *Daphnia* acute toxicity test in general without influencing the results?
2. Can the *Daphnia* acute toxicity test be miniaturized to about 50 % (amount of daphnids and substance) without influencing the results compared to the standard test design?
3. Does the increased surface:volume ratio in a miniaturized test design influence the toxicity? Especially, does it reduce the toxicity of AgNP as a potentially adsorbing substance?
4. Does the outcome of a prolonged test duration of 96 h differ between $K_2Cr_2O_7$ as a reference substance and AgNP since the reaction kinetics and the toxicity of NP can change with time?
5. Can the prolonged *Daphnia* acute toxicity test be conducted without feeding? Does starving or feeding influence the results?

2. Materials & Methods

Culturing

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, which was renewed twice a week. They were fed with the green algae *Raphidocelis subcapitata* (#61.81, SAG, Göttingen, Germany) on a basis of 150 µg C Daphnid^{-1} day^{-1}.

Test setup

Four different test setups with Potassium dichromate ($K_2Cr_2O_7$) were compared. The standard test (**ST-G**) according to the OECD guideline 202 (OECD 2004) was performed in 20 ml glass beakers with 10 ml of test solution and 5 neonates replicate^{-1} and 4 replicates of each concentration. The same test setup (**ST**) was conducted in 6-well microtiter plates (Greiner bio-one, #657102, Frickenhausen, Germany). The adopted test (**MT**) was performed in 24-well microtiter plates (Sarstedt, #83.1836, Newton, USA) with 2 ml test solution and 1 neonate replicate^{-1} with 10 replicates per concentration. Further, the adopted test with additional algae (**MT-F**) as a feeding control was performed. Algae were provided on a basis of 25 µg C neonate^{-1} day^{-1}. The used microtiter plate types consisted of polystyrene (PS). Since the ST-M test series was performed at a different time it could not be compared directly to the other series. Therefore, it was compared to an MT reference conducted parallel. This was easier to perform; moreover, differences were expected between these two but not between ST and ST.

The AgNP were tested with three different designs. The standard test (**AgNP-ST-P**) according to OECD-guideline 202 was conducted in polystyrene cups (Sarstedt, #73.1056, Nümbrecht, Germany) with 10 ml of test solution, 5 neonates replicate^{-1} and 4 replicates per concentration. It was compared to the same test setup performed in 6-well microtiter plates (**AgNP-ST**) and the miniaturized test design (**AgNP-MT**) performed in 24-well microtiter plates as described for $K_2Cr_2O_7$. Ionic controls with silver nitrate ($AgNO_3$) were conducted in MT (**Ag^+-MT**) and ST-M (**Ag^+-ST-M**).

Test preparation

$K_2Cr_2O_7$ (Sigma Aldrich, Steinheim, Germany; CAS 7778-50-9) was weighted and dissolved directly in Elendt M7 medium to prepare the stock solution. From this highly concentrated stock (around 2,5 g L^{-1}), a second stock with 10 mg L^{-1} was made. Stocks were kept at 4 °C and aged between 3 and 8 days to achieve an equilibrium and equal reactivity. The test concentrations were prepared directly before the beginning of the test from the 10 mg L^{-1} stock. The daphnids were exposed to nominal $K_2Cr_2O_7$-concentrations of 0, 50, 100, 250, 500, 750, 1000, 1500, 2000, and 3000 µg L^{-1}. The test vessels were filled with a repetitive pipette (HandyStep® electronic, Brandt, Wertheim, Germany), ensuring equal volumes in each replicate.

The NM-300K AgNP (detAgNP) dispersion (10.16% silver; uncoated particle in dispersion with 4% TWEEN 20 and 4% polyoxyethylene glycerol trioleate; NM-series of representative nanomaterials provided by JRC; ras materials GmbH, Germany) was diluted with milli-Q water to a concentration of 2% silver and placed in an ultrasonication bath (HF 45 kHz, Utrasonic Cleaner Bath, VWR, Leuven, Belgium) for 15 minutes. Ultrasonication was repeated before each use. A stock dispersion (10 mg Ag L^{-1}) was prepared by weighting and diluting the NM-300K dispersion in Elendt M7 medium. The stock dispersion was stored at 4 °C and aged for at least 2 days to achieve an equilibrium and equal reactivity. Test concentrations were diluted

in Elendt M7 medium directly before the test. Nominal concentrations equal to or higher than 30 µg Ag L^{-1} were diluted directly from the stock. Lower concentrations were prepared by dilution series of higher test concentrations. Tests were conducted with nominal Ag concentrations of 0, 5, 10, 20, 30, 50, 100, and 200 µg L^{-1}.
For the silver nitrate tests (Ag^+), a stock with 1 g Ag L^{-1} (1.5748 g L^{-1} $AgNO_3$; purum p.a., ≥ 99.0 %, Fluka, Buchs, Switzerland) and 10 mg Ag L^{-1} (dilution 1/100 from 1 g Ag L^{-1}) were prepared in 1 % HNO3 and stored in the dark. Tests dilutions were prepared in Elendt M7 medium directly from the 10 mg Ag L^{-1} stock to minimize loss of silver via adsorption. The tested nominal concentrations for Ag^+-MT were 0, 2, 3, 3.5, 3.75, 4, 4.5, and 5 µg L^{-1} and 0, 2, 2.5, 2.6, 2.7, 2.8, 2.9, 3, 3.1, 3.2, 3.3, and 3.7 µg L^{-1} for Ag^+-ST-M.
24 hours before the start of the test all neonates were collected from the cultures for synchronization. After 18 to 20 hours all newborn neonates from these were caught with a glass pipette and counted. Prior to the tests the neonates were fed with living algae (*Raphidocelis subcapitata*), providing them a minimum of 150 µg C daphnid^{-1}. After 4 hours they were put into the prepared test vessels.

Test procedure
Twenty-two to twenty-four hours after the synchronization, the neonates were put into the test vessels with a pasteur glass pipette in a small volume of Elendt M7 medium, ensuring lowest possible dilution. The tests were performed in a climate controlled chamber with a 16:8 h light-dark rhythm. The positions of the test vessels were changed randomly once a day. The immobilization of the daphnids was investigated visually after 24, 48, 72 and 96 h. According to OECD standards (OECD 2004), tests were only counted valid with (1) an immobilization ≤ 10% in the control after 96 h, (2) with a temperature of 20 ± 1 °C, (3) with a pH between 6 and 9 (actually pH was always between 6.4 and 7.8) and a variation lower than 1.5 units, and (4) with an O_2 above 3 mg L^{-1} after the tests. (3) and (4) were only randomly measured, but at least once per test trial, since they never exceeded the validation criteria.
At least five valid repeats of each test design were performed with $K_2Cr_2O_7$ (three for ST-M-G). Seven valid repeats were performed with AgNP in MT, three in ST-P, and five in ST. Six valid repeats with $AgNO_3$ were conducted in MT and ST. Depending on the amount of neonates available, between two and six tests could be started at one day. Neonates from independent cultures were used for simultaneously performed test trials. Tests were not completely independent since only one stock dispersion was used for simultaneous tests.

Analytics of AgNP
The size of AgNP in the stock dispersion (10 mg Ag L^{-1}) was measured using dynamic light scattering (DLS; Delsa^{TM}Nano C by Beckman Coulter, Krefeld, Germany). Particle size was measured in 24-well microtiter plates under test conditions over a period of 72 h. Mean hydrodynamic particle diameter was 54.0 ±12 nm (± mean peak width). UV/VIS scans (Cadas 200 by Hach Lange GmbH, Germany) showed a peak at 412 nm with no shoulder at longer wavelengths indicating that no agglomeration occurred.
The Ag concentrations were quantified by graphite furnace atomic absorption spectrometry (GF-AAS) using an Unicam 989 QZ AA Spectrometer with autosampler FS-90. After *aqua regia* digestion each sample was measured in three replicates. *Aqua regia* digestion was chosen due to the chloride content in Elendt M7 medium. The excess of chloride prevents precipitation of silver chloride ($AgCl_s$) and ensures the formation of soluble higher silver chloride complexes ($[AgCl]_{aq}$, $[AgCl_2]^-_{aq}$, …). Basic sample volume was 100 µL. Samples were digested by adding 80 µL of concentrated HCl (37%) and 20 µL of concentrated HNO_3 (65%). After drying the samples, 1 mL of diluted *aqua regia* (100 mL, consisting of 11 mL conc. HNO_3

(65%) and 44 mL conc. HCl (37%) and approx. 45 mL bidest. water) was added. Following MacDougall et al. (1980), the quantification limit in GF-AAS was estimated to 0.56 µg Ag L^{-1} (and the detection limit to 0.17 µg Ag L^{-1}). The above described digestion procedure increased the reliable quantification limit to approx. 5 µg Ag L^{-1} in the untreated samples. Exemplary Ag measurements were performed with nominal concentrations of 100 and 200 µg Ag L^{-1}. These concentrations were chosen because at lower concentrations the Ag content decreased below the quantitative detection limit of 5 µg Ag L^{-1} after 96 h. A concentration measurement of the stock dispersion performed later revealed 15 % lower Ag concentrations than indicated by the manufacturer (*preliminary data*). Therefore, the measurement in 50, 100, and 200 µg Ag L^{-1} was actually made with 15 % lower concentrations. This was taken into account in the results analysis and EC_{50} calculations. The test was run using the same conditions and duration like the *Daphnia* tests in three replicates, but without neonates. Samples were taken daily and prepared and measured immediately via AAS.

Calculation of adsorption quantities of AgNP

Assuming the adsorption of a monolayer of AgNP on the internal surface of the container, the surface would adsorb approximately 9.5 µg Ag cm^{-2}. This amount was estimated by basic geometric consideration of a hexagonal array of spheres with the diameter of 15 nm (electron microscopic data, see Klein et al. 2011) consisting of silver (density 10.5 g cm^{-3} (CRC 2012)). However, in Elendt M7 medium the AgNP show a hydrodynamic diameter of approximately 53,4 nm (by DLS), which can be attributed to a surrounding halo of the stabilizing agents used in NM300K (Polyoxyethylene Glycerol Trioleate and Polyoxyethylene (20) Sorbitan mono-Laurat (TWEEN 20) (Klein et al. 2011). Considering the combined adsorption of the AgNP with their stabilizers, the adsorbed amount of silver on the internal container surface is approximately 0,81 µg Ag cm^{-2}.

Statistics

EC_{50} values were calculated separately for each test using the statistics software R (Version 2.13.2, http://www.r-project.org) with a logistic regression. Mean EC_{50} value calculation and graphical plotting were made with GraphPad Prism 5.00 (GraphPad Software, San Diego, California, USA). Significance tests were calculated with a two-way ANOVA (repeated measurements) using GraphPad Prism.

3. Results

Potassium Bichromate

Comparisons of the miniaturized 24-well microtiter test (MT) with the standard test in glass beakers (ST), the standard test in 6-well microtiter plates (ST), and the miniaturized test system with additional algae as feeding control (MT-F) are shown in Fig. 51. The corresponding EC_{50} values are given in Table 51 and statistics in Table 5.2.

The graphs for MT and ST-G are almost congruent (Fig. 5.1a) with increasing toxicity over time, also in the prolonged test span of 72 and 96 h. Statistics (Table 5.2) indicate that EC_{50} values were similar in both test designs with no significant interaction, meaning that the stress response over time was equal in both test systems.

ST (Fig. 5.1b) was performed to study the possible use of 6-well microtiter plates for the standard test according to OECD-guideline 202. The toxicity of $K_2Cr_2O_7$ in ST compared to MT was similar at all time points, which was confirmed by statistical analysis (Table 5.2).

Possible starving effects on the daphnids – especially in the prolonged time span – were investigated by comparative tests between MT and MT with additional algae (MT-F) (Fig. 5.1c). Overall, the influence of the stressor was nearly identical, which was confirmed by statistics. Again, toxicity increased over time. Interestingly, the mean EC_{50} values after 96 h were not congruent anymore. Although statistics did not calculate significant differences, this might indicate a trend of $K_2Cr_2O_7$ being more toxic in presence of algae.

Silver nanoparticles

Silver nanoparticles (AgNP) were tested with the standard test design in PS cups (AgNP-ST-P), in 6-well microtiter plates (AgNP-ST) and in the miniaturized system (AgNP-MT). Toxicity in all three test setups increased over time. The standard test always revealed similar EC_{50} values, independent of the test vessels used (Table 5.1, Fig. 5.2a). Neither the test design nor the interaction between AgNP-ST-P and AgNP-ST revealed significant effects (Table 5.2). The mean EC_{50} values in AgNP-MT were equal to AgNP-ST-P after 24 h, but from 48-96 h the mean EC_{50} values in AgNP-MT were considerably lower than in the other two test systems (Table 5.1), indicating that detAgNP act more toxic in the miniaturized test. The interaction between AgNP-ST-P and AgNP-MT was highly significant (Table 5.2), indicating differing toxicity in the two test designs over time.

The ionic silver control (Ag^+) with silver nitrate was conducted in the MT and ST-M design. Statistics revealed significantly different toxicity between the two test designs (Table 5.2). Ag^+ (Table 5.1, Fig. 5.2b) was significantly more toxic in ST (EC_{50} ~2.35 µg Ag L^{-1}) than in MT (EC_{50} ~3.0 µg Ag L^{-1}). Between 24 and 48 h toxicity increased only slightly in both test designs. In general, toxicity of $AgNO_3$ after 24 h was about 10 to 15-times higher than toxicity of AgNP (Table 5.1, Fig. 5.2).

Since the toxicity of AgNP strongly depends on their sorption characteristics and the release of Ag^+ ions, the contact surface of the test vessels was calculated. This was compared to the test solutions' volume, giving a SVR. Table 3 illustrates that glass beakers used for ST and PS cups used for AgNP-ST-P have similar SVR as well as water column heights. 6-well microtiter plates have a similar SVR but a much lower water column height compared to the ST test vessels. The water column height of 24-well microtiter plates and 6-well microtiter plates is similar, but the former have a much higher SVR compared to all other test vessels, i.e. a larger relative contact surface.

Fig. 5.3 shows the AAS measurements of 50, 100, and 200 µg Ag L^{-1} in the three test vessels. For 200 µg Ag L^{-1} (Fig. 5.3a, b) concentrations were between 35.3 and 44.5 % lower than the nominal concentrations, for 100 µg Ag L^{-1} (Fig. 5.3c, d) concentrations were between 26.5 and

30.5 % lower, and for 50 µg Ag L^{-1} (Fig. 5.3e, f) concentrations were between 13.7 and 22.4 % lower directly after putting the test solutions into the test vessels (T0). About 15 % loss can be explained by the lower concentration specifications of the stock dispersion than those given by the manufacturer (see above). The Ag concentrations in all test vessels decreased over time (Fig. 5.3a, c, e), consequently increasing the adsorbed partition (Fig. 5.3b, d, f). After 96 h between 73 and 93 % of the original Ag was not present anymore in the water column compared to T0, independent of the test vessel (Fig. 5.3b, d, f). The Ag concentration decrease, respectively the loss of Ag from the water column, was consistently higher in AgNP-MT, which was expected due to the higher SVR.

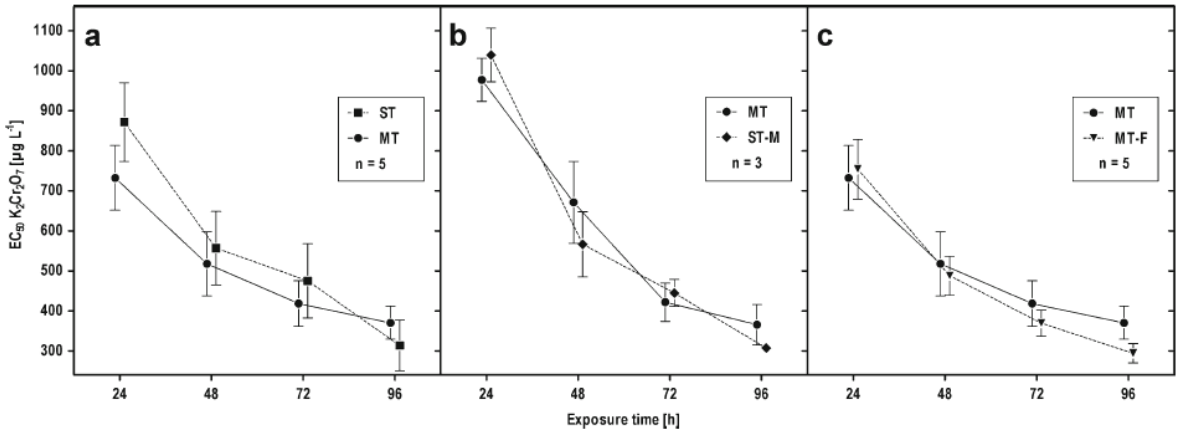

Figure 5.1. Acute toxicity of $K_2Cr_2O_7$ to *D. magna* with mean EC_{50}-values (± SE) over 96 h. The figure is an extract of Baumann et al., 2014 (doi:10.1007/s11356-013-2094-y), so abbreviations deviate from the ones used in this work. The miniaturized microtiter plate (MT) test is compared to (**a**) the standard test design (ST, here: ST-G), (**b**) ST performed in 6-well microtiter plates (ST-M, here: ST), and (**c**) MT with additional algae as feeding control (MT-F). All concentrations are given as nominal concentrations of $K_2Cr_2O_7$ in µg L^{-1}.

The amount of potentially adsorbed detAgNP in the different test vessels was calculated and compared. Table 5.4 shows the total amount of Ag in AgNP-ST-P/AgNP-ST and AgNP-MT test vessels at the lowest (5 µg L^{-1}) and highest (200 µg L^{-1}) nominal AgNP concentrations. Assuming adsorption of a monolayer of AgNP on the internal surface of the test vessels, the minimum and maximum AgNP adsorption amounts are shown. Since the minimum calculated adsorbed amount is about 9-times higher in AgNP-ST-P and about 14-times higher in AgNP-MT than the total amount of AgNP in the highest test concentration of 200 µg Ag L^{-1}, potentially all AgNP in all tested concentrations could have been adsorbed to the surfaces, independent of the test vessel type or test design. Although all Ag could theoretically have adsorbed to the container surface, 10 to 20 % remained in the water column after 96 h (Fig. 5.3).

4. Discussion

Miniaturization

The main aim of this study was the economization of the *Daphnia* acute immobilization standard test according to OECD-guideline 202 (OECD 2004). The test was simplified by transferring it to 24-well microtiter plates with only one neonate per well in a 2 mL volume.
The main advantage of the adaptation was to save 50% of both animals and test substance. Since only one neonate had to be investigated in a well, the time for the daily check of the test was clearly shortened, because immobilized daphnids could be detected immediately. In the standard test design 5 neonates have to be examined together, making it more difficult to define the immobilized ones. Taken together, this results in a substantial reduction of test substances costs, of the disposal after the test, and of the complexity in hatching of daphnids. By this, more independent tests can be performed at the same time.

Table 5.1. Acute immobilization of *Daphnia magna* for each test design and test substance (treatment) with the number of test repeats (N) and the mean EC_{50} ± 95% CI (µg L^{-1}). AgNP and $AgNO_3$ concentrations correspond to the metallic silver content. ST-G, ST-P = standard test in glass or plastic beakers, respectively, ST = ST performed in 6-well microtiter plates, MT = miniaturized microtiter plate test, and MT-F = feeding control of MT.

Treatment	N	24 h	48 h	72 h	96 h
$K_2Cr_2O_7$					
ST-G*	5	872.1 ± 273.0	556.8 ± 254.7	475.2 ± 257.2	313.9 ± 175.7
MT*	5	732.6 ± 224.6	518.0 ± 222.8	418.5 ± 158.3	370.5 ± 115.7
MT-F*	5	754.2 ± 207.9	488.1 ± 133.8	369.7 ± 91.1	294.0 ± 67.0
ST**	3	1039.3 ± 287.7	566.5 ± 351.1	444.9 ± 144.8	307.4 ± 36.2
MT**	3	977.8 ± 232.3	671.2 ± 441.8	421.9 ± 205.3	366.2 ± 217.9
AgNP					
ST-P	3	34.5 ± 31.1	33.6 ± 29.4	32.7 ± 28.9	27.6 ± 34.7
ST	5	37.4 ± 9.4	33.7 ± 9.9	33.1 ± 9.3	25.3 ± 7.5
MT	7	35.1 ± 4.6	22.4 ± 3.8	21.2 ± 3.8	15.9 ± 3.6
$AgNO_3$ (Ag^+)					
ST	6	3.05 ± 0.23	3.01 ± 0.23		
MT	6	2.39 ± 0.34	2.32 ± 0.31		

* first test trial; **second test trial

With only one daphnid per replicate, the exposure scenario was clearly changed in comparison to 5 daphnids in the standard test (OECD 2004). The same volume per daphnid (2 mL) is given

in both test designs, but the overall accessible volume is reduced from 10 to 2 mL, which might affect the results. However, the comparison of the standard test setup ST with the miniaturized test system MT revealed no significant differences between the two test designs (Table 5.1 & 5.2, Fig. 5.1a). In all designs toxicity increased over time. We conclude that the individualization of the daphnids from 5 to one neonate per replicate as well as the reduction of the absolute replicate volume from 10 to 2 mL did not affect the toxicity of $K_2Cr_2O_7$. Furthermore, the reduced volume did not affect the neonates' fitness, as the control survival was not reduced in the MT design (data not shown).

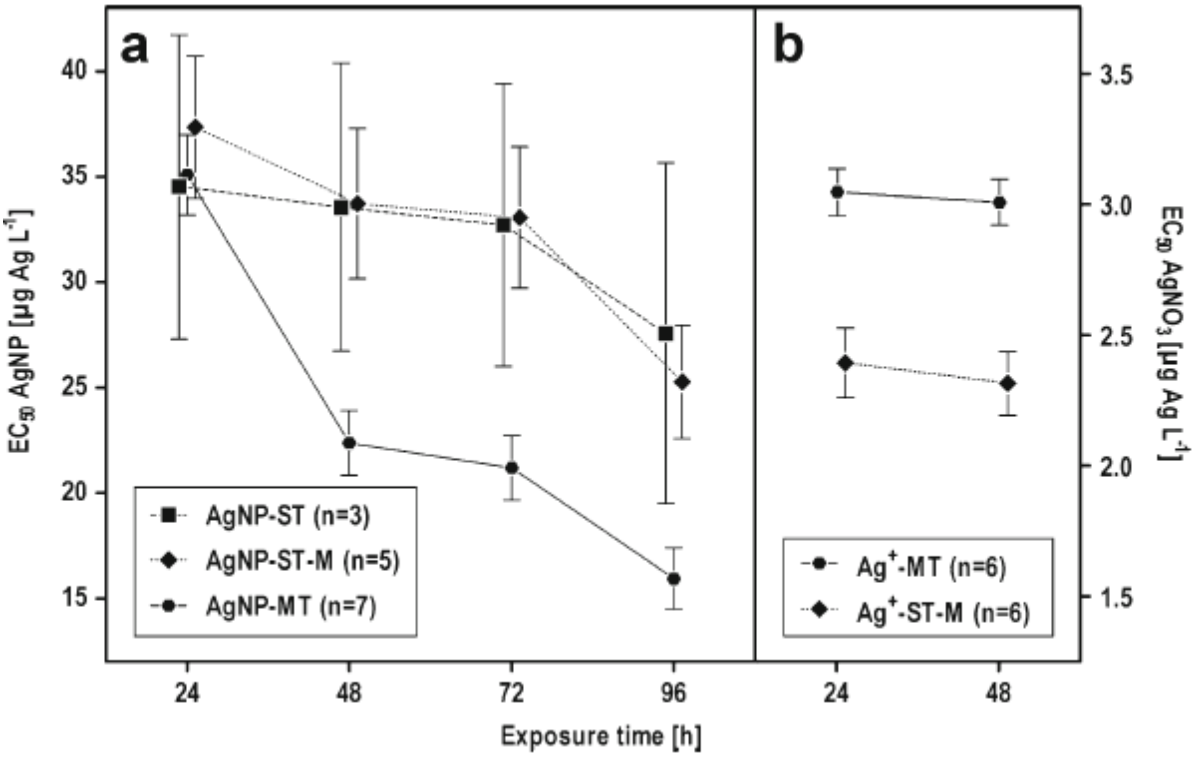

Figure 5.2. Acute toxicity to *D. magna* of **(a)** silver nanoparticles over 96 h and **(b)** silver nitrate (Ag^+) over 48 h. The figure is an extract of Baumann et al., 2014 (doi: 10.1007/s11356-013-2094-y), so abbreviations deviate from the ones used in this work. Mean EC_{50}-values (± se) are compared in the standard test design (AgNP-ST, here: AgNP-ST-P), the standard test performed in 6-well microtiter plates (AgNP-ST-M, here: AgNP-ST), and the miniaturized test design performed in 24-well microtiter plates (AgNP-MT). Concentrations are indicated in µg Ag L^{-1}.

Also the different SVR did not affect the toxicity of $K_2Cr_2O_7$, although in MT it was increased from around 2.1 to 3.5 cm^2 mL^{-1} (Table 3). In solution $K_2Cr_2O_7$ forms chromium (VI) anions. The adsorption of Cr(VI) is strongly pH-dependent. At the given aerobic test conditions at ph 7-8 it remains highly mobile. No or only very low adsorption rates could be expected (Richard and Bourg 1991; European Commission 2005). In contrast, the toxicity of other, highly adsorbing substances might be influenced by the increased SVR.

Because of their physico-chemical properties, AgNP as well as Ag^+ ions (released from the AgNP) tend to adsorb to surfaces (Welz and Sperling 1997; Lau et al. 2012). For the standard

test (ST-P) small PS cups replaced the normally used glass beakers since the adsorption of Ag depends on the surface materials (personal observation), which might affect the comparison to the test performed in PS microtiter plates (MT).
The tests with detAgNP revealed similar EC_{50} values in ST and MT after 24 h. After longer exposure the EC_{50} values were about 1.5-times lower in MT than in ST (Table 1, Fig. 2 a), which is approved by a highly significant interaction between the test designs (Table 2). The ionic silver control (Ag^+; Table 1, Fig. 2 b) revealed significantly higher toxicity of ST than in MT (Table 2). Due to the strong adsorption affinity of Ag^+ ions and the increased SVR in MT this was expected. Therefore, the increased toxicity of detAgNP in MT was completely contradictory to our hypothesis.

Table 5.2. P-values of two-way ANOVAs (time, design) for comparing the different tests designs. MT = miniaturized microtiter plate test, ST-G/-P = standard test performed in glass/plastic beakers, respectively, ST = standard test performed in 6-well microtiter plates, and MT-F = feeding control of MT. Numbers given indicate the p-values; ** p < 0.01, *** p < 0.001; ns = not significant.

	$K_2Cr_2O_7$			**AgNP**		**$AgNO_3$**
	MT vs. ST-G	MT vs. ST	MT vs. MT-F	ST-P vs. ST	MT vs. ST	MT vs. ST
test design	ns	ns	ns	ns	ns	**
	0.6752	0.7492	0.6763	0.9693	0.1107	0.0014
interaction (design vs. time)	ns	ns	ns	ns	***	ns
	0.0519	0.4306	0.2794	0.5897	0.0003	0.3145

The exemplary AAS measurements in detAgNP treatments showed decreasing Ag concentrations over time in all test designs (Fig. 5.3), indicating strong Ag sorption. In MT the loss of Ag from the water column was even faster and higher than in the two others, which should be linked to the enlarged SVR. From the calculations in Table 5.4 it can be assumed that there were not enough AgNP to build a closed monolayer on the test vessel walls, since this would require an at least 9-times higher AgNP concentration than in the highest test concentration of 200 µg Ag L^{-1}. Most likely the adsorbed AgNP were singly distributed over the walls. Although all AgNP could be theoretically adsorbed to the test vessel walls in all test designs (Table 5.4), the AAS measurement after 96 h showed small Ag concentrations from 7% to 27% of the original concentrations at T0 (Fig. 5.3), with still increasing toxicity (Fig. 5.2a). From the analytics and theoretical assumptions it cannot be clarified why the detAgNP were more toxic in AgNP-MT.
Since the toxicity of AgNP is closely linked to released Ag^+ ions (Radniecki et al. 2011; Navarro et al. 2008; Bowman et al. 2012), a higher dissolution of detAgNP must have occurred in MT. Possibly the adsorption of detAgNP chemically destabilized their integrity, resulting in an increased dissolution of Ag^+ ions. Due to the larger SVR, more AgNP were adsorbed in MT, which is slightly supported by the AAS measurements (Fig. 5.3). Since more AgNP were adsorbed, the higher overall release of Ag^+ ions increased the toxicity in MT. The higher toxicity should therefore result from the increased shift from the nanoparticular form of silver to the more toxic ionic form. Since this assumption is highly speculative, the Ag measurement via

AAS has to be refined, to be able to measure lower Ag contents and to distinguish between AgNP and Ag^+ ions at these low concentrations.
Toxicity of AgNP not only depends on adsorption and dissolution. Also agglomeration and sedimentation can influence the bioavailability (Klaine et al. 2008). These parameters are affected by surface functionalizations (Liu et al. 2012), the dissolved organic matter (Kennedy et al. 2012) or the pH (Klaine et al. 2008; Bhatt and Tripathi 2011). With the applied size measuring methods (DLS and UV/VIS) agglomeration and sedimentation tendency could only be measured in the stock solution, since test concentrations were far below the detection limits. In the stock (containing 10 mg Ag L^{-1} in Elendt M7 medium) detAgNP did not agglomerate (data not shown). Therefore, the observed effects of detAgNP should not be linked to agglomeration. However, the limited analysis cannot completely excluded agglomeration and sedimentation during the tests.
Concerning the miniaturization of the *Daphnia* acute toxicity test, our results on $K_2Cr_2O_7$ are similar to those of Powell et al. (1996). A miniaturization is possible and the use of microtiter plates is an appropriate alternative to the OECD test design (OECD 2004), but adsorption might affect bioavailability. Powell et al. (1996) showed that even a more radical miniaturization with a volume of only 1 ml per neonate is possible. The use of microtiter plates is not new in aquatic ecotoxicology. Several commercially available microbiotest use microplates, e.g. the TOXKIT™ tests (MicroBioTestsInc. 2013) using the brine shrimp *Artemia franciscana* (ARTOXKIT M™, 24-well, 10 larvae in 1 mL, 3 replicates), the estuarine rotifer *Brachionus plicatilis* (ROTOXKIT M™, 36-well, 5 rotifers in 0.3 mL, 6 replicates), and also daphnids (DAPHTOXKIT™). The D*aphnia* kits follow the OECD guideline 202 (OECD 2004). Neonates are hatched from dormant eggs (ephippia), which allows culture and maintenance free testing. Combining this effort with our adaptations would result in an enormous economization of the *Daphnia sp.* acute toxicity test. With only one animal per replicate and the performance in 24-well microtiter plates, our test design also corresponds to the new proposal of the fish embryo test (OECD 2012). The draft proposes five different concentrations (plus control) of a test substance with 20 replicates. In our design we preferred 10 replicates giving us the possibility of testing more finely graduated concentrations with the same amount of animals.

Table 5.3. Comparison of test volume, contact surface area, ratio between surface and volume (SVR), and water column height of the four different test vessels used.

test vessel	volume (cm^3)	surface (cm^2)	SVR (cm^{-1})	water column (cm)
glass beaker	10	21.16	2.12	2.19
PS cup	10	20.79	2.08	1.96
6-well microtiter plate	10	21.17	2.12	1.01
24-well microtiter plate	2	7.01	3.50	0.98

Alternative Test Design

Alternatively, the standard test was performed in 6-well microtiter plates (ST) with $K_2Cr_2O_7$ and detAgNP. ST is still easier to handle than ST-G/-P in small beakers. The comparison between MT and ST revealed similar EC_{50}-values for $K_2Cr_2O_7$ without significant differences (Table 5.1 & 5.2, Fig. 5.1b). Also detAgNP were similarly toxic in ST-P and ST (Fig. 5.2 a, Table 5.2). Therefore, only three repeats of ST-P were performed with detAgNP, which explains large standard error values. Equal toxicity should be due to equal SVR in ST-P and ST (Table 5.3). Overall, ST-G/-P and ST can be regarded interchangeable The water column of ST and MT is reduced, which leaves less height for the neonates to swim. Effects of $K_2Cr_2O_7$ did not differ significantly between ST-G and MT as well as between ST and MT, nor did effects of detAgNP between ST-P and ST. It can thus be postulated that the reduced water column did neither affect the fitness of the neonates nor change the sorption or ratio of AgNP and Ag^+ ions. Especially for adsorbing substances 6-well microtiter plates can be an appropriate alternative to ST-G/-P beakers by combining the standard test with the advantages of microtiter plates. Based on these results, the ionic silver control was only performed in ST and compared to MT (Fig 5.2b, Table 5.2).

In ST the bottom surface is highly increased, providing an enlarged contact area for agglomerating and sedimenting NM. The lowered water column considerably increases the contact probability of daphnids and sedimented NM. If such a modified exposure scenario is desirable remains to be discussed.

Prolongation

We also investigated the prolongation of the *Daphnia* acute immobilization test from 48 h to 96 h. Less acute toxic substances, especially nanoparticles (NP), tended to show no or only slight toxic effects after the 48 h test period. Filser et al. (2013) investigated iron oxide NP. Although no significant toxic effect was found within the prolonged test period, immobilization slightly increased at the highest concentration of 100 mg iron L^{-1} after 96 h. Titanium dioxide NP had no effect in the tested concentrations (0.5-8 mg L^{-1}) within the standard test interval, but after 72 and 96 h the immobilization was dramatically increased in all concentrations (Dabrunz et al. 2011). Between 72 h and 96 h the immobilization was even doubled, resulting in 100% immobilization. This indicates that at least for some NM, but probably also for any other less acute toxic substance, the standard test duration might underestimate potential environmental risks.

Our reference tests with $K_2Cr_2O_7$ showed increasing toxicity over time within the 48 h standard test span, but toxicity continued to increase also in the prolonged test span of 96 h, indicated by the logarithmic decrease of EC_{50} values (Table 5.1, Fig. 5.1). Also detAgNP toxicity increased in all test designs (Table 5.1, Fig. 5.2) although the increase was higher in AgNP-MT than in AgNP-ST-P and AgNP-ST. EC_{50} values of detAgNP decreased logarithmically until 72 h. Between 72 to 96 h the toxicity over time was enforced, which might be due to nano-specific characteristics. Since toxicity of AgNP is closely linked to the release of Ag^+ ions the results might indicate an increased dissolution of AgNP.

Fig. 5.1c compares the influence of the prolongation from 48 h to 96 h under feeding conditions in the miniaturized test with $K_2Cr_2O_7$. Even though the differences are not significant (Table 2), in presence of algae the toxicity of $K_2Cr_2O_7$ (MT-F) tended to slightly increase after 96 h (Table 5.1). One might expect that feeding increases the neonates' fitness and decreases toxicity of $K_2Cr_2O_7$. An opposite effect is already known for daphnids and endosulfan (Barry et al. 1995). The authors postulated that increasing food levels directly increased the bioavailability of the substance because of sorption to the algae. Rose et al. (2002) explained similar effects of fenoxycarb on daphnids with their filtering rates: depending on the available amount of food,

the daphnids regulate their filter feeding rate, with lower filtering rates at lower food levels (McMahon and Rigler 1965; Haney 1985). Finally, food affects development and thereby the uptake and elimination kinetics (Pieters et al. 2006). For $K_2Cr_2O_7$, adsorption should not play an important role since it is low under the given conditions (Richard and Bourg 1991; European Commission 2005). Since no food was provided in MT, lower filtering rates and delayed development might have decreased the uptake of $K_2Cr_2O_7$, resulting in decreased toxicity. Even though the prolongation might have provoked starving of the neonates, this did not additionally increase the toxicity of $K_2Cr_2O_7$ in MT. The presence of algae can also increase the pH due to photosynthesis. Especially in a miniaturized test this could lead to high peaks of pH. Random measurements of pH showed only moderate pH increases of 0.6 units, with values between 6.8 and 7.8 (data not shown). This should not harm the daphnids, which was also confirmed by equal control mortality in MT and MT-F (data not shown). If a more stable pH is desired, the use of buffers is possible (Rendal et al, 2012).

Table 5.4. Comparison of the nominal total Ag content in the standard test (AgNP-ST-P = PS cup; AgNP-ST= 6-well microtiter plate) and used in the miniaturized test (AgNP-MT = 24-well microtiter plate) at the lowest and highest test concentration (5 and 200 µg L^{-1}) and the minimal (min) and maximal (max) calculated Ag amount needed to build a closed monolayer of AgNP on the test vessel walls (Ag adsorption potential). The minimum Ag amount was calculated on the basis of the mean hydrodynamic diameter of 54.0 nm in Elendt M7 medium, whereas the maximum Ag amount was calculated with the primary particle diameter of 15 nm.

	total Ag content [µg]		**AgNP adsorption potential [µg]**	
test (volume)	5 µg L^{-1}	200 µg L^{-1}	min	max
AgNP-ST-P/-ST (10 mL)	0.05	2.0	17.0	199.5
AgNP-MT (2 mL)	0.01	0.4	5.7	66.5

However, the *Daphnia* acute toxicity test cannot be prolonged even more without feeding. The neonates undergo their first molting 24-48 h after start of the test, also without a food source. Yet for the second molting between 72-96 h they depend on food – otherwise their development is inhibited, and most animals die before onset of the second molting (personal observation). We also encountered sporadic mortality higher than 10% in the controls between 72 and 96 h (data not shown), indicating that this is the maximum test duration without feeding. We never encountered increased mortality in the controls up to 72 h, making this an appropriate prolongation period. A test duration of 96 h without feeding is possible, but with the risk of an increased amount of invalid tests due to molting-linked mortality in the second molting.

In summary, acute tests can underestimate the toxicity of a substance not only because of their short duration, but also by the lack of food. This supports the importance of forcing the investigation of long-term effects, e.g. with the EPA 10-day chronic toxicity test (U.S.EPA 1994) or the *Daphnia magna* reproduction test (OECD 1998)

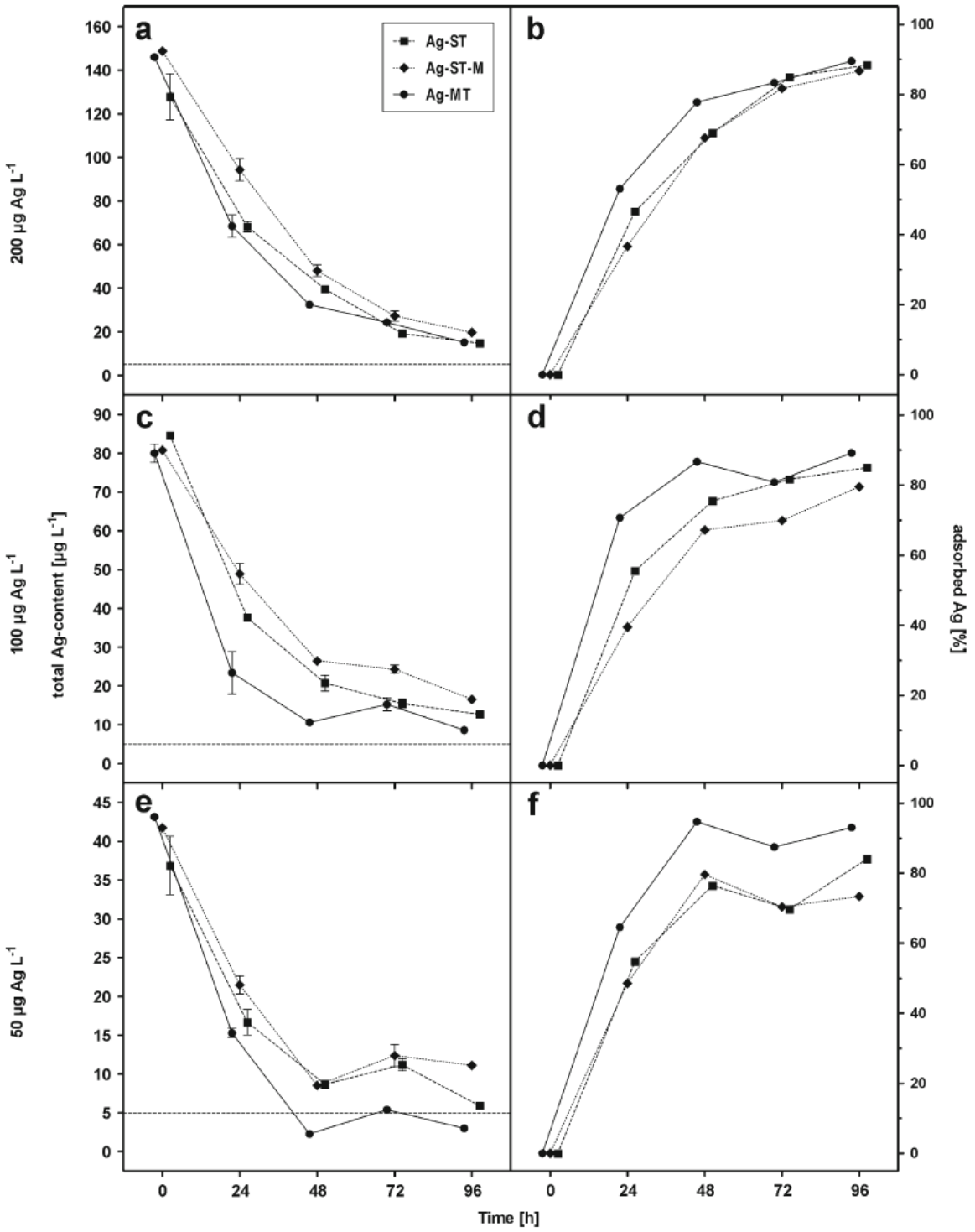

Figure 5.3. AAS measurement of the total silver contents and adsorption of silver on a percentage basis in Elendt M7 medium over 96 h with nominal concentrations of 200 µg Ag L^{-1}(**a, b)**, 100 µg Ag L^{-1} (**c, d**), and 50 µg Ag L^{-1} (**e, f**) with AgNP-ST = PS cup, AgNP-ST-M = 6-well microtiter plate, and AgNP-MT = 24-well microtiter plate. The figure is an extract of Baumann et al., 2014 (doi: 10.1007/s11356-013-2094-y), so abbreviations deviate from the ones used in this work: AgNP-ST = AgNP-ST-P, AgNP-ST-M = AgNP-ST.
a, c, e: Comparison of the absolute silver concentrations in the three different test vessels over time. Data represent mean values ± SE of three independent samples. Dotted line at 5 µg L^{-1} indicates the lower limit for a reliable quantitative silver measurement of the used method. **b, d, f**: Graphs show the adsorbed silver partition on a percentage basis over 96 h versus the starting concentration at T0.

Toxicity of test substances

$K_2Cr_2O_7$ is a standard reference substance for testing the sensitivity of *Daphnia* laboratory cultures. It is suggested by OECD guideline 202 (OECD 2004). The guideline prescribes a 24 h EC_{50} between 0.6 and 2.1 mg L^{-1} for *D. magna*. An ISO ring test generated a mean EC_{50} (24 h) of 1.12 mg L^{-1} (ISO 1996). Persoone et al. (2009) obtained data from seven different European laboratories with mean EC_{50} (24 h) ranging from 0.8 to 1.43 mg L^{-1}. The EC_{50} (24 h) in our tests was around 0.8 mg L^{-1} in the first (Table 5.1, Fig. 5.1a, c) and around 1.0 mg L^{-1} in the second test series (Table 5.1, Fig. 5.1b), which is in the range of both the OECD guideline and the study by Persoone et al. (2009). The difference between our two tests series should result from non-simultaneous testing. Gersich et al. (1986) reported greater variability when the assays were carried out on different occasions.

Since toxicity of AgNP is linked to the release of Ag^+ ions, their toxicity mainly depends on surface properties like relative surface area, charge, and functionalization. Therefore, data on AgNP toxicity is variable. 48 h EC_{50} range from one to three-digits in the µg L^{-1} range (Allen et al. 2010; Asghari et al. 2012; Hoheisel et al. 2012). Our results (48 h: 22 to 34 µg Ag L^{-1}; Table 5.1, Fig. 5.2a) are in the mid-range of literature values.

The ionic silver control (Ag^+) with $AgNO_3$ revealed mean EC_{50} from 2.3 to 3.0 µg L^{-1} (Table 5.1, Fig. 5.2b), strongly depending on the SVR. The EC_{50} were about 10 to 15 times lower than those of the detAgNP treatments (based on mass concentration). Similar toxicity and relations for AgNP and $AgNO_3$ were found by Blinova et al. (2013). Comparable $AgNO_3$ toxicity was also reported by others (Karen et al. 1999; Asghari et al. 2012; Allen et al. 2010). This clarifies that the sensitivity of our *D. magna* clone to Ag^+ was normal. $AgNO_3$ tests had lasted only 48 h because in pilot studies toxicity did not increase within the prolonged test span (*data not shown*).

5. Conclusions

The tested miniaturization is an appropriate alternative to the standard test with many advantages because of its high economization potential. Reducing the amount of test solution reduces the cost for test substances and their deposition. By saving animals the effort for hatching and controlling the daphnids is reduced. Accordingly, more tests can be performed at the same time. Alternatively, more concentrations can be tested for a more solid EC_{50} calculation. Furthermore, the prolonged and miniaturized design in 24-well microtiter plates opens the possibility to measure individual oxygen consumption over four days. Although this sublethal effect was not investigated, it might be a promising future endpoint.

A major disadvantage of a miniaturization is the increased SVR. Due to sorption processes, the toxicity of test substance can be significantly different. Our results show that the changed SVR significantly affected $AgNO_3$ and AgNP toxicity. This might also point to a general issue in aquatic ecotoxicology. Tests are standardized, but test volumes are always individual for each test species. Different EC_{XX} concentrations might not exclusively result from different sensitivity of test species or e.g. different ionic strength of the test medium. Also reactions on test vessel walls followed by changed bioavailability of the test substance due to different SVR have to be taken into account. This clarifies the importance of a thorough analysis of test substances under test conditions, especially of NM. Concerning our results, analytical methods have to be refined for a better understanding of AgNP sorption and dissolution processes.

This study also aimed to investigate advantages and disadvantages of prolonging the *Daphnia sp.* acute test to 96 h. In general, a maximum prolongation to 96 h without additional feeding is possible. The acute toxicity of both $K_2Cr_2O_7$ and AgNP always continued to increase after

the 48 h standard duration in every test design. This indicates that a duration of 48 h might underestimate acute toxicity, especially of substances with low acute toxicity. For NP, this assumption is supported by the results of Filser et al. (2013) and Dabrunz et al. (2011). Since a prolongation to 96 h may decrease control survival, additional feeding is to be recommended which also displays a more realistic scenario. However, it has to be taken into account that feeding might also affect toxicity and test conditions do not further correspond to the OECD standard test (OECD 2004). Actually, the *Daphnia sp.* acute toxicity test was developed to evaluate lethal or immobilizing concentrations of harmful substances. Also a prolongation to 96 h cannot replace long-term sublethal tests, but it helps to render more precisely the range of concentrations for chronic testing. If chronic tests like the *Daphnia magna* reproduction test (OECD 1998) can also be economized by miniaturization leaves to be tested.

Acknowledgments
The authors thank S. Devin for the advices in statistical analysis of the data. J. Baumann was financially supported by the Hans Böckler Foundation with a doctoral scholarship.

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6 How test vessel properties affect the fate of silver nitrate and sterically stabilized silver nanoparticles in two different test designs used for acute tests with *Daphnia magna*

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Under review

Contributions of Yvonne Sakka:

- Performance of all experiments
- Quantification of silver in the different test designs
- Performance of statistical data analysis
- Preparation of all figures and tables
- Writing and final improvement of the manuscript

Jan Köser supported the silver measurements and the interpretation of the obtained results by applying the method of T-SAR. Juliane Filser supported the statistical analysis and revised the manuscript.

How test vessel properties affect the fate of silver nitrate and sterically stabilized silver nanoparticles in two different test designs used for acute tests with *Daphnia magna*

Abstract
The relation between test conditions such as medium composition or pH on silver nanoparticle (AgNP) behavior and its link to toxicity is one of the major topics in nanoecotoxicological research in the last years. In addition, the adaptation of the ecotoxicological standard tests for nanomaterials is intensely discussed to increase comparability and reliability of results. Due to the limitation of test material production volumes and the need for high throughput screening, miniaturization has been proposed for several test designs. In the present study, the effect of a miniaturization of the acute *Daphnia* immobilization test on AgNP behavior was investigated. For this purpose, available, adsorbed and dissolved silver fractions were measured using AgNP and silver nitrate in two test designs: a standard test design (ST) and a miniaturized test design (MT) with reduced test volume and less animals.
Despite the increase in surface area in relation to the test volume in MT, more AgNP attached to the standard test (ST) vessel surface, so that in this case, exposure concentrations were significantly lower compared to the MT assessment. Ionic silver concentrations resulting from AgNP dissolution were similar in both test designs. The same was observed for ionic silver concentrations in silver nitrate ($AgNO_3$) treatments, but adsorbed silver was also higher in ST treatments. Assessing the Structure-Activity-Relationships revealed that surface properties such as hydrophobicity, potential binding sites or surface roughness were of higher importance than surface:volume ratios for both test substances.

Key words
silver nanoparticles, adsorption, surface interaction, stabilizer, hydrophobicity

1. Introduction

Silver nanoparticles (AgNP) are one of the most used and best investigated nanomaterials (Kahru and Dubourguier 2010). In addition, an increasing number of studies investigates possible exposure scenarios from different AgNP sources (Gottschalk et al. 2013; Wigger et al. 2015). This makes AgNP a good model for systematically investigating the fate of nanoparticles in the environment. Many studies with AgNP have addressed the relation between toxicity and behavior – measuring either the colloidal or chemical stability of the used nanomaterial during the test (Fabrega et al. 2009a; Jin et al. 2010; Baalousha et al. 2013; Topuz et al. 2014a) or how test conditions, e.g. media composition, pH or food level, affect fate and resulting toxicity (Allen et al. 2010; Angel et al. 2013; Gondikas et al. 2012; Lee et al. 2011; Levard et al. 2012; Mackevica et al. 2015; Reinsch et al. 2012; Sharma et al. 2014; Zhang et al. 2012).

Surface chemistry is likely to play a role in explaining differences in toxicity between coatings or at different environmental conditions. Studies addressing the development of AgNP coated surfaces show that surface chemistry processes determine the coating pattern and efficiency (Bandyopadhyay et al. 1997; Kleimann et al. 2006; Yang et al. 2007; Michna et al. 2011). For example, AgNP attachment varied with hydrophobicity of the surface (Song et al. 2011), or the test medium composition (Thio et al. 2012). However, in an ecotoxicological context, the influence of the surfaces on AgNP attachment has rarely been studied and information on the exact test setup including also illumination or used test vessels size is often lacking (Sekine et al. 2015). If considered, results support the importance of taking surface chemistry processes into consideration of AgNP fate (Sekine et al. 2015; Malysheva et al. 2016). Consequently, systematic investigation and analytics may be a helpful tool to predict and understand interactions between nanomaterials and natural surfaces like roots, particles (clay, particular organic matter) or organisms in the environment. To detect and analyze such relationships, simple laboratory tests are a good starting point for identifying and understanding the processes driving the fate of the used nanomaterial during the test and in the environment.

Due to the complexity of processes that may take place for different nanomaterials, it has often been asked whether standards developed for ecotoxicological tests can be applied to nanoparticles (NP) as well (Handy et al. 2008; Baun et al. 2009; Dhawan and Sharma 2010; Handy et al. 2012). An additional problem arises from the high number of differently sized, coated or charged NP that demand for rapid and cost effective testing. Consequently, several authors developed miniaturized test designs of OECD standard tests to save material, time and consequently money (Baumann et al. 2014; Engelke et al. 2014; Filser et al. 2014). The reduction in size of test vessels, however, may exert a strong influence on the fate of the used NP. For AgNP, these processes may include: increased adsorption due to an increase in relative surface area, decreased photoreduction of free silver ions due to a smaller surface area exposed to light, and increased reactivity in surface-AgNP mediated processes (Baumann et al., 2014; Sekine et al., 2015). This underlines the need for analytics in all test designs to clearly evaluate benefits and drawbacks.

Recently, we compared *Daphnia magna* toxicity of sterically stabilized AgNP in three different test designs: a) a standard test according to OECD guideline No. 202 (Organisation for Economic Co-operation and Development 2004) in glass beakers, b) the same test in large multiwell plates, and c) a miniaturized design in small multiwell plates (Baumann et al., 2014). No differences in toxicity or in actual silver concentrations were observed when the two standard test designs were compared. Surprisingly, toxicity was higher in the miniaturized design. As differences between actual concentrations of silver in all test designs were small, the present study investigated the fate of the sterically stabilized AgNPs in the two multiwell plate tests (standard and miniaturized) in detail. Our main focus was to link AgNP fate and

toxicity. For this purpose, fate of AgNP as well as $AgNO_3$ was analyzed daily in both test designs and results were related to the previously observed toxicity pattern for AgNP. We aimed at identifying differences in 1) the fate of AgNP and of $AgNO_3$; 2) the influence of the test design on the fate of both AgNP and $AgNO_3$, and 3) released ionic silver in AgNP treatments and its relation to toxicity.

2. Material & Methods

Chemicals and test solutions

Silver nanoparticles (detAgNP, ras materials, Regensburg, Germany; OECD standard material NM-300K) were uncoated, spherical, and delivered with a nominal concentration of 10.16 wt% in a non-ionic detergent solution containing each 4% polyoxyethylene sorbitan monolaurate, (TWEEN 20) and polyoxyethylene glycerole trioleate (trade name TAGAT® TO (Hensel et al. 1996)) resulting in a volume:volume ratio of about 1:8 for Ag:detergents. AgNP stock dispersions were diluted with demineralized water (Milli-Q plus, Merck Millipore) water to a nominal silver content of 2% and ultrasonicated for 15 min before use (Sonorex RE100H, BANDELIN electronic). Recent analytics of a long-term series of silver content measurements of NM-300K stock dispersions proved that the actual silver content was 79 ± 5 % of the nominal content (Koeser, personal communication), so all nominal AgNP concentrations in the present study were corrected using this value. Test stock dispersions of 7.9 mg Ag L^{-1} were prepared using 2-fold Elendt M7 and AgNP stock dispersion in equal amounts and Elendt M7 medium for further dilution. Test stock dispersions were equilibrated at 4 °C in the dark for 7-8 days before use. Silver nitrate, $AgNO_3$, (purum p.a., ≥ 99.0, Fluka) stock solution of 10 mg Ag L^{-1} was made in 1% HNO_3 to minimize losses during storage. Test solutions/dispersions were prepared fresh by dilution with Elendt M7. pH values were adjusted to 6.0-7.5 using NaOH (Titrisol, Merck). Freshly filled test vessels were stored at test conditions (20 °C, light) for about 2 h before the start of the experiment to minimize differences in starting conditions between single vessels.

Preparation of test vessels

Following Baumann et al. (2014), each test design was conducted in a specific multiwell plate: for the standard test design (ST), 6 well cell culture plates (No. 657102, Greiner Bio-One) were used while 24 well cell culture plates (No. 83.3922, Sarstedt) were chosen for the miniaturized design (MT). To use independent samples, plates were cut into pieces: 1 well per piece for 6 well plates and 2x2 wells per piece for 24-well plates. All parts were washed with deionized water and air dried before use. Contamination by dust was prevented by lint-free tissue (Kimwipes, VWR International) covering.

Test organisms

Daphnia magna (Clone B, Bayer) were originally obtained from Helmholtz Center for Environmental Research (UFZ, Leipzig, Germany) and have been cultured in our laboratory since several years. Culture conditions were 20 ± 1 °C with a 16:8 h light-dark cycle (climate controlled chamber, BK 6160, Heraeus Holding) in Elendt M7 medium. Media exchange took place twice a week and 1.5 mg C d^{-1} $animal^{-1}$ of the green algae *Raphidocelis subcapitata* (no.61.81, culture collection of algae SAG) were used as food source with each media exchange.

Experimental design

Two test designs were used as previously described (Baumann et al., 2014), ST with 10 mL of test solution/dispersion and 5 neonates, and MT with 2 mL and 1 neonate. Test concentrations were 16 and 40 µg Ag L^{-1} for AgNP, and 20 and 50 µg Ag L^{-1} for $AgNO_3$ treatments. Differences to the previous design (Baumann et al., 2014) were the use of multiwell plate pieces and no addition of neonates in $AgNO_3$ treatments, as the used concentrations would have caused 100% mortality within the first few hours of the experiment. Tests were run at 21.1 ± 0.1 °C using a 16:8 h light-dark-cycle in a climate room for 96 h. All tests were run with 4 replicates. To control for changes in toxicity, acute tests were run as duplicates in each design.

Sampling procedure and silver analysis

For $AgNO_3$, two silver fractions were distinguished: silver present in the test medium (Ag_m), and silver sorbed to the test vessel surface (Ag_s). For AgNP, a third silver fraction was included: silver present in the test medium as ions (dissolved silver, Ag_i). Samples were taken directly at the beginning of the experiment and every 24 h for 4 days, except for Ag_i where day 3 was not included. Sampling always followed the same routine with Ag_m and Ag_i samples as a first step directly from the test medium, followed by emptying test vessels and refilling with diluted aqua regia (100 mL prepared with double distilled H_2O, 44 mL conc. HCl,, and 11 mL conc. HNO_3), and sampling of Ag_s from the aqua regia after 5 min (a detailed description of the complete sampling protocol can be found in the Online Resource, Part S1).

All samples were analyzed using a graphite furnace atomic absorption spectrometer (GF-AAS; GF 90 and Solaar 989QZ, Unicam). Control samples of new, empty test vessels and from Elendt M7 medium were taken using the same protocol. The results were in the range of the background levels of the device (data not shown).

Particle characterization

Size, zeta-potential, and colloidal stability were measured in the AgNP test stock dispersion due to the low concentrations used for the experiment. Size was measured by dynamic light scattering (Beckman-Coulter DelsaNanoC, Beckman Coulter) with a backscattering angle of 165°; 4 min equilibration time and 25 °C. Evaluation of the correlation functions was done using the cumulants method giving the z-average of the hydrodynamic diameter (HDD) and the polydispersity index (PDI). The non-negative-least-square algorithm (NNLS) was used for the calculation of the intensity-weighted HDD distribution. For simplification only the main peak was recorded. Zeta-potential measurements were done using a Flow Cell in the same device with a scattering angle of 15°, 4 min equilibration time and 25 °C.

Colloidal stability was also assessed by measuring the surface plasmon resonance (SPR) using an UV-vis spectrophotometer (350-800 nm; Cadas200, HACH LANGE). To compare the AgNP stability in both test designs, size and peak wavelength were measured in samples from stock dispersion filled into the two different multiwell plates (ST and MT) stored under test conditions. Samples were taken immediately after the start of the test (2 h), after 24 h and at the end of the test (96 h).

For visual comparison of AgNP attachment in both tests, pictures of test vessel surfaces were taken in duplicates: Squares of 1 ± 0.1 cm^2 from the bottom plate of both types of test vessels were placed into the corresponding wells containing either 10 mL (ST) or 2 mL (MT) of a 40 µg Ag L^{-1} test dispersion. Wells were then transferred directly to test conditions. Pictures were taken using scanning electron microscopy (SEM, SUPRA40, Zeiss) after 15 min (day 0) and after 96 h (day 4).

Data treatment and statistics

After measurement as concentration, Ag_s was transformed into amount per available surface area to account for differences in available surface area in the two test designs. Statistics were run with R, version 3.0.3 (R Core Team, 2014) for each type of samples (Ag_m, Ag_i, Ag_s). Data sets were analyzed using linear models (lm) or general linear models (glm) with test design, nominal test concentration, substance, and day as factors. If conditions for lm or glm were not fulfilled, Kruskal-Wallis tests were run for investigating the single factor effects. Due to the high number of influencing factors, data sets were subsequently split between substances and therein between single days for investigating the effect of the test design in detail.

Interpretation of results using "Thinking in terms of Structure-Activity-Relationships" (T-SAR)

The methodology of "Thinking in terms of Structure-Activity-Relationships" (T-SAR) was used to estimate the interactions of the test substances and the test vessels surfaces (Fernández et al. 2011). For AgNP the interactions of the stabilizers TWEEN 20 and TAGAT® TO with the surface of the test vessels were also taken into account. Here, the focus was the identification of the lipophobic and hydrophobic parts of the structures as shown in Fig 6.4 and in Fig 6.5 as blue (lipophobic) and yellow (hydrophobic) parts and their interaction potentials with each other.

3. Results & Discussion

Particle characterization

The mean hydrodynamic diameter of detAgNP according to non-negative-least-square (NNLS) calculation was 41.2 ± 2.6 nm with a zeta-potential of -15.7 ± 1.3 mV (Table 6.1). The peak of the SPR was at 412 nm in the stock dispersion and decreased to about 410 nm during the test (Supporting Information, Table S6.7), indicating rather stable size distributions over time. The main peak size according to NNLS remained stable throughout the test, while the z-average of the HDD decreased in both test designs and the polydispersity index increased. The decrease in z-average of the HDD was more pronounced in the MT test design (SI, Table S6.7).

The values show low surface charge combined with comparatively high stability and are in line with other studies using non-charged organics as coating or stabilizer for AgNP (Sharma et al. 2014). The measured negative surface charge, despite using uncharged stabilizers, is most likely caused by reactions of the outer layers of silver with oxygen or chloride present in the stock dispersion and Elendt M7 test medium and subsequent formation of a $AgCl/Ag_2O$ shell (Reicho 2008; Selmani et al. 2014).

The reduction of z-average HDD and peak of the SPR wavelength indicate sedimentation of large detAgNP aggregates that are no longer included in the size measurement. This explanation is supported by the decreasing amount of total silver in both test designs (SI, Table S6.2), as sedimented silver would also be missing in the AAS sampling of Ag_s (due to the emptying of the vessels). The higher degree of sedimentation in the MT design is most likely attributed to the lower number of neonates that could retain aggregates in solution by creating water flows with their swimming movements.

Table 6.1: z-average hydrodynamic diameter with corresponding polydispersity index, and NNLS main peak HDD, as well as zeta-potential with corresponding conductivity of detAgNP in the test stock dispersion. All values are given as mean ± se (n = 10 for size measurements, n = 3 for zeta-potential measurements).

z-average Hydrodynamic Diameter [nm]	Polydispersity Index (PI)	NNLS Main Peak Hydrodynamic Diameter [nm]	Zeta-Potential [mV]	Conductivity [µS m-1]
368 ± 82	0.188 ± 0.031	41.2 ± 2.6	-15.7 ± 1.3	0.715 ± 0.001

SEM pictures of the bottom squares showed no good resolution due to the low conductivity of the polymer (Figure 6.1). Thus, pictures were not used for detailed analysis of the particles. However, it can be seen that the MT surface was smoother than the ST surface and that coverage with detAgNP was poor in both cases after 4 days of exposure. detAgNP attached to the surface appear as spherical shapes, indicating that particles did either not dissolve or dissolved too fast to be detectable on the pictures. MT multiwell plates were hydrophilized with oxygen while ST multiwell plates were not treated after production (personal communication with Sarstedt Inc. and Greiner Bio-One GmbH, respectively). As reduction of roughness due to plasma treatments of polymer surfaces has been observed elsewhere (Großmann 2009), smoothing of surfaces in MT due to hydrophilization is possible. However, the difference in roughness may bias the calculation of the surface area and thus the surface:volume ratio

(SVR), as completely smooth surfaces were assumed for calculation of available surface area in both test designs resulting in a possible underestimation of surface area in case of ST. This would lead to a reduction in SVR difference between both test designs.

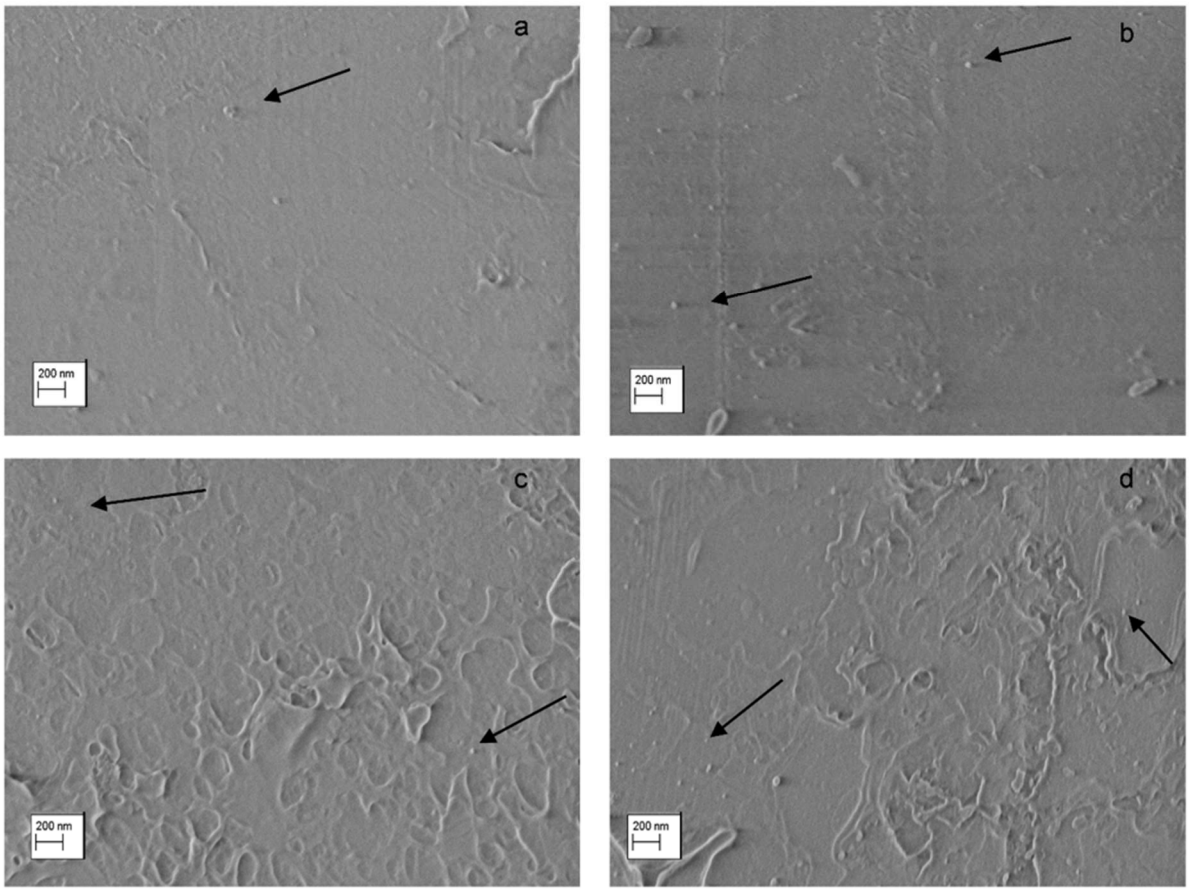

Figure 6.1 REM pictures of the test vessel surfaces in the two test designs. a) MT design after 15 min (day 0); b) MT design after 4 days; c) ST design after 15 min (day 0); d) ST design after 4 days. Arrows mark places where AgNP were visible best.

Fate of $AgNO_3$ in the two test designs

In all $AgNO_3$ treatments, Ag_m concentrations decreased significantly throughout the test period (Kruskal-Wallis-Test, $p<0.001$) alongside with an increase in Ag_s (Figures 6.2a, b and 6.3a, b; lm, $p<0.001$). If the sum of total silver (Ag_s + Ag_m) per day is calculated (details listed in SI, Table S6.1), it can be seen that total silver decreases throughout the test. As Ag_m is similar in both designs while Ag_s is higher in ST than in MT (lm, $p<0.001$), the fraction of silver not included in the measurement is higher in MT than in ST. This loss of silver cannot be explained by *Daphnids*, as no animals were added to $AgNO_3$ treatments, or by AgCl precipitation, as the chosen silver concentration was below the limiting concentration for AgCl precipitation in Elendt M7 medium (calculated using PHREEQCi; version 3.376-11094 and the database minteq.v4; Parkhurst, 2016). It is possible that loss of silver was caused by incomplete re-dissolution of Ag_s with the used protocol. Sekine et al. (2015) were able to maintain higher recovery rates throughout their experiments when measuring radioactive silver directly at the test vessel walls, supporting the assumption of silver remaining at the surface of the test vessels in the present study. Silver ions in solution do not bind directly to polystyrene (PS), but

to ions bound to the PS surface (Lieser et al. 1988). Oxygen plasma treatment can increase the number of binding sites for metallic silver in PS (Burger and Gerenser 1992). As MT multiwell plates were treated with oxygen, it is possible that the reduced recovery in the MT test design was caused by the increased number of binding sites for silver.

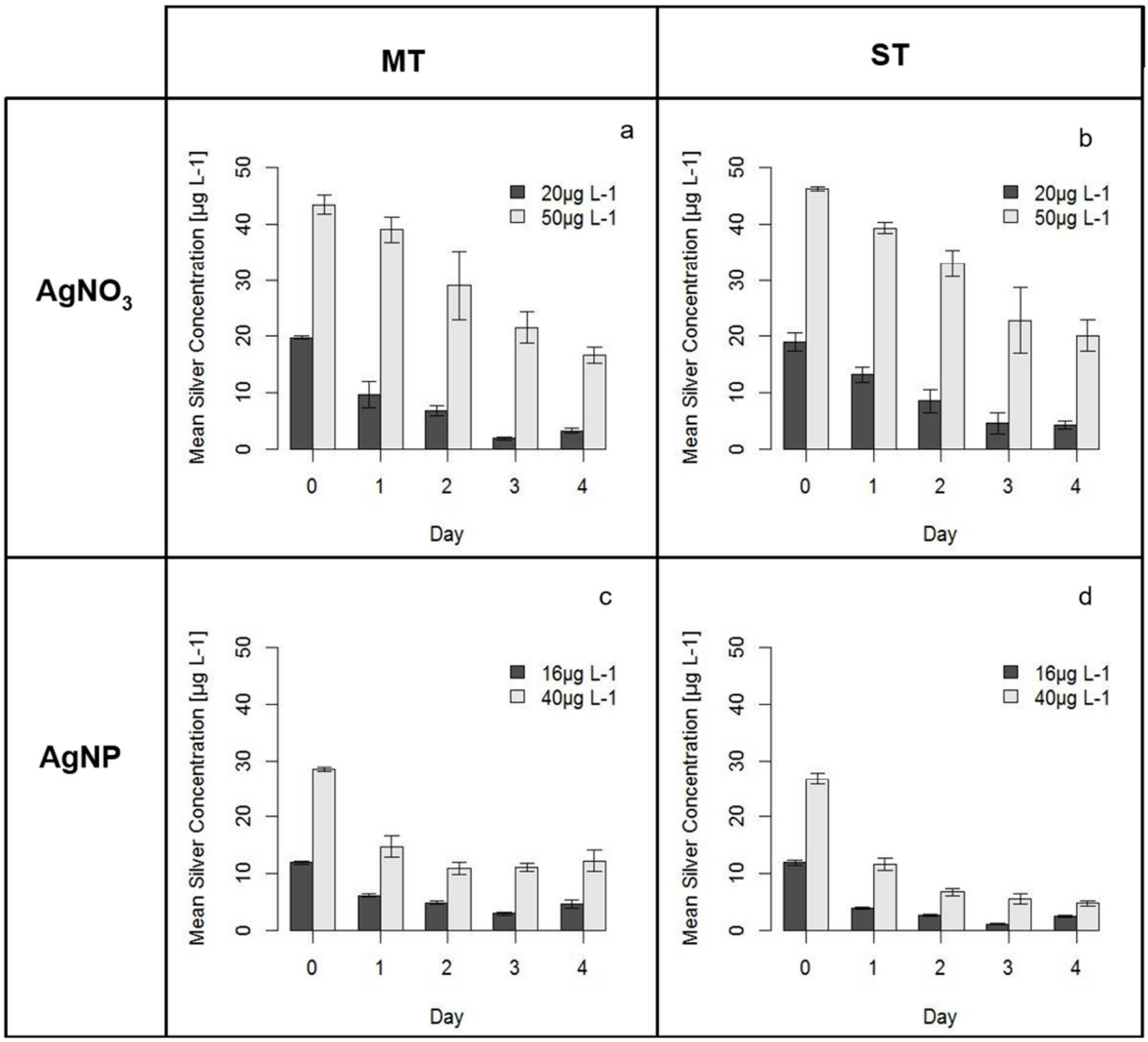

Figure 6.2 Available silver concentration (Ag_m) during the experiment in all treatments (mean ± se, n=4). a) miniaturized test design with $AgNO_3$ as test substance; b) standard test design with $AgNO_3$ as test substance; c) miniaturized test design with detAgNP as test substance; d) standard test design with detAgNP as test substance. Light grey bars show results for 50 and 40 µg Ag L^{-1} in $AgNO_3$ and in detAgNP treatments, respectively; dark grey bars show results for 20 and 16 µg Ag L^{-1} in $AgNO_3$ and in detAgNP treatments, respectively.

The difference between test designs observed for Ag_s was not detectable for Ag_m (Kruskal-Wallis-Test, p=0.34). It is likely that the reduction of roughness in MT (Figure 6.1) reduced the available surface area in this test design, thus causing smaller differences in SVR than theoretically expected. To determine the extent of this change, systematic measurements of surface roughness are necessary, but could not be done in this study. In addition,

photoreduction of silver [Ag(+1) → Ag(0)] and subsequent attachment of the newly formed, uncoated AgNP to the surface may have reduced Ag_m concentrations (Sekine et al., 2015; Sharma et al., 2014). As the ST design has a larger top surface area, the process would have reduced differences between test designs as well. The results illustrate the complexity of processes taking place in a comparatively well-known system like acute tests with $AgNO_3$ and emphasize the importance of surface chemistry based processes.

Differences between AgNP and $AgNO_3$

The general pattern of silver distribution (decrease in Ag_m, increase in Ag_s) was similar in $AgNO_3$ and AgNP treatments (Figures 6.2 and 6.3). However, Ag_m values were significantly higher for $AgNO_3$ than for AgNP treatments (Kruskal-Wallis-Test, $p<0.001$) when expressed as percent of test concentration. For Ag_s, similar silver levels were measured for both test substances (Figure 6.3), but there was a clear difference in development over time between $AgNO_3$ and AgNP treatments (lm, $p<0.001$). In addition, their response to changes in test design differed (see also section 3.2 and 3.4). The reduction in SVR did not affect Ag_m in the expected pattern for both test substances. Instead, surface properties such as binding sites and roughness or hydrophobicity and surface charge could explain better how $AgNO_3$ and AgNP adsorb to the surfaces. The difference between AgNP and $AgNO_3$ illustrates that extrapolations from $AgNO_3$ to AgNP may be misleading. However, the comparison of ionic and nanoparticular silver may help to identify and explain effects caused by the (nano-)particle nature, like changes in responses to light, ions or possible surface interactions.

Fate of AgNP in the two test designs

As described above, Ag_m concentrations decreased over time while amounts of Ag_s increased (Figure 2c, d and 3c, d; Ag_m: lm, $p<0.001$, Ag_s: Kruskal-Wallis-Test, $p < 0.001$). For Ag_m, significantly higher concentrations were measured in MT than in ST (40 µg Ag L^{-1}: from day 2 onwards; 16 µg Ag L^{-1}: from day 1 onwards, see SI, Table S6.2). Correspondingly, Ag_s levels were lower in MT than in ST (Kruskal-Wallis-Test, $p < 0.001$). The comparison between the used concentration levels showed a proportional relation for Ag_m as well as for Ag_s (SI, Table S6.1).

The results of the T-SAR suggested that the surface in the ST design is rather uncharged and also partly hydrophobic (Figure 6.4 and 6.5). The hydrophilization with oxygen, however, is likely to decrease hydrophobicity due to the formation of hydroxyl and carboxy functional groups on the surface (Figure 6.4 and 6.5). In addition, the reaction with oxygen is also likely to introduce weak negative charges to the MT surface wherever oxygen is bound to it. As detAgNP had a weak negative charge as well (Table 6.1), detAgNP may have been repulsed by the MT test vessel surface. At the ST surface, much less repulsion should occur: only the functional groups of the polymerization starter can be expected to charge the surface of this design. Differences in surface charge played an important role in the attachment of AgNP to silica (Thio et al. 2012) or polymer surfaces (Sekine et al. 2015).

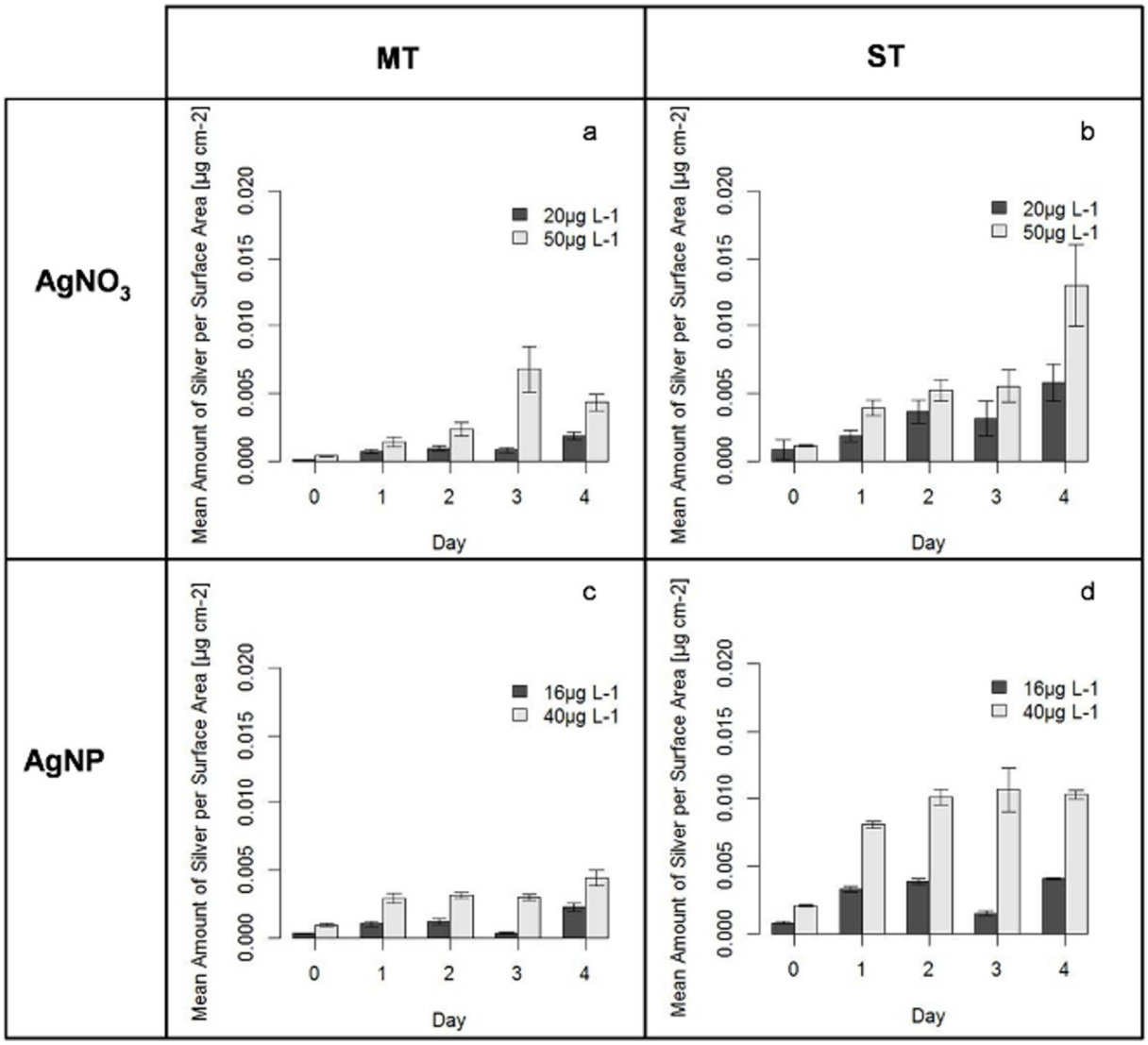

Figure 6.3 Amount of adsorbed silver (Ag_s) per surface area during the experiment in all treatments (mean ± se, n=4). a) miniaturized test design with $AgNO_3$ as test substance; b) standard test design with $AgNO_3$ as test substance; c) miniaturized test design with detAgNP as test substance; d) standard test design with detAgNP as test substance. Light grey bars show results for 50 and 40 µg Ag L^{-1} in $AgNO_3$ and in detAgNP treatments, respectively; dark grey bars show results for 20 and 16 µg Ag L^{-1} in $AgNO_3$ and in detAgNP treatments, respectively.

Ions present in the test medium may have also contributed to attachment or destabilization of AgNP (Jin et al. 2010). As the medium was identical in both treatments, an interaction between the stabilizers and the dissolved ions should have affected the colloidal stability in the same fashion on both treatments. Still, it is possible that a reaction between the formed polar or charged groups on the hydrophilized MT surface and ions in the test medium took place and reduced a possible difference in surface potential. In this case, surface charge could not explain the reduced loss in the MT test design. Measurements of the test vessel's surface charge would be required to address the influence of ions dissolved in the test medium on electrochemical repulsion, but this was out of the scope of this study.

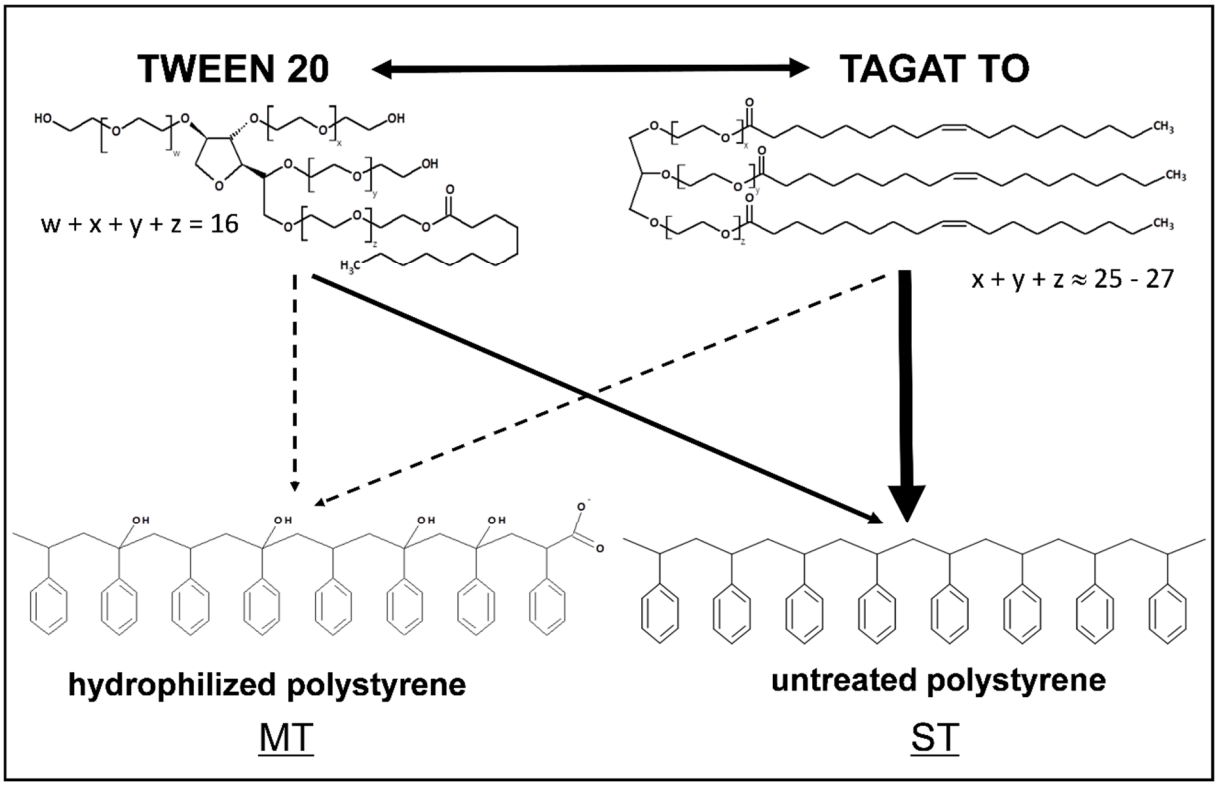

Figure 6.4 Estimation using T-SAR of the binding affinities between the two stabilizers (TWEEN 20 and TAGAT® TO) and the surfaces in the two test designs (MT and ST) in presence of H_2O. Dashed arrows mark lipophobic interactions, continuous arrows mark hydrophobic interactions. The thickness of arrows indicates the binding affinity between the linked components.

Aside electrostatical repulsion, also differences in hydrophobicity have been suggested as explanatory variable for differences in AgNP attachment (Malysheva et al. 2016). In this context, the stabilizer of the AgNP becomes a crucial part, as the silver core itself is lipophobic. According to the SAR analysis, TAGAT® TO is clearly more hydrophobic than TWEEN 20 (Figure 4). Attachment of non-ionic surfactants to polystyrene (PS) has been shown to increase with increasing hydrophobicity of the surfactant (Jódar-Reyes et al. 2005). However, TWEEN 20 is able to form self-organized monolayers on untreated polystyrene (Shen et al. 2011). It is consequently likely that both stabilizers attach to untreated PS (like in ST). Even though the completeness of the hydrophilization in the MT vessels was not tested in this study, it is likely that the hydrophobic surface area in this test design is smaller than in their ST counterpart. The interaction between lipophobic parts of the stabilizers and the hydrophilized PS is possible, but due to the large number of water molecules present in the test dispersion, we expect this to play only a minor role in this scenario. We summarized these conclusions in a binding affinity scheme for both stabilizers and surfaces (Figure 6.4) for better understanding of the suggested processes.

However, the binding of the stabilizers to the silver core is not reported by the manufacturer and methods for their measurement directly on the nanoparticles are not easy to assess. The development of such measurements is part of current research, especially in the field of nanoparticle corona research, but in the present work the methods were limited to particle characterization, AAS and T-SAR. Still, the constant main peak HDD is a strong indicator for the coverage of the AgNP by the stabilizers. When AgNP are synthesized in the presence of

TWEEN 80, TWEEN 80 fast covers AgNP nuclei during AgNP formation by producing a bilayer around the silver core (Kvitek et al. 2008). We can assume a similar coverage for the TWEEN 20-TAGAT® TO stabilized AgNP, as the molecules have a similar amphiphilic structure.

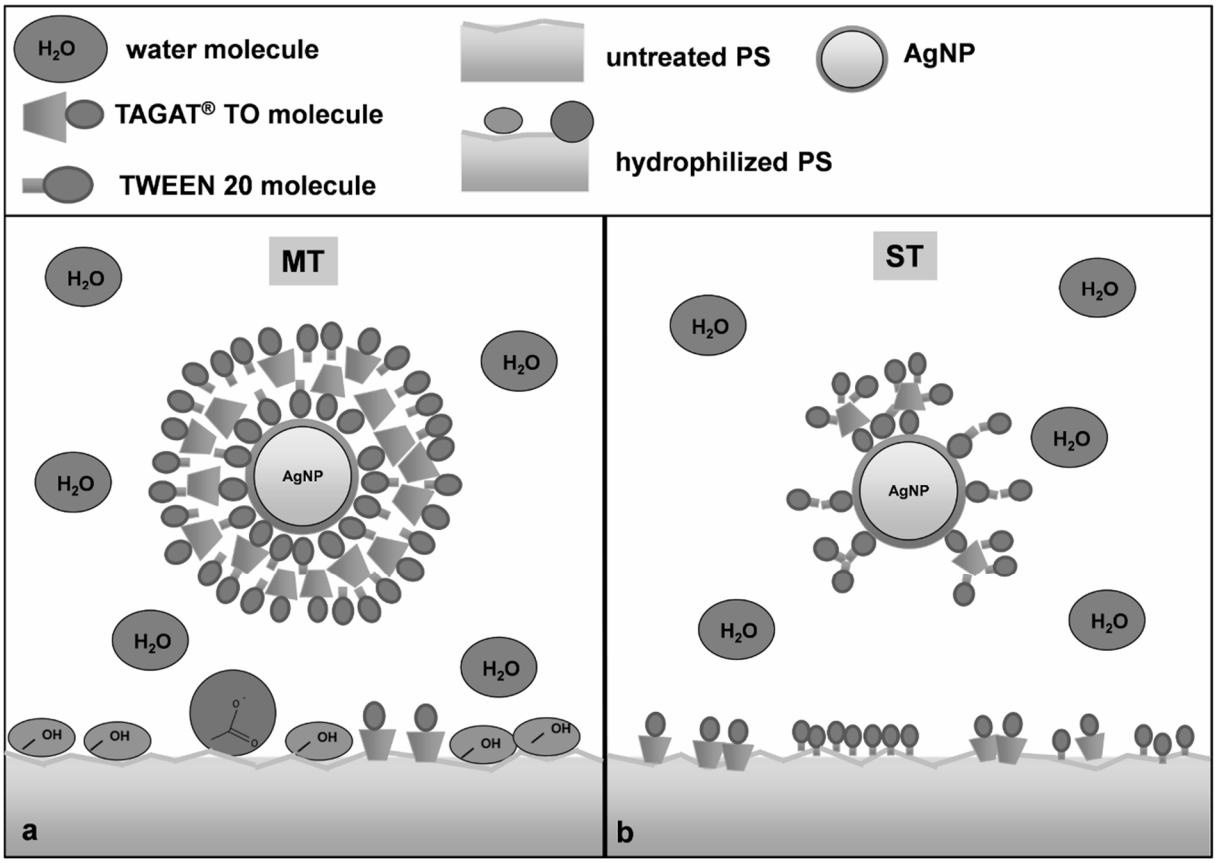

Figure 6.5 Suggested detAgNP stabilizer structure in the two different test designs MT and ST after interactions between water molecules, stabilizer molecules and the test vessel surface in each test design. Symbols are explained in the legend above. **a)** In MT, water molecules can interact with the lipophobic outer layer of the stabilizers around the AgNP core, but as energetically favoured binding sites at the test vessel surface are few, the stabilizing layer remains intact, and no interaction between water molecules and the detAgNP core takes place. **b)** In ST, the water molecules can replace stabilizers in the layer, as binding sites for both stabilizers at the hydrophobic surface of the test vessel are abundant. This attachment may be even favored due to the high hydrophobicity of TAGAT® TO and due to monolayer formation in case of TWEEN 20. This process will in turn reduce the stabilizing layer around the detAgNP core, reducing the colloidal stability of the detAgNP

A possible explanation for the observed fate based on the interaction between test vessel surfaces and the stabilizers would then be the following: the interaction between the untreated PS surface and both stabilizers is favored in comparison to the coverage of the detAgNP particle surface. The attachment of the stabilizers to the test vessel surface reduces the stabilizing shell around the detAgNP and thus increases the attachment of destabilized detAgNP to the PS in the ST design (Figure 6.5). In case of a hydrophilized PS surface, attachment of detAgNP also occurs, but to a lower extent, due to electrostatical repulsion and higher stability of the stabilizing shell. This theoretical model is supported by the stronger

agglomeration in the ST design as indicated by the higher z-average HDD in this design compared to MT.
Modifications of the coating due to interactions with a surface have not been investigated to our knowledge, but cysteine was able to replace PVP as coating agent on AgNP (Gondikas et al. 2012). The effect of hydrophobicity on AgNP attachment has also been suggested as explanation for multiwell plates used for algae tests and bPEI coated AgNP (Malysheva et al. 2016). In this case, however, hydrophobicity was not important for citrate coated AgNP, indicating the high importance of stabilizers for attachment of AgNP on a given surface. This is further supported by the result that attachment to glass beads not only depends on the hydrophobicity of the glass bead coating, but also on the coating of the AgNP (Song et al., 2011) and by studies aiming to produce NP monolayers where both, the coating and the surface properties affect NP deposition (Bright et al. 1998; Kleimann et al. 2006; Yang et al. 2007; Flores et al. 2010; Michna et al. 2011; Topuz et al. 2014b). Bandyopadhyay et al. (1997) demonstrated that even the orientation of the linker molecule at the surface may alter AgNP deposition.
However, also the surface or exudates of the *Daphnia* need to be taken into consideration for explaining AgNP behavior. Excreted proteins were shown to attach to the AgNP surface on top of the original covering, thus altering their colloidal stability (Nasser and Lynch, 2016). Still, the density of animals was identical in both test designs, so that any attachment, uptake or excretion should have been comparable in both designs. As discussed above, swimming behavior most likely reduced sedimentation in the ST design. It is possible that it also affected the number of collisions between AgNPs and the surface. However, the test vessel's surface area per animal in the ST design was smaller than in the MT design, (4.23 cm^2 vs. 7.01 cm^2, respectively), so that the movement should cause less collisions in the ST test vessel. Still, more investigations on effects of differences in neonate numbers and AgNP behavior are needed to identify relations between sedimentation and swimming behavior, as well as excreted biomolecules and colloidal stability.
Whether electrostatic repulsion or stabilizer-surface interactions drove the fate of detAgNP in MT and ST cannot be distinguished in the present study. Experiments using AgNP stabilized by either TWEEN 20 or TAGAT® TO only and systematic variation of surface charges and hydrophobicity are required to identify the underlying mechanisms. However, the results illustrate how much information about AgNP behavior can be gathered from simple ecotoxicological tests.
In the environment, long-term transformations of the NP core material may alter fate and behavior to a large extent as well, as has been shown for PVP coated AgNP in an artificial wetland (Lowry et al. 2012). A combined assessment of coating and core material interactions with the environment on long and short-term time scales is consequently needed to evaluate bioavailability of AgNPs or potential sources and sinks in the environment.
The differences in sorption behavior between ST and MT also demonstrate that the vessel's material properties have to be taken into account in detail. Consequently, surface-coating interactions need to be included in the investigation of AgNP fate and stability alongside with pH, ion composition or biomolecules.

Dissolved silver concentrations and AgNP toxicity

Significantly higher Ag_i concentrations were measured in the 40 µg Ag L^{-1} than in the 16 µg Ag L^{-1} (glm, $p<0.001$). Day and test design did not influence the measured concentration (Figure 6). Between 2% and 3% of the nominal concentration could be found as Ag_i at both nominal detAgNP concentrations (SI, Table S6.4).

Ag_i levels are in line with the results of other studies where release of silver ions was included (Fabrega et al. 2009b; Jin et al. 2010; Silva et al. 2014), but some variation can be found when the coating is considered (Sekine et al. 2015). For TWEEN 80 stabilized AgNPs, ion release was not observed in two studies (Kvitek et al. 2008; Soukupová et al. 2008), but a constant 3% level of silver ion concentration was reached within the first 6 h in a 15 day experiment (Li and Lenhart 2012). Due to the presence of TWEEN 20 and stable ion concentrations during the test, it is likely that detAgNP behaved similar to TWEEN 80 stabilized AgNP and that dissolution already occurred in the stock dispersion.
Ag_i can be expected to behave in the same fashion as $AgNO_3$ (see also section 3.2). It is thus likely that at least some part of the silver measured as Ag_s also includes ionic silver, as our sampling with acid does not differentiate between these kinds of silver. Even though the influence of Ag_i on Ag_s can be expected to be small due to its low concentrations in the present work, a higher amount of Ag_i would most likely reduce the differences in Ag_s between the two test designs that are caused by the differences in AgNP adsorption as suggested by the processes presented in Figure 6.5.

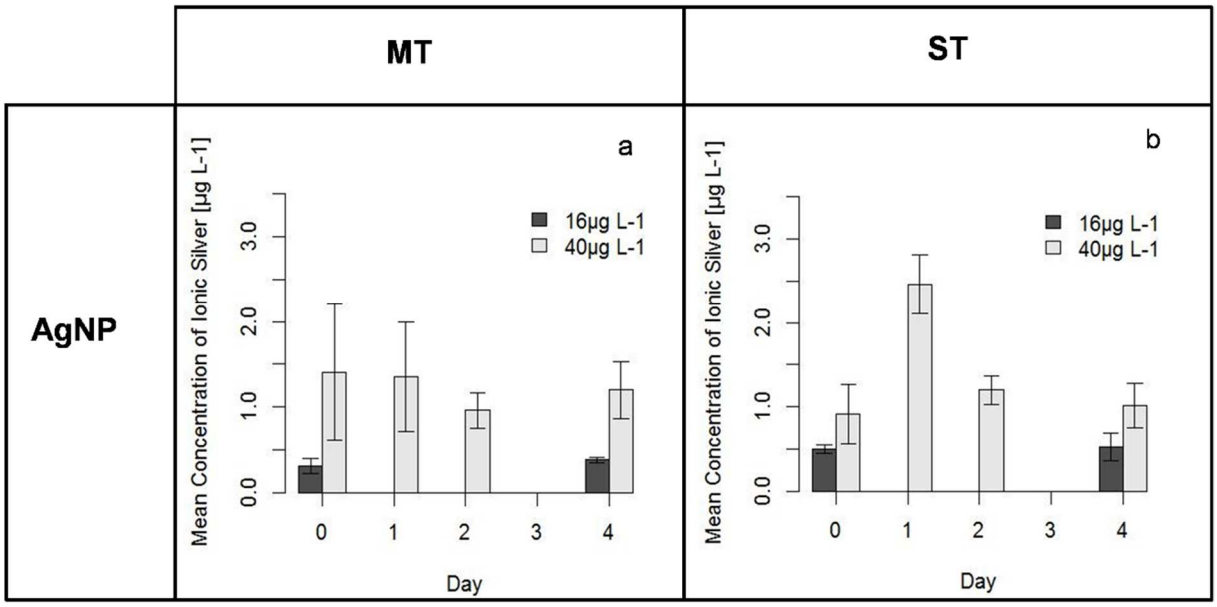

Figure 6.6 Dissolved silver concentration (Ag_i) during the acute test in the two different test designs used (mean ± se; n=4 in 40 µg Ag L^{-1} and in 16 µg Ag L^{-1} on day 0; n=3 in 16 µg Ag L^{-1} on day 4). a) miniaturized test design (MT); b) standard test design (ST). Light grey bars show results for 40 µg Ag L^{-1}; dark grey bars show results for 16 µg Ag L^{-1}.

As most *Daphnia* were alive at the time of sampling (SI, Table S6.5), they may have influenced the dissolution of detAgNP during the test. However, Ag_i values did not differ with time, so a clear direction, increase or decrease of dissolution, of such influence is not detectable. It is still possible that both, detAgNP and Ag_i, were taken up by or adsorbed to the surface of the *Daphnia*. Uptake and sorption of citrate coated AgNP are reported to be about 60 µg/g dry weight at 40 µg L^{-1} with about 50% soft tissue and exoskeleton silver each for adult *D. magna* (Zhao and Wang 2010). As the recovery of detAgNP in the present study was below 100% (SI, Table S6.1), some fraction of silver may have been lost to the test organisms. As we expected the values to be below the quantification limit of the AAS (1 µg Ag L^{-1}), especially in the MT design, the degree of this loss was not determined here. In another mass-balance experiment

with neonate *Daphnia* and the same AgNP as in this study, 15-30 ng Ag were measured per *Daphnia* after 72 h at 30 µg Ag L^{-1} (Sakka et al., 2016). However, animals were fed during this mass-balance experiment and other results show that dietborne uptake is much higher than waterborne uptake (Zhao and Wang 2011). Even though it is likely that some silver as AgNP or Ag_i was lost from the test system by uptake of and attachment to *Daphnia*, an estimation is currently not possible. Losses in a similar range were also observed in all $AgNO_3$ treatments where animals were not added to the test vessels. Consequently, it is more likely that sedimentation of silver chloride in case of $AgNO_3$ or AgNP agglomerates are responsible for the increase of silver lost from the test system, as discussed for the particle characterization. Other studies investigating the attachment of AgNP to test vessel surfaces used radiotracing to quantify silver (Sekine et al. 2015; Malysheva et al. 2016), which seems to be a more reliable method. However, when fate of AgNP and toxicity shall be related, the same AgNP needs to be used in both studies, so that radiolabelling is more suited for systematic investigations of AgNP attachment.

The acute toxicity of detAgNP was similar to Baumann et al. (2014), with EC_{50} values between 20 and 50 µg Ag L^{-1} compared to 25 µg Ag L^{-1} (Baumann et al., 2014) for ST and between 0 and 20 µg Ag L-1 in comparison to 16 µg Ag L^{-1} (Baumann et al., 2014) in MT, revealing again higher mortality in MT than in ST. This can be explained by the difference in Ag_m concentrations (Figure 6.2c, d) rather than by the presence of ionic silver. This is also supported by the increasing difference in EC_{50} values with increasing test duration observed by Baumann et al. (2014), as differences in Ag_m in the present study became more pronounced during the experiment. Toxicity from AgNP rather than Ag^+ has also been reported elsewhere (Choi et al. 2009; Fabrega et al. 2009b; Ribeiro et al. 2014; Silva et al. 2014).However, Baumann et al. (2014) found slightly lower Ag_m concentrations in MT than in ST. As this difference increased with decreasing concentration, it is likely that this was caused by higher dilution of the samples during AAS preparation in their study and a resulting higher uncertainty of measurements, especially at lower concentrations (use of 1:10 instead of 1:5 in the present study). The difference between the present study and Baumann et al. (2014), as well as the above discussed losses of silver emphasize the importance of highly sensitive analytics for silver and AgNP. In addition, the results illustrate the need for ongoing analytics throughout the test.

4. Conclusions

The presented data underline the importance of analyzing the exposure concentrations throughout the test with suited methods, and they also revealed the importance of hydrophobicity in determining the reactions between the test vessel surface and the stabilizer of AgNP: The higher hydrophobicity of the untreated PS surface reduced the stabilizing coverage of the AgNP and thus decreased the available AgNP concentration in the test medium, finally resulting in lower toxicity to *Daphnia magna*. The high influence of the properties of the test vessel's surface emphasize the importance of taking all elements of a test system, especially all surfaces as well as stabilizers or dispersants, into consideration for interpreting nano-ecotoxicological tests. In addition to being a good example for influencing factors on AgNP fate, interactions between the test vessel surface and the used stabilizers can help to interpret interactions with biological surfaces: The high influence of lipophobic-hydrophobic interactions is likely to influence the bioavailability and attachment of AgNP for and to organisms. To evaluate these effects, systematic research on detergent coated and stabilized NPs and differently hydrophobic surfaces are required.

Acknowledgements

Silver nanoparticles (OECD material NM-300K) were provided by the UMSICHT project granted by the German Ministry for Education and Research (BMBF 03X0091). The authors thank Petra Witte from the working group of historical geology and palaeontology of the university Bremen for making the SEM pictures of the prepared well-slides.

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Supporting Information

Table of contents of the supporting information

S6.1: Protocol of Sample Taking and Sample Preparation for Silver Analysis

1. sampling of 100 µL in the middle of the test vessel for Ag_m
2. <u>for AgNP treatments only:</u>
   sampling of 500 µL in the middle of the test vessel with subsequent ultrafiltration (13,600 g for 30 minutes, ultracentrifugation cups: Vivaspin 500, MWCO 3000PES membrane, Sartorius Stedim Biotech GmbH, Göttingen, Germany; centrifuge: Mini Spin Plus, Eppendorf AG, Hamburg, Germany) and subsampling of

   100 µL of the filtrate for Ag_i
3. emptying of the test vessel and refilling with the same volume of diluted aqua regia (1 L prepared with double distilled H_2O, 440 mL conc. HCl., and 110 mL conc. HNO_3), sampling of 100 µL in the middle of the test vessel after 5 min for Ag_s.
4. addition of 10 µL 1% HNO_3 to all Ag_m and Ag_i samples to hinder adsorption during storage
5. storage in the dark until sample preparation for measurements
6. addition of 80 µL HCl and 20 µL HNO_3 to each sample
7. mixing for 5 s
8. heating overnight at 56 °C (Thermomixer compact, Eppendorf AG, Hamburg, Germany)
9. resuspension in diluted aqua regia: 300 µL for 40 or 50 µg Ag L^{-1} Ag_m and Ag_s samples; 200 µL for all 16 or 20 µg Ag L^{-1} and all Ag_i samples
10. mixing for 30 s
11. centrifugation (10,000 rpm for 5 min; Mini Spin Plus, Eppendorf AG, Hamburg, Germany)
12. use of the supernatant (250 or 150 µL for 300 and 200 µL resupsensions, respectively) for GF-AAS measurements

Nitric acid, HNO_3, (puriss p.a., ≥ 99.0, 65 %) was obtained from Sigma-Aldrich (Munich, Germany) and hydrochloric acid, HCL, (p.a., 37 %) from VWR (Darmstadt, Germany).

Table S6.1: Mean amount of silver for Ag_m and Ag_s with standard errors (n=4) for each test day and both substances in ST and MT.

Test design	Sub-stance	Nominal concen-tration	Day	Mean amount of silver [µg]	
				Ag_m	Ag_s
ST	$AgNO_3$	20 µg Ag L^{-1}	0	0.191 +/- 0.016	0.019 +/- 0.015
			1	0.132 +/- 0.014	0.040 +/- 0.009
			2	0.085 +/- 0.021	0.077 +/- 0.018
			3	0.046 +/- 0.019	0.067 +/- 0.028
			4	0.044 +/- 0.007	0.123 +/- 0.028
		50 µg Ag L^{-1}	0	0.462 +/- 0.003	0.024 +/- 0.001
			1	0.393 +/- 0.010	0.084 +/- 0.012
			2	0.329 +/- 0.023	0.111 +/- 0.017
			3	0.228 +/- 0.058	0.117 +/- 0.025
			4	0.201 +/- 0.028	0.275 +/- 0.064
	AgNP	20 µg Ag L^{-1}	0	0.119 +/- 0.004	0.017 +/- 0.002
			1	0.040 +/- 0.002	0.069 +/- 0.005
			2	0.027 +/- 0.002	0.082 +/- 0.004
			3	0.0160 +/- 0.0003	0.033 +/- 0.004
			4	0.025 +/- 0.002	0.086 +/- 0.002
		50 µg Ag L^{-1}	0	0.267 +/- 0.010	0.044 +/- 0.002
			1	0.117 +/- 0.011	0.171 +/- 0.005
			2	0.067 +/- 0.006	0.214 +/- 0.012
			3	0.056 +/- 0.009	0.226 +/- 0.035
			4	0.048 +/- 0.004	0.218 +/- 0.007

Table S6.1 continued:

Test design	Sub-stance	Nominal concen-tration	Day	Mean amount of silver [µg]	
				Ag_m	Ag_s
MT	$AgNO_3$	20 µg Ag L^{-1}	0	0.0396 +/- 0.0004	0.0010 +/- 0.0001
			1	0.0194 +/- 0.0046	0.0052 +/- 0.0010
			2	0.0137 +/- 0.0018	0.0068 +/- 0.0012
			3	0.0038 +/- 0.0005	0.0060 +/- 0.0012
			4	0.0066 +/- 0.0009	0.0132 +/- 0.0019
		50 µg Ag L^{-1}	0	0.0869 +/- 0.0033	0.0030 +/- 0.0003
			1	0.0780 +/- 0.0045	0.0103 +/- 0.0025
			2	0.0581 +/- 0.0120	0.0167 +/- 0.0043
			3	0.0431 +/- 0.0055	0.0476 +/- 0.0119
			4	0.0334 +/- 0.0028	0.0306 +/- 0.0043
MT	AgNP	20 µg Ag L^{-1}	0	0.0239 +/- 0.0006	0.0023 +/- 0.0003
			1	0.0122 +/- 0.0005	0.0074 +/- 0.0012
			2	0.0097 +/- 0.0005	0.0084 +/- 0.0015
			3	0.0060 +/- 0.0005	0.0027 +/- 0.0015
			4	0.0095 +/- 0.0015	0.0161 +/- 0.0019
		50 µg Ag L^{-1}	0	0.0569 +/- 0.0007	0.0068 +/- 0.0009
			1	0.0296 +/- 0.0037	0.0206 +/- 0.0025
			2	0.0218 +/- 0.0020	0.0221 +/- 0.0015
			3	0.0224 +/- 0.0014	0.0211 +/- 0.0016
			4	0.0246+/- 0.0037	0.0315+/-0.0040

Table S6.2: Mean percentages of silver for Ag_m and Ag_s with standard errors (n=4) for each test day and both substances in ST and MT. Percentages are given as percent of nominal concentrations.

Test design	Sub-stance	Nominal concen-tration	Day	Percentage distribution between compartments		
				Ag_m	Ag_s	sum
ST	$AgNO_3$	20 µg Ag L^{-1}	0	95.5 +/- 8.0	9.5 +/- 7.5	105 +/- 15.5
			1	66.0 +/- 7.0	20.0 +/- 4.5	86.0 +/- 11.5
			2	42.5 +/- 10.5	35.0 +/- 9.0	77.5 +/- 19.5
			3	23.0 +/- 9.5	33.5 +/- 14.0	56.5 +/- 23.5
			4	22.0 +/- 3.5	61.5 +/- 14.0	83.5 +/- 17.5
		50 µg Ag L^{-1}	0	92.4 +/- 0.6	4.8 +/- 0.2	97.2 +/- 0.8
			1	78.6 +/- 2.0	16.8 +/- 2.4	95.4 +/- 4.4
			2	65.8 +/- 4.6	22.2 +/- 3.4	88.0 +/- 8.0
			3	45.6 +/- 11.6	23.4 +/- 5.0	69.0 +/- 16.6
			4	40.2 +/- 5.6	55 +/- 12.8	95.2 +/- 18.4
	AgNP	20 µg Ag L^{-1}	0	75.3 +/- 2.5	10.8 1.25	86.1 +/- 3.75
			1	25.3 +/- 1.25	43.7 +/- 3.1	69.0 +/- 4.4
			2	17.1 +/- 1.25	51.9 +/- 2.5	69.0 +/- 3.75
			3	10.1 +/- 1.9	20.9 +/- 2.5	31.0 +/- 4.4
			4	15.8 +/- 1.25	54.4 +/- 1.25	70.2 +/- 2.5
		50 µg Ag L^{-1}	0	67.4 +/- 2.5	11.1 +/- 0.5	68.5 +/- 3.0
			1	29.5 +/- 2.75	43.2 +/- 1.25	72.7 +/- 4.0
			2	16.9 +/- 1.5	54.0 +/- 3.0	70.9 +/- 4.5
			3	14.1 +/- 2.25	57.1 +/- 8.75	71.2 +/- 11.0
			4	12.1 +/- 1.0	55.1 +/- 1.75	67.2 +/- 2.75
MT	$AgNO_3$	20 µg Ag L^{-1}	0	99 +/- 1.0	2.5 +/- 0.25	101.5 +/- 1.25
			1	48.5 +/- 11.5	13 +/- 2.5	61.5 +/- 14.0
			2	34.25 +/- 4.5	17 +/- 3.0	51.25 +/- 7.5
			3	9.5 +/- 1.25	15 +/- 3.0	24.5 +/- 4.25
			4	16.5 +/- 2.25	33 +/- 4.75	49.5 +/- 7.0
		50 µg Ag L^{-1}	0	86.9 +/- 3.3	3 +/- 0.3	89.9 +/- 3.6
			1	78 +/- 4.5	10.3 +/- 2.5	88.3 +/- 7.0
			2	58.1 +/- 12	16.7 +/- 4.3	74.8 +/- 16.3
			3	43.1 +/- 5.5	47.6 +/- 11.9	90.7 +/- 17.4
			4	33.4 +/- 2.8	30.6 +/- 4.3	64.0 +/- 7.1

Table S6.2 continued:

Test design	Sub-stance	Nominal concen-tration	Day	Percentage distribution between compartments		
				Ag_m	Ag_s	sum
MT	AgNP	20 µg Ag L^{-1}	0	75.4 +/- 1.9	7.3 +/- 0.9	82.7 +/- 2.8
			1	38.5 +/- 1.6	23.3 +/- 3.75	61.8 +/- 5.4
			2	30.6 +/- 1.6	26.5 +/- 4.7	57.1 +/- 6.3
			3	18.9 +/- 1.6	8.5 +/- 4.7	27.4 +/- 6.3
			4	30.0 +/- 4.7	50.8 +/- 5.9	80.8 +/- 10.6
		50 µg Ag L^{-1}	0	71.9 +/- 0.9	8.6 +/- 1.1	80.5 +/- 2.0
			1	37.4 +/- 4.7	26.0 +/- 3.2	63.4 +/- 7.9
			2	27.6 +/- 2.5	27.9 +/- 1.9	55.5 +/- 4.4
			3	28.3 +/- 1.8	26.7 +/- 2.0	55.0 +/- 3.8
			4	31.1 +/- 4.7	39.8 +/- 5.1	70.9 +/- 9.8

Table S6.3: Test statistics of daily comparisons between the available silver concentrations, Ag_m, in the two test designs (MT and ST). All remaining factors and their corresponding p-values of the minimal adequate model were given. Minimal adequate models were obtained by stepwise reduction.

Substance	Concentration	Day	Factors	p-value
$AgNO_3$	20	0	Test design	0.664
		1	Test design	0.249
		2	Test design	0.48180
		3	Test design	0.291
		4	Test design	0.278781
	50	0	Test design	0.05714
		1	Test design	0.6857
		2	Test design	1
		3	Test design	0.8857
		4	Test design	0.4857
AgNP	20	0	Test design	0.96
		1	Test design	0.00978 *
		2	Test design	0.000842 *
		3	Test design	0.00125 *
		4	Test design	0.0398 *
	50	0	Test design	0.3429
		1	Test design	0.3429
		2	Test design	0.02857 *
		3	Test design	0.02857 *
		4	Test design	0.02857 *

Table S6.4: Test statistics of daily comparisons between the amounts of adsorbed silver per area, Ag_S, in each test vessel in the two test designs (MT and ST). All remaining factors and their corresponding p-values of the minimal adequate model were given. Minimal adequate models were obtained by stepwise reduction. Significant influences are marked with an asterisk.

Substance	Day	Factor	p-value
AgNO3	0	Test design	0.05834
		Nominal Concentration	0.0116 *
	1	Test design	0.000669 *
		Nominal Concentration	0.004333 *
	2	Test design	0.000977 *
		Nominal Concentration	0.036311 *
	3	Test design	0.018781 *
		Nominal Concentration	0.000295 *
		Test design: Nominal Concentration	0.045557 *
	4	Test design	9.73e-05 *
		Nominal Concentration	0.000841 *
AgNP	0	Test design	3.65e-06 *
		Nominal Concentration	5.27e-07 *
	1	Test design	1.73e-07 *
		Nominal Concentration	6.76e-07 *
	2	Test design	2.38e-08 *
		Nominal Concentration	2.33e-07 *
	3	Test design	1.41e-07 *
		Nominal Concentration	9.20e-10 *
	4	Test design	5.21e-06 *
		Nominal Concentration	2.10e-06 *

Table S6.5: Relative concentration of dissolved silver in relation to test design and nominal concentration. Dissolved silver given as percent of nominal concentration as mean value +/- se; n=4 for 50 µg Ag L^{-1} and day 0 20 µg Ag L^{-1}; n=3 for day 4 20 µg Ag L^{-1} in MT and n=2 for day 4 20 µg Ag L^{-1} in ST.

Nominal Concentration	Day	Relative concentration of released ionic silver [%] during the test	
		MT	ST
20 µg Ag L^{-1}	0	0.020 +/- 0.006	0.032 +/- 0.003
	1	NA	NA
	2	NA	NA
	4	0.024 +/- 0.002	0.033 +/- 0.011
50 µg Ag L^{-1}	0	0.036 +/- 0.020	0.023 +/- 0.009
	1	0.034 +/- 0.016	0.062 +/- 0.009
	2	0.024 +/- 0.005	0.030 +/- 0.004
	4	0.030 +/- 0.008	0.026 +/- 0.007

Table S6.6: Mortality of *Daphnia* in test wells of 50 µg Ag L^{-1} AgNP treatments when silver samples were taken. No mortality occurred in 20 µg Ag L^{-1} test wells of AgNP treatments.
1 animal was added in MT, 5 animals in ST, in the beginning of the experiment (day 0). Dead test organisms are noted as bold.

Test Design	Day	Replicate	Status of Test Organisms
MT	1	A	alive
		B	alive
		C	alive
		D	alive
	2	A	alive
		B	alive
		C	alive
		D	alive
	3	A	alive
		B	alive
		C	alive
		D	**dead**
	4	A	alive
		B	alive
		C	**dead**
		D	**dead**
ST	1	A	all alive
		B	all alive
		C	all alive
		D	all alive
	2	A	all alive
		B	all alive
		C	all alive
		D	all alive
	3	A	all alive
		B	**1 dead**, 4 alive
		C	all alive
		D	all alive
	4	A	all alive
		B	all alive
		C	all alive
		D	all alive

Table S6.7: Cumulative hydrodynamic diameter with corresponding polydispersity index, as well as NNLS-main peak diameter and adsorption peak wavelength of detAgNP in the test stock dispersion under test conditions in the two test designs. All values are given as mean ± se (n = 30 for size measurements, n = 3 for adsorption wavelength).

		Cumulative method		**NNLS method**	
Time	**Design**	**z-average [nm]**	**Polydispersity Index (PDI)**	**Main Peak Diameter [nm]**	**Absorption Peak Wavelength [nm]**
2h	**MT**	297 ± 33	0.213 ± 0.013	64.6 ± 2.9	411.7 ± 0.3
	ST	307 ± 30	0.196 ± 0.009	59.4 ± 2.6	411 ± 0
24h	**MT**	151 ± 21*	0.218 ± 0.016	49.0 ± 1.1	410.7 ± 0.3
	ST	445 ± 175*	0.196 ± 0.011	50.3 ± 1.4	411 ± 0
96	**MT**	46.5 ± 1*	0.353 ± 0.005	53.6 ± 4.5	409.7 ± 0.3
	ST	111 ± 25*	0.332 ± 0.016	60.7 ± 6.3	410 ± 0

* No overlapp of mean values, if standard errors are considered.

7 Behavior and chronic toxicity of two differently stabilized silver nanoparticles to Daphnia magna

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Published in: Aquatic Toxicology, 117, p. 526-535, 2016
doi: 10.1016/j.aquatox.2016.06.025

Contributions of Yvonne Sakka:

- Performance of all experiments
- Performance of statistical data analysis
- Preparation of all figures and tables
- Writing and final improvement of the manuscript

Lars M. Skjolding and Aiga Mackevica supported the development of the experimental design, the practical work at DTU and the finalization oft he manuscript. Juliane Filser initialised this work and revised the manuscript. Anders Baun supervised the work at DTU and revised the manuscript.

Behavior and chronic toxicity of two differently stabilized silver nanoparticles to *Daphnia magna*

Abstract
While differences in silver nanoparticle (AgNP) colloidal stability, surface potential, or acute aquatic toxicity for differently stabilized AgNP have often been reported, these have rarely been studied in long-term ecotoxicity tests. In the current study, we investigated the chronic toxicity of AgNP to *Daphnia magna* over a 21-day period with two different stabilizers (citrate and detergent), representative for charge and sterical stabilizers, respectively. This was coupled with a series of short-term experiments, such as mass balance and uptake/depuration testing, to investigate the behavior of both types of AgNP during a typical media exchange period in the *D. magna* test for chronic toxicity. As expected, the sterically stabilized AgNP were more stable in the test medium, also in the presence of food; however, a higher uptake of silver after 24 h exposure of the charge stabilized AgNP was found compared to the detergent stabilized AgNP (0.046 ± 0.006 µg Ag µg DW^{-1} and 0.023 ± 0.005 µg Ag µg DW^{-1}, respectively). In accordance with this, the higher reproductive effects and mortality was found for the charge stabilized than for the sterically stabilized silver nanoparticles in 21-d tests for chronic toxicity. (LOEC was 19.2 µg Ag L^{-1} for both endpoints for citrate coated AgNP and >27.5 µg Ag L^{-1} (highest tested concentration for detergent stabilized AgNP). This indicates a link between uptake and toxicity. The inclusion of additional short-term experiments on uptake and depuration is recommended when longer-term chronic experiments with nanoparticles are conducted.

Keywords
silver nanoparticle, D*. magna*, chronic toxicity, stabilizer effect, uptake

1. Introduction

Silver nanoparticles (AgNP) are currently the nanoparticles (NP) used in the highest number of consumer products (The Nanodatabase, 2016), with a range of different applications mainly due to their antimicrobial action (Vance et al., 2015; Wigger et al., 2015). This widespread use in combination with the inherent toxic potential has raised concern about their human health and environmental effects (Hartemann et al., 2015) which has in turn resulted in a large number of experimental studies and reviews on the toxicity of AgNP (Fabrega et al., 2011; Hansen and Baun, 2012; Kim et al., 2013; Lapresta-Fernández et al., 2012; Levard et al., 2012; Sharma et al., 2014).

A number of studies have focused on the relation between AgNP aquatic toxicity and AgNP properties, especially on the effect of colloidal stability of the used AgNP, and the identity of the NP stabilizer has been shown to be a highly influential factor on colloidal stability (Kvitek et al., 2008; Silva et al., 2014; Tejamaya et al., 2012). Sterically stabilized AgNP show a very broad spectrum of colloidal stability in aqueous media; ranging from very stable, like for PVP-stabilized, to relatively unstable, like PEG or TWEEN 80-stabilized AgNP, with the degree of instability varying with media composition and stabilizer identity (Levard et al., 2012; Sharma et al., 2014; Silva et al., 2014; Tejamaya et al., 2012). Charge stabilized AgNPs become less stable with increasing ionic strength of the test medium, especially in presence of divalent cations (Levard et al., 2012; Sharma et al., 2014; Silva et al., 2014; Tejamaya et al., 2012). The colloidal stability in the test medium has a strong impact on the behavior and fate of the tested AgNP in a given system (Sharma et al., 2014), directly affecting the exposure conditions of the test organism.

However, the relation between colloidal NP stability in aqueous media and toxicity is less clear. Higher toxicity of more stable AgNP has been found in several studies (Asghari et al., 2012; Kvitek et al., 2008; Morones et al., 2005). However, higher toxicity was observed for aggregated citrate coated AgNP than for non-aggregated PVP coated AgNP of similar primary particle size (Angel et al., 2013). An increase in size due to low colloidal stability in the test medium also caused higher uptake of AgNP by *D. magna* (Zhao and Wang, 2010), but this was found not to be the only factor causing the observed differences in toxicity in another study (Allen et al., 2010). Surface charge has often been reported as an important factor influencing the NP toxicity (El Badawy et al., 2010; Kim et al., 2013; Levard et al., 2012), and surface charge combined with colloidal stability in the test medium were sufficient to explain the observed toxicity to *E. coli* and *Daphnia magna* in case of PVP, bPEI and citrate coated AgNP (Silva et al., 2014).

No general trends were reported for other properties such as hydrophobicity of the stabilizers or presence/absence of certain reactive groups in the coatings (Kim et al., 2013; Levard et al., 2012). In addition, the stabilizer itself changed the toxicity as well as the mode of action of the investigated nanoparticles in other experimental studies (Baumann et al., 2014a; Bozich et al., 2014; Dominguez et al., 2015).

While the relation between AgNP properties and short-term aquatic toxicity has been the focus of several studies, chronic toxicity is somewhat overlooked. To our knowledge, only one study used more than one AgNP type in a chronic *Daphnia* test (Blinova et al., 2013). The investigation of longer-term exposure to low concentrations is of special interest, as predicted environmental concentrations of AgNP are in the range of ng L^{-1}, so much lower than the concentration levels used in chronic tests that are in the µg L^{-1} range (Batley et al., 2013; Fabrega et al., 2011; Gottschalk et al., 2013). Besides, most acute tests do not include feeding. For *Daphnia* tests feeding implies the addition of algae to the test system. Though algae are an important part of the environmental ecosystem, their presence in the test system may

influence the AgNP behavior and alter the exposure conditions in comparison to the acute setup. In addition, AgNP behavior and toxicity are strongly affected by the materials chosen for the given test system (Sekine et al., 2015). Consequently, the question of how stabilizers influence on chronic toxicity can hardly be answered by comparing different studies with differently stabilized AgNPs, but needs to be tested in the same test setup.
In the present study, we investigated whether chronic toxicity of two differently stabilized AgNP can be linked to their behavior in the test medium. In addition to measurements of colloidal stability in the test medium, the fate of both AgNP, citrate coated (citAgNP) and detergent stabilized (detAgNP), in the test system was analyzed using mass-balance analysis during a typical media exchange period to assess differences in AgNP behavior under test conditions in more detail. The relation between AgNP behavior in tests and toxicity was further analyzed by comparing uptake and depuration of both AgNP by *D. magna* on a short-time scale and by measuring silver body burden at the end of the chronic test. We expected citAgNP to be of lower colloidal stability in the ionic rich test medium, Elendt M7, resulting in lower silver concentrations in the aquatic phase, lower uptake and lower toxicity compared to detAgNP. Aside from this difference in toxic intensity, we expected the sensitivity of the different endpoints of the chronic test; such as reproduction, growth or mortality; to be similar between the two AgNPs.

2. Materials & Methods

Chemicals and Test Dispersions

The AgNP were citrate coated AgNP (citAgNP) from Cline Scientific AB (Gothenburg, Sweden) and sterically stabilized AgNP (detAgNP) from ras materials GmbH (Regensburg, Germany; reference material NM-300K). Both AgNP were of spherical shape and of similar initial size (20-30 nm). citAgNP were supplied as 20 mg Ag L^{-1} suspension and used directly to prepare test dispersions. detAgNP were delivered as 10.16 wt% Ag suspension and were diluted twice before use: first, to a suspension of 2 wt% Ag with Milli-Q water (Merck KGaA), second to a suspension of 10 mg Ag L^{-1} using Elendt M7 medium prepared according to OECD guideline No. 211 (Organisation for Economic Co-operation and Development, 1998) after sonication (15min, water bath, UR 1, Retsch) of the Milli-Q diluted suspension. This 10 mg Ag L^{-1} AgNP dispersion in Elendt M7 medium was used as a stock for all test dispersions with detAgNP without additional sonication. Both AgNP stock dispersions were stored at 4°C in the dark until use. All experiments were conducted using Elendt M7 medium as diluting agent for the AgNP dispersions and the pH was kept stable in the range of 7.9 and 8.3 (SensION+ pH3, Hach-Lange).

Test Organisms

Daphnia magna were originally collected in Birkedammen, Denmark, in 1978, and were cultured continuously afterwards in the laboratory of the Environmental Engineering Department of the Technical University of Denmark. Elendt M7 medium with a density of one individual per 100 mL at 20°C with 16:8 h light-dark cycle and daily feeding by an automatic pump with the green algae *Raphidocelis subcapitata* were used for culturing. Algae were cultured using aeration and permanent illumination until an approximate cell density of 10^6 cells mL^{-1}. For feeding, algae were concentrated at 4°C for two to three days by settlement and counted using a particle counter (Coulter Counter Z2, Beckmann Coulter). The carbon

content was calculated according to relationship provided in Hailing-Sørensen et al. (1996), where 10^4 cells mL^{-1} correspond to 0.1 mg C L^{-1}.

Particle Characterization

To estimate how both AgNPs behave during the chronic test, particles were characterized by measuring both particle size, as hydrodynamic diameter, and zeta potential in the test medium using a Zetasizer Nano ZS (Malvern Instruments Ltd.) with 173° backscattering angle at 25 °C with an equilibration time of 60 s and three repeats per sample. Samples were prepared to represent the conditions in the chronic test as closely as possible using the same volume of Elendt M7 medium, temperature and light intensity as in the chronic test. However, no food could be added to the samples, as the algae would have disturbed the particle measurements. In addition, the used silver concentration of 1 mg Ag L^{-1} was higher than what was used in all further experiments, but being at the lower limit of detection for particle characterization with the used device. Samples for size and surface potential measurements were taken at 0, 1, 2, 4, 6, 24 and 48 h in duplicates.

To visually compare the shape and the behavior of the two AgNP, Transmission Electron Microscopy (TEM) images of the stock dispersions were made using a Tecnai T20 G^2 (FEI). The samples were prepared by drying a 4 µL drop of each AgNP dispersion on a copper grid covered with carbon film (Agar Scientific).

Experiments

All experiments were conducted at 20 ± 0.2°C in a climate room with a 16:8 light-dark-cycle with 0.15 mg C L^{-1} d^{-1} $animal^{-1}$ of a *Raphidocelis subcapitata* culture resuspended in Elendt M7 as food source. For all experiments, < 24 h old neonates were used. To avoid spatial effects, all test vessels were rotated daily.

Samples for silver analysis were prepared using 65% nitric acid and stored in the dark at 4°C until measurement. Medium silver concentrations as well as silver in *D. magna* and at the beakers were measured using Inductively Coupled Plasma Mass Spectrometry (ICP-MS) (7700x Series ICP-MS, Agilent Technologies Inc.). Samples of ionic silver in the test medium were measured by graphite furnace atomic adsorption spectrometry (C GF-AAS; GF 90 and Solaar 989QZ, Unicam) after ultracentrifugation (Vivaspin 500, 3000kD filters, Sartorius Stedim Biotech GmbH).

Mass Balance Analysis

For mass balance analysis, a three-day media exchange period was used (72 h). Test conditions were set as described above (2.4). Two testing concentrations (10 and 30 µg Ag L^{-1}) were used for both AgNP types in triplicates. Apart from that, a control sample of the pure test medium and samples of all test dispersions at the start of the experiment were taken. Sampling was done after 24 and 72 h and included: 1) silver in the test medium, 2) ionic silver in the test medium, 3) silver in/on the test organisms, and, 4) silver attached to the test vessel surface. For each sampling, one beaker was chosen for samples 1 to 4 and 9 additional beakers were used for sample 3 to pass the detection limit of the ICP-MS. Medium samples (1) were taken from the middle of the beaker and prepared as described above (2.4). Ionic silver samples were taken from the same location and centrifuged at 10,000 g for 30 min (Multifuge X3R, Thermo Fisher Scientific) using ultracentrifugation cups (Vivaspin 500, Sartorius Stedim Biotech GmbH). A subsample from the filtrate was acidified with 1% HNO_3 and stored in the dark until measurement. Ten *D. magna* were collected with gauze, and digested using concentrated HNO_3 in the dark. Silver adsorbed to the beaker was sampled as follows: beakers were emptied, rinsed two times with concentrated HNO_3. A subsample of the washing solution was used for analysis and immediately diluted with Milli-Q water. All samples

for ICP-MS (1,3,4) were stored for 48 h in the dark before diluting all samples to a content of 1% HNO_3 with Milli-Q water for measurements.

Uptake and Depuration Experiment

For testing the silver uptake, neonates were exposed to a nominal concentration of 30 µg Ag L^{-1} for 24 h in triplicates. The test conditions were as described above (2.4), except the animal density which was 10 neonates in 100 mL. Sampling took place after 2, 4, 6, and 24 h during uptake and depuration. Before the start of the depuration, all remaining neonates were rinsed in Elendt M7 to remove silver loosely attached to their carapaces and to reduce transfer of AgNP to the clean medium. Samples of the test medium were taken during the uptake phase to be able to relate silver in the test organisms to silver present in the test dispersion. A control without silver was also included and sampled at the end of the experiment.

Chronic *Daphnia* Test

The chronic *Daphnia* test was conducted according to the OECD guideline No. 211 (Organisation for Economic Co-operation and Development, 1998). Feeding took place with every media exchange every two to three days. To overcome variance in exposure conditions due to differences in test dispersion preparation, a time-related protocol was used including all steps from test dispersion preparation to transfer of neonates. To minimize losses of AgNP from the test medium, two sets of the required number of beakers were pre-soaked with their corresponding test dispersion for 24 h. These beakers were used in turns throughout the test and cleaned manually after use to remove any loosely attached AgNP and surplus feeding algae. 5 nominal concentrations (5, 10, 15, 30, and 60 µg Ag L^{-1}) and a control were used for both AgNP. Mortality and reproduction were controlled daily; number of molts was counted with every media exchange. At the end of the test, animals were collected individually, and the size was measured using a camera (Go-5, Q-Imaging Inc.) connected stereomicroscope (M7_6, Leica Microsystems), and picture analysis software (Q Capture Pro. 6.0, version 6.0.0.605 by Media Cybernetics Inc. and Q-Imaging Inc.). Afterwards, animals were carefully dried, weighed and acid digested for ICP-MS analysis of their silver content (body burden). Analyzed endpoints were mortality, molting, size and silver content at the end of experiment, and reproduction (cumulative number of alive and aborted neonates, number of reproductions, mean number of alive neonates per reproduction, and onset of reproduction including the number of alive and aborted neonates). All neonates without movement within 15 s and all unhatched eggs were counted as aborted neonates in this study. One sample of the actual silver concentration in the test medium was taken in the beginning and the end of every of the 9 media exchanges. Due to the large number of medium samples, only the highest AgNP concentration of each AgNP was measured by ICP-MS. The actual concentrations for these two data sets were calculated as time weighted mean as described in the guideline (Annex 6, OECD No. 211,Organisation for Economic Co-operation and Development, 1998). All other concentrations were calculated on the basis of the proportional reduction of the time weighted mean from the nominal concentration at the highest silver concentration for each AgNP.

Data analysis

The measured endpoints were related to either the nominal concentration (mass-balance analysis), actual concentration (uptake and depuration experiment) or the time-weighted mean concentration (chronic test). In the mass balance analysis, results were corrected for background (control measurements), dilution, and origin in each compartment. In addition, resulting amounts were related to corresponding initial concentrations. A similar correction was

used in the uptake and depuration experiment (background, number of neonates per sample, dilution) and resulting amounts per *Daphnia* were also related to the corresponding media concentration during the uptake phase. Body burden from the chronic test were also related to the corresponding calculated concentrations (2.4.3, Table 3).
All data were analyzed using R, version 3.1.2, (R Foundation for Statistical Computing, 2015). Data were checked for homogeneity of variance using Levene's test and normality of errors (Shapiro-Wilk test). If both criteria were met, linear models (lm) or analysis of variance (ANOVA) were used with nanoparticle type, time and concentration as independent variables. Transformations were used to meet these criteria in two cases: square-root transformation for results of silver body burden in the mass balance analysis and log-transformation for silver body burden in the chronic test. If conditions for those models were not met after transformation, general linear models or non-parametric tests (Kruskal-Wallis test for more than two factor levels or Wilcoxon tests) with suitable subsets of the data were used. Some endpoints of the chronic Daphnia test did not show a linear relation to silver concentration, and were analyzed by general additive models. In this case, factors were added stepwise to the model to be able to separate effects from concentration and AgNP. ANOVAs were used to test whether significant improvement was achieved by increased complexity. For NOEC/LOEC determination in the chronic *Daphnia* test, datasets of each AgNP were analyzed separately by ANOVA or Kruskal-Wallis test with corresponding post-hoc analysis.
An overview on the statistical analysis is given in the Supporting Information (SI, section S7.2, Table S7.1-S7.4).

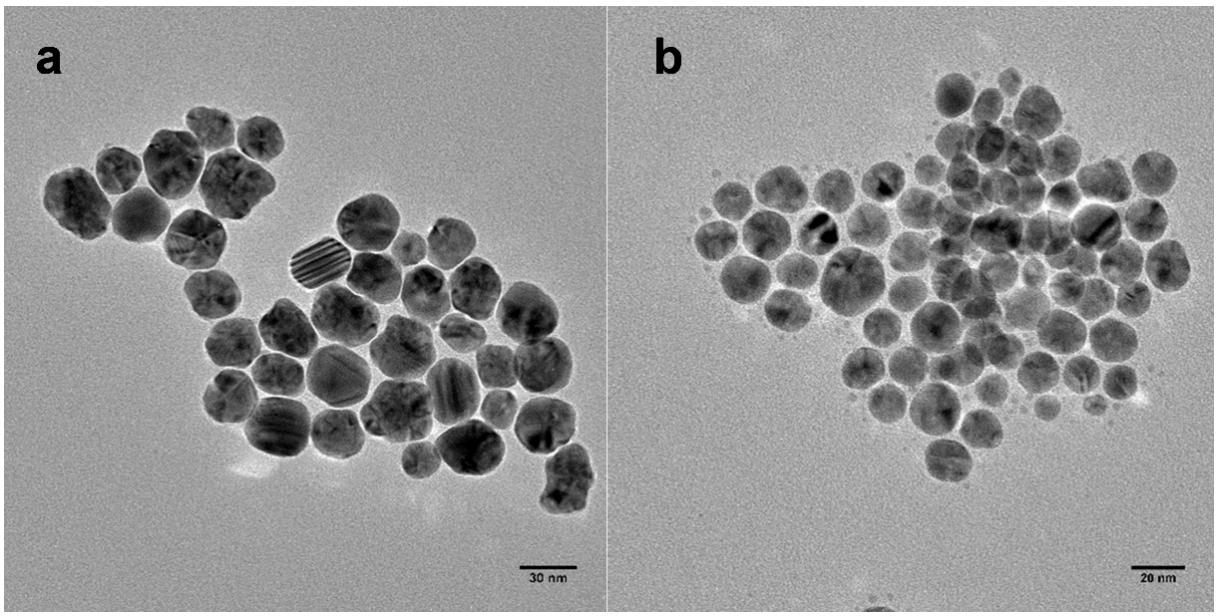

Figure 7.1 TEM image of citAgNP (a) and of detAgNP (b) in the corresponding stock dispersions.

3. Results

Particle Characterization

TEM analysis confirmed the spherical shape and similar core size for both AgNP stock dispersions (Figure 7.1). In the test medium, the hydrodynamic diameter (HDD) directly after

dispersion preparation was larger for citAgNP (143 ± 13 nm) than for detAgNP (96 ± 1 nm) (Table 7.1).
The HDD of citAgNP increased to 502 ± 311 nm during the 48 h of measurements, but results became increasingly uncertain, as the related polydispersity index increased to values around 1. The HDD of detAgNP was about 100 nm throughout, but some sedimented agglomerates were visible after about 24 h of exposure that were not included in the measurements (as indicated by the PDI in the stable range of 0.2 to 0.3). The surface potential was around -15 mV for citAgNP and -6 mV for detAgNP in all measurements whereas conductivity was similar for both AgNP types (Table 7.1).

Table 7.1 Hydrodynamic diameter as z-average, and corresponding polydispersity index; zeta-potential and conductivity at different times after mixing in OECD M7 medium. All values are given as mean values with standard errors (n=3).

Nanoparticle	Time	Hydro-dynamic Diameter [nm]	Polydispersity Index	Zeta-Potential [mV]	Conductivity [mV]
citAgNP	**0h**	143 ± 13	0.27 ± 0.01	NA	NA
	1h	200 ± 2	0.37 ± 0.004	-14.6 ± 0.03	0.732 ± 0
	2h	241 ± 9	0.45 ± 0.02	-15.2 ± 0.05	0.737 ± 0.001
	4h	277 ± 12	0.60 ± 0.06	-15.1 ± 0.39	0.719 ± 0.026
	6h	293 ± 11	0.70 ± 0.06	-15.7 ± 0.30	0.728 ± 0.009
	24h	345 ± 74	0.91 ± 0.09	-14.4 ± 0.18	0.709 ± 0.003
	48h	502 ± 311	0.81 ± 0.19	-14.4 ± 0.33	0.716 ± 0.014
detAgNP	**0h**	96 ± 1	0.25 ± 0.01	-5.98 ± 0.18	0.703 ± 0.004
	1h	98 ± 6	0.24 ± 0.02	-6.28 ± 0.01	0.766 ± 0.004
	2h	101 ± 2	0.23 ± 0.01	-6.18 ± 0.32	0.750 ± 0.020
	4h	101 ± 3	0.27 ± 0.03	-3.15 ± 1.71	0.505 ± 0.253
	6h	108 ± 5	0.26 ± 0.03	-6.36 ± 0.11	0.772 ± 0.006
	24h	104 ± 1	0.29 ± 0.01	-6.59 ± 0.08	0.748 ± 0.008
	48h	99 ± 3	0.28 ± 0.01	-6.43 ± 0.19	0.703 ± 0.039

NA: Not analyzed

Mass balance analysis

Ionic silver concentrations were below the detection limit of the AAS (1 µg Ag L^{-1}) in all samples. Total silver in the test medium, on the test vessel, and in the test organism were always above the detection limit of the ICP-MS (0.5 µg Ag L^{-1}).
At the beginning of the experiment, the amount of silver was higher for citAgNP (309 ± 9 ng Ag and 1123 ± 23 ng Ag) than for detAgNP (204 ± 3 ng Ag and 763 ± 7 ng Ag) at both nominal concentration levels, 10 and 30 µg Ag L^{-1}.

Table 7.2 Total and relative amounts of silver in the different compartments of the test system over time. Relative amounts are expressed in percent relative to the initially added amount of silver for the two different types of AgNP[1].

nominal concentration [µg Ag L^{-1}]	**AgNP**	**Time** [h]	**Compartment**	**Total amount of silver** [ng Ag]	**Relative amount of silver** [%]
10	citAgNP	24	Medium	159 ± 9 [a]	51.1 ± 2.9
			Beaker	148 ± 92	47.9 ± 29.9
			Daphnia	0.43 ± 0.06	0.138 ± 0.018
		72	Medium	46 ± 4	14.9 ± 1.1
			Beaker	116 ± 13 [b]	37.6 ± 4.2
			Daphnia	0.62 ± 0.09	0.199 ± 0.028
	detAgNP	24	Medium	101 ± 14 [a]	49.3 ± 6.6
			Beaker	29 ± 4	14.4 ± 2.0
			Daphnia	0.30 ± 0.07	0.144 ± 0.032
		72	Medium	67 ± 25	32.8 ± 12.0
			Beaker	69 ± 9 [b]	33.7 ± 4.3
			Daphnia	0.84 ± 0.14	0.408 ± 0.069
30	citAgNP	24	Medium	498 ± 53	44.4 ± 4.8 [a]
			Beaker	309 ± 19 [c]	27.5 ± 1.2 [b]
			Daphnia	1.00 ± 0.32	0.089 ± 0.039
		72	Medium	134 ± 4	11.9 ± 0.3
			Beaker	412 ± 8 [d]	36.7 ± 0.7 [c]
			Daphnia	3.49 ± 0.24 [e]	0.311 ± 0.021
	detAgNP	24	Medium	482 ± 24	63.2 ± 3.2 [a]
			Beaker	88 ± 5 [c]	11.5 ± 0.6 [b]
			Daphnia	0.49 ± 0.05	0.064 ± 0.006
		72	Medium	144 ± 33	18.9 ± 4.3
			Beaker	183 ± 13 [d]	24.0 ± 1.6 [c]
			Daphnia	2.08 ± 0.32 [e]	0.272 ± 0.042

[1]Total and relative amounts were corrected for background, dilution, and sampling procedure. The relative amounts were calculated in relation to the initially added amount of silver in the corresponding treatment. All values are given as mean values with standard errors (n=3). Letters (a-e) indicate significant differences between citAgNP and detAgNP in the corresponding compartments of the same sampling ($p<0.05$).

The amount of silver in the aqueous phase decreased significantly over time for both AgNP ($p=0.004$, Kruskal-Wallis test; Table 7.2, Figure S7.1). Medium amounts did not differ between the two types of AgNP, except for the direct comparison at 10 µg Ag L^{-1} after 24 h (Table 7.2), but when related to the initially added amount of silver, medium concentrations were significantly higher for detAgNP ($p=0.009$, lm) at the higher concentration (Figure S7.1). Correspondingly, losses to the beaker increased over time with different slopes for both concentrations (concentration:time, $p=0.005$, linear model) (Figure S7.1). At both concentrations, citAgNP had significantly higher silver residuals at the beaker than detAgNP (10 µg Ag L^{-1}: $p=0.006$, lm; 30 µg Ag L^{-1}: $p<0.001$, lm) that were also reflected in the direct comparisons (Table 7.2). When residuals at the beaker were expressed relative to initially added amounts of silver, AgNP did not affect the proportion on the beaker at the lower concentration (SI, Table S7.1), but were significantly higher for citAgNP at the higher concentration ($p<0.001$, lm) (Table 7.2, Figure S7.2, SI).
The silver content measured in *D. magna* increased with increasing exposure time with different slopes for the used concentrations (concentration:time, $p<0.001$, lm with square-root transformation) and were significantly higher for citAgNP at the higher concentration level ($p=0.008$, lm) (Figure S7.1) that was most pronounced after 72 h (Table 7.2). Expressed as proportion of initially added silver, uptake of detAgNP at the lower concentration was significantly higher with different uptake patterns over time (AgNP, $p=0.033$, AgNP:time, $p=0.041$, lm), but the differences were not significant in a direct comparison (Table 7.2)

Uptake and Depuration Experiment
The body burden of silver in *D. magna* increased during 24 h for both AgNP (Figure 7.2, Table S7.2). No difference in uptake between citAgNP and detAgNP was observed within 6 h of exposure, but after 24 h, the body burden of silver was significantly ($p=0.049$) higher for citAgNP (0.69 ± 0.09 ng Ag *D. magna*$^{-1}$/ 0.046 ± 0.006 ng Ag µg DW^{-1}) than for detAgNP (0.35 ± 0.08 ng Ag *D. magna*$^{-1}$/ 0.023 ± 0.005 µg Ag ng DW^{-1}). The same pattern was detected when body burden was expressed in relation to the actual medium concentration for each sampling time. After transfer into Elendt M7 without AgNP, the difference in body burden between the two types of AgNP was still detectable after 1 h of depuration (0.32 ± 0.06 ng Ag *D. magna*$^{-1}$ for citAgNP compared to 0.15 ± 0.15 ng Ag *D. magna*$^{-1}$ for detAgNP), but not significant anymore. Body burden of silver decreased rapidly within the first 6 h of depuration and reached silver body burden similar to control levels for both AgNP after 24 h (Figure 7.2).

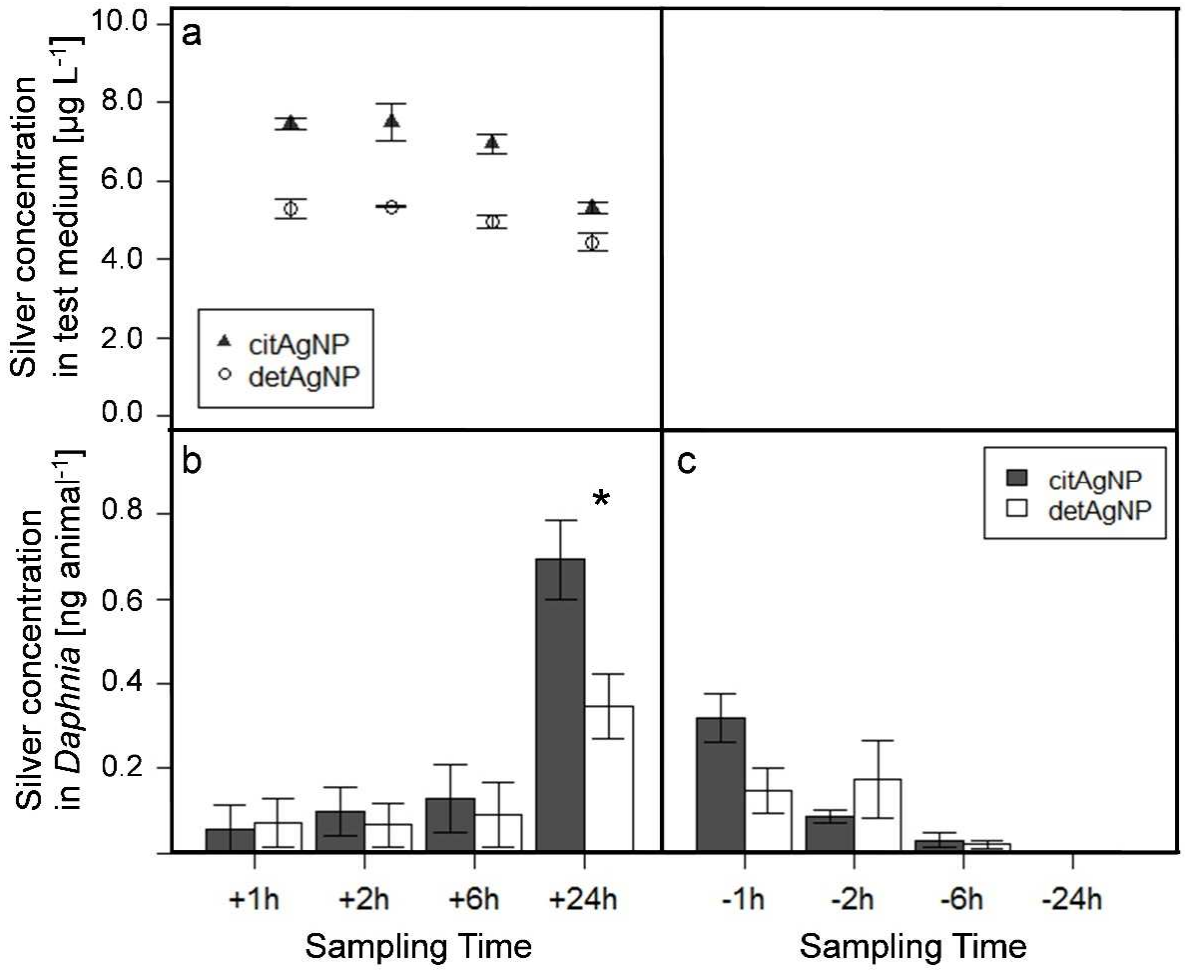

Figure 7.2 Actual silver concentration in the test dispersion during the uptake phase and silver body burden of *D. magna* during the uptake and depuration phases for both AgNP. All values are given as mean values with standard errors (n=3). Pluses indicate sampling during the uptake phase, minuses indicate sampling during the depuration phase. Asterisks marks significant differences between body burden of citAgNP and detAgNP. a) silver concentration during the uptake phase, b) amount of silver per *Daphnia* during the uptake phase, and c) during the depuration phase.

Chronic *Daphnia* Test

The time weighted mean concentrations of silver differed strongly from the nominal values and between the used AgNP (Table 7.3), so time weighted mean values were used for all data analysis and figures.

According to the OECD guideline 211 requirements, the control group matched the validity criterion for mortality (≤ 20%), but not for reproduction, as mean reproduction in controls was only about 36 ± 3 neonates *D. magna*$^{-1}$ instead of the recommended 60 neonates *D. magna*$^{-1}$. No response to AgNP exposure was found for molting, size and wet weight (Table 7.4). Mortality increased at the highest AgNP concentrations (19.2 and 38.5 µg Ag L^{-1} for citAgNP and 27.5 for detAgNP) with significantly higher values above 19.2 µg Ag L^{-1} for citAgNP compared to control (p=0.011, glm) and without significant differences between concentrations for detAgNP (Table 7.4; SI Table S7.5). Reproduction showed a hormesis-like response to increasing silver concentrations (Figure 7.3a) with higher number of neonates at concentrations up to 10 and 15 µg Ag L^{-1} for citAgNP and detAgNP, respectively. However,

the positive effect was significant only for citAgNP at 6.4 µg Ag L^{-1} ($p<0.05$, Kruskal-Wallis post-hoc analysis; Table 7.4).

Table 7.3 Nominal and calculated silver concentrations in the test dispersions of the chronic *Daphnia* test.

AgNP	Nominal Concentration [µg Ag L^{-1}]	Time weighted mean (*) and calculated concentrations [µg Ag L^{-1}]
citAgNP	5	3.2
	10	6.4
	15	9.6
	30	19.2
	60	38.5*
detAgNP	5	2.3
	10	4.6
	15	6.9
	30	13.8
	60	27.5*

* indicate the time weighted mean based on silver concentration measurements (n=9). Calculations of the time weighted mean were done according to the description in the guideline (OECD No.211, Annex 6). The proportional relation between time weighted mean and nominal concentration in the measured concentration of each AgNP was used to calculate all other actual concentrations (citAgNP: 64%, detAgNP 46%).

The start of reproduction did not change with increasing AgNP concentration (Table 7.4) while the number of broods, the number of neonates per brood and consequently the total number of neonates varied. At low AgNP concentrations, the number of neonates per brood increased (Figure 7.3b), while the number of broods was similar to control levels (Figure 7.3c, Table 7.4). At the highest silver concentrations, the number of neonates decreased below control levels (Figure 7.3a, Table 7.4). In addition, the number of broods decreased signifcantly for citAgNP ($p<0.001$, ANOVA). The initial positive effect of AgNP on reproduction was supported by the reduction of the number of aborted neonates at low concentrations (Table 7.4). However, differences between single concentrations were not significant for detAgNP (Table 7.4), but silver concentration significantly decreased number of broods ($p=0.046$, Kruskal-Wallis rank sum test) and increased neonates per brood at low concentrations ($p=0.023$, ANOVA).

Table 7.4 Overview on all investigated biological endpoints and their response to AgNP exposure in terms of statistically significant relationships ($p<0.05$). Significant differences to control values are given for citAgNP only, as no significant differences to control values were found for detAgNP.

Endpoint:	**Significant influence by:**				**Significant differences to control values at: [µg Ag L^{-1}]**	
	Concen-tration	**Nano-particle**	**Inter-action**	**p-value**	**increase**	**decrease**
Reproduction related:						
Offspring at 1st reproduction	Yes	Yes	No	0.0285	None	None
Aborted offspring[1] at 1st reproduction	No	No	No	>0.005	--	--
Cumulative number of offspring	Yes	Yes	No	<0.001	6.4	19.2
Cumulative number of aborted offspring[1]	Yes	No	NA	0.004	6.4; 9.1	None
Offspring per reproduction	Yes	Yes	No	<0.001	6.4	None
Number of reproductions	Yes	Yes	Yes	<0.001	None	19.2
Time to 1st offspring	No	No	No	>0.005	--	--
Growth related:						
Molting	No	No	No	>0.005	--	--
Size	No	No	No	>0.005	--	--
Wet weight	No	No	No	>0.005	--	--
Survival related:						
Mortality	NA	NA	NA	NA	None	19.2

NA: The data did not meet the criteria for statistical analysis. --: A comparison to control was not made due to missing responses to AgNP exposure. None: despite a significant effect of AgNP exposure, differences to control values were not significant.

[1] all offspring without visible movement within 15 s and unhatched eggs were considered as aborted.

The body burden of silver at the end of the chronic test increased significantly with increasing concentration ($p<0.001$, lm with log-transformation) without significant difference between both AgNP ($p=0.75$, lm with log-transformation) when expressed as silver content per wet weight of animal. In the proportional analysis, significantly ($p=0.021$, ANOVA) higher body burden were measured for citAgNP (41 ± 3 %) than for detAgNP (33 ± 2 %) after 21 d of AgNP exposure (Table 7.5).

4. Discussion

The observed increase in size of citAgNP over time is in line with literature on agglomeration behavior of citAgNP in presence of divalent cations and in OECD *Daphnia* medium (Baalousha et al., 2013; Tejamaya et al., 2012) and with previous results for this type of AgNP in Elendt M7 medium (Mackevica et al., 2015). For detAgNP in the same medium, colloidal instability and increasing agglomerate size as well as relatively stable small aggregates of about 60 nm size have been reported (Baumann et al., 2014b; Cupi et al., 2015; unpublished data). In the present study, agglomerate sizes were constant, but a bit larger than reported for the stable dispersions. As the same medium was used in all studies, it is more likely that differences in colloidal stability in the test medium are linked to test dispersion preparation protocols or colloidal stability in the stock dispersion. The large differences between studies for detAgNP underline the high importance of particle characterization in the test media.

The different agglomeration behavior between citAgNP and detAgNP meets our expectations on their behavior during the typical media exchange period of 48 h as well as differences between charge and sterically stabilized AgNP in various media in other studies (reviewed in: Levard et al., 2012; Sharma et al., 2014).

The assumption that in the case of citAgNP, large aggregates sediment during the media exchange period is supported by the link between reduced colloidal stability in the test medium and increased amounts of silver at the beaker in the mass balance analysis. In addition to agglomeration, attachment to the beaker may have been increased by electrostatic bonds between divalent cations and charged AgNP. This additional sorption mechanism may have increased especially the loss of citAgNP from the test medium.

The main difference between the AgNP behavior in the particle characterization and the short-term experiments are the results for body burden. Due to lower colloidal stability in Elendt M7, citAgNPs were expected to be lost from the medium and to be taken up to a lesser extent. However, body burdens were higher for citAgNP than for detAgNP in both short-term experiments, except for the low concentration in the mass balance analysis, indicating higher uptake of the more agglomerated citAgNP at least at high exposure concentrations. As these differences were also significant when body burdens were related to actual media concentrations in the uptake and depuration experiment, the higher uptake cannot only be explained by higher silver medium concentrations for citAgNP at the same nominal concentration. This is supported by the result for body burden in the chronic test where body burden for citAgNP were significantly higher than for detAgNP when expressed as proportion of the calculated concentration.

However, the reduced colloidal stability of citAgNP could have caused higher uptake directly in case of sedimentation: *Daphnia* are feeding on sedimented algae as well and in case of higher citAgNP concentrations here, uptake of citAgNP may have taken place via ingestion of sedimented citAgNP agglomerates or attachments of those to sedimented algae. In this case, reduced colloidal stability would have increased internal exposure due to the feeding behavior of the test animal. Even though amounts of sedimented AgNP cannot be directly quantified,

the amount of silver lost from the test system can serve as good approximation: as beakers were emptied after measuring silver in the medium and before measuring silver attached to the test vessel, sedimented AgNP would be lost from the measurement.

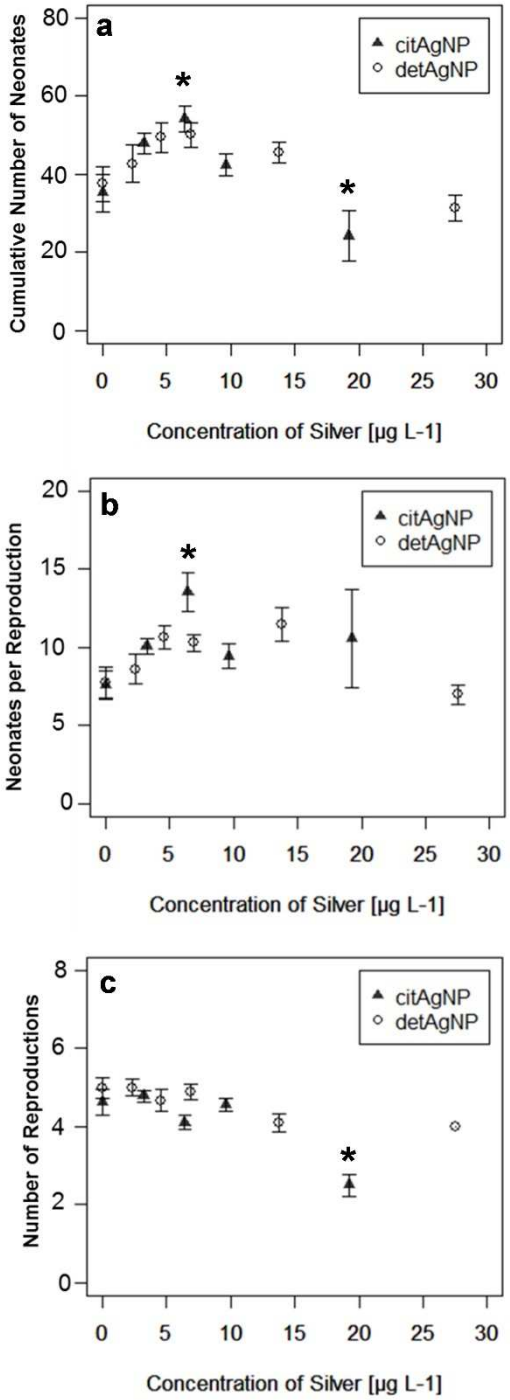

Figure 7.3 Relation between concentration of silver in the test dispersion and reproduction for both AgNP All values are given as mean values for all surviving adults (SI, Table S5) with standard errors. * mark significant differences to the control for citAgNP ($p<0.005$). No significant differences were observed in case of detAgNP. a) cumulative number of neonates, b) number of neonates per reproduction, c) number of reproductive events.

The comparison of 24 h and 72 h results of the mass-balance analysis can thus provide information on the degree of losses due to sedimentation. A comparison of both AgNPs revealed that only at 10 mg Ag L^{-1}, losses of citAgNP from the test system were clearly higher than for detAgNP (about 46 % compared to a rather constant amount, respectively). The differences in body burdens, however, were more pronounced at higher concentrations. Even though the feeding from sedimented AgNP agglomerates remains a likely route of uptake, other reasons need to be responsible for differences in body burden between the used AgNPs. These reasons may be linked to: 1) attachment to algae, 2) the possibility to be actively filtered from the water column, and 3) attachment to the carapace of *D. magna*.

Table 7.5: Mean silver body burden of *D. magna* in relation to silver concentration and AgNP expressed as amount per animal and proportion of actual concentration per animal.

Nanoparticle	**Silver Concentration [µg Ag L^{-1}]**	**Body burden as amount [µg Ag $Daphnia^{-1}$]**	**Body burden as proportion of the actual silver concentration [% $Daphnia^{-1}$]**
citAgNP	0	Not detectable	Not detectable
	3.21	1.25 ± 0.20	0.39 ± 0.06
	6.41	2.81 ± 0.36	0.44 ± 0.06
	9.61	3.75 ± 0.50	0.39 ± 0.05
	19.23	1.37 ± 0.69	0.43 ± 0.04
	38.45	NA	NA
detAgNP	0	Not detectable	Not detectable
	2.29	0.77 ± 0.08	0.34 ± 0.04
	4.59	1.67 ± 0.20	0.36 ± 0.05
	6.88	2.04 ± 0.32	0.30 ± 0.05
	13.76	4.56 ± 0.43	0.33 ± 0.03
	27.52	4.04 ± 2.33	0.31 ± 0.08

All values are given as mean with standard errors (n according to SI, Table S5). NA indicates treatments without surviving adult Daphnia at the end of the test.

Attachment of nanoparticles to algae has been shown for several NPs (Bouldin et al., 2008; Röhder et al., 2014; Sadiq et al., 2011; Van Hoecke et al., 2008), but only one study investigated whether surface charge affected attachment to algae (Ma and Lin, 2013).
Studies investigating uptake of NPs report higher levels for dietary than for waterborne uptake (Zhao and Wang, 2010), as well as reductions of uptake in presence of algae (Skjolding et al., 2014). However, some studies on uptake in relation to colloidal stability in the test medium show that agglomerates are taken up in larger amounts than their smaller counterparts (Asghari et al., 2012; Kwon et al., 2014) which may be explained by active filtration of these agglomerates as suggested in other studies (Rosenkranz et al., 2009; Zhao and Wang, 2010). In the present study, citAgNP pass the active filtration limit for *D. magna* (of about 300-500 nm;

(Brendelberger, Heinz, 1991; Geller and Müller, 1981; Kwon et al., 2014) after about 24 h of characterization while detAgNP remain below, so that active filtration would be an exclusive mechanism for citAgNP.
Attachment to the carapax has been reported for several NPs with different core materials and coatings (Asghari et al., 2012; Baumann et al., 2014a; Bozich et al., 2014; Dabrunz et al., 2011; Zhao and Wang, 2012). In most cases, it caused reduction or even inhibition of molting (Baumann et al., 2014a; Bozich et al., 2014; Dabrunz et al., 2011) which was not the case in the present study indicating low levels of attachment for the AgNP to the exoskeleton here.
Even though additional experiments, e.g. with radioactive isotopes of silver as described elsewhere (Coutris et al., 2012; Sekine et al., 2015), would be necessary to prove the reasons for higher body burden of citAgNP, a higher uptake due to ingestion seems most likely at the moment. Overall, the results of the short-term experiments illustrate that the organisms added to the test system (algae and/or *Daphnids*) affected the behavior of both AgNP causing results contradictory to the conclusion from the particle characterization.

The reduced reproduction in the chronic test can be explained by a deviating feeding regime, as amount of food is one of the main factors driving the reproduction (Enserink et al., 1993): the actual carbon content of the used algae (SI, section S1) was about 0.5 times lower than the carbon content based on the used equation.
However, previous results with citAgNP (Mackevica et al., 2015) showed that differences in feeding regime resulted in a similar toxicity pattern, with lower toxicity at higher food concentrations. As we were mainly interested to compare the two differently stabilized AgNP, we consider the current study to still reflect the toxicity of the used AgNP in an adequate manner.
Toxic effects of both AgNP on survival and reproduction were detected, but not on the time to first offspring (Table 4). In contrast to previous results with citAgNP (Mackevica et al., 2015), animal size was not affected in the present study. However, the behavior of citAgNP differed between the studies, probably causing this difference in toxicity pattern. PVP and collargol coated AgNP have also been reported to not affect size during chronic exposure (Blinova et al., 2013), while size was the most sensitive endpoint in case of carbonate coated AgNP (Zhao and Wang, 2011). Still, effects on reproduction and survival were noted in all studies.
This is similar to results for ionic silver which highly affects survival and reproduction (Bianchini and Wood, 2003) but not growth or molting (Naddy et al., 2007). However, in the present study, dissolved silver levels were below the detection limit (< 1 µg Ag L^{-1}) indicating that observed effects were most likely caused by the used AgNP themselves, not by released ions.
The NOEC values for reproduction in the present study were higher than the highest concentration tested, except for the effect of citAgNP on the number of broods (NOEC: 9.6 µg Ag L^{-1}; LOEC: 19.2 µg Ag L^{-1}). For citAgNP, mortality was 100% at the highest concentration (38.5 µg Ag L^{-1}) and the LOEC was the same as for number of broods, indicating mortality to be equally sensitive as reproduction. As mortality was 60 % at the highest concentration of detAgNP, it is likely that a similar picture can be detected for detAgNP when higher concentrations are tested. In our previous study, the LOEC for reproduction was at a nominal concentration of 40 µg Ag L^{-1}, which is in good agreement with the present results, but the LOEC for mortality was lower (nominal: 20 µg Ag L^{-1}) indicating higher sensitivity of mortality than reproduction. Higher sensitivity for mortality than for reproduction has also been reported for collargol and PVP coated AgNP (Blinova et al., 2013), while growth was the most sensitive endpoint in case of carbonate coated AgNP (Zhao and Wang, 2011). The endpoints affected by 21 d AgNP exposure were similar for both AgNP in the present study, which is in line with Blinova et al. (2013) where PVP and collargol coated AgNP varied in toxicity, but not in affected

endpoints. Systematic investigations on stabilizer effects on AgNP toxicity are needed to identify whether this is a general pattern in AgNP toxicity.

Besides comparing the toxicity of the used AgNP, one aim of the present study was to link AgNP behavior and toxicity by using the results of particle characterization, short-term experiments and chronic exposure. The short-term experiments as well as the results for body burden in the chronic test show higher uptake of citAgNP than of detAgNP, which is in line with the higher toxicity observed for this kind of AgNP and may be caused by negative effects of AgNP after ingestion. Several studies addressed the uptake and the resulting effect of various NP on the midgut epithelium of *Daphnia* (Asghari et al., 2012; Feswick et al., 2013; Heinlaan et al., 2011; Khan et al., 2014; Kwon et al., 2014; Lovern et al., 2008; Rosenkranz et al., 2009; L. M. Skjolding et al., 2014; L.M. Skjolding et al., 2014) with different results related to the colloidal stability of NP: in some studies, mainly single NPs were found in the gut (Heinlaan et al., 2011; Khan et al., 2014; Lovern et al., 2008), while another study showed that more agglomerated NP caused higher damage in to the midgut epithelium (Kwon et al., 2014). However, the midgut epithelium is protected by the peritrophic membrane (PTM) and citAgNP were mainly larger than the PTM permeability of 130 nm (Avtsyn and Petrova, 1986; Hansen and Peters, 1997) while detAgNP remained below. According to this size limit, detAgNP should have caused higher toxicity to the midgut epithelium despite the lower body burden.

A possible explanation may be that the sterically stabilization hindered direct contact between the AgNP core and the midgut epithelium, while the charged stabilization of citAgNP may have even enabled interactions between citAgNP and the midgut cells. The presence/absence of certain chemical groups in the stabilizer or the stabilizer identity has been shown to cause differences in toxicity also in other studies (Baumann et al., 2014a; Kim et al., 2013; Moyano and Rotello, 2011). However, it is also possible that ingested citAgNP acted more toxic due to higher dissolution rates. Even though the pH in the midgut is in the neutral to basic range (Hasler, 1935; von Elert et al., 2004), a dissolution of AgNP after ingestion cannot be excluded and due to their internalization, also very low levels of silver ions would cause severe effects on the test organism. The body burden at the lowest concentration causing a negative effect on reproduction and survival was 8.2 µg g wet weight $^{-1}$. The chronic NOEC for ionic silver has been reported being below 3.0 to 5.0 µg Ag L^{-1} (Bianchini and Wood, 2003; Naddy et al., 2007) which is clearly lower than the body burden reported in the present study. Measurements of fate inside the digestive tract and cell toxicity studies assessing the toxic mechanism of the used AgNP are required to identify the exact reason for the observed difference in toxicity. However, we were able to link the difference in toxicity to differences in body burden which were related to the effect of the test organisms on the AgNP behavior and also to differences colloidal stability.

5. Conclusion

In the present study, charge stabilized AgNP (citAgNP) were more toxic than sterically stabilized AgNP (detAgNP) which related well to their body burden measured in all experiments (citAgNP > detAgNP), and can be explained by differences in the direct contact between AgNP, feeding algae and *D. magna*: even if differences in silver concentrations in the medium are considered, body burden as well as toxicity of citAgNP remained higher. These results illustrate the importance of stabilizer mediated interactions between test organisms and nanoparticles.

These differences in body burden at the end of the chronic test were already detectable in the used short-term experiments (uptake and depuration and mass-balance analysis) making them a helpful tool for systematic investigation of the effect of the stabilizer on (Ag)NP behavior

under conditions more similar to environmental and test conditions than characterization in test medium. Measuring the behavior of (Ag)NP in the presence of food is of special interest for all consumers, like the *D. magna* in the present study, to interpret results of long-term experiments and the role of the food, like the algae, in uptake of nanoparticles.

Acknowledgements
This work is part of the project ENVNANO (Environmental Effects and Risk Evaluation of Engineered Nanoparticles) supported by the European Research Council (grant no. 281579) and was supported by an Impulse Grant of the University of Bremen as part of the Excellence Initiative. detAgNP (OECD material NM-300K) were provided by the UMSICHT project (BMBF 03X0091). We thank Signe Qualmann for her support in the laboratory during the chronic test, and during the final sampling, and Susanne Kruse for measuring the ICP samples.

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Supporting Information

Table of Contents:

S7.1 Carbon content of algal food

The used assumption of 0.1 mg C per 10^4 algae mL^{-1} (Hailing-Sørensen et al. ;1996) was tested after the chronic *Daphnia* test by carbon measurements of subsamples of the algae suspensions used for feeding (carbon combustion followed by laser CO_2 detection; I-05/RP and D-03GTE, C-mat 5500, all from Ströhlein Instruments). Measurements were run for each algal suspension used for feeding using 4 replicates and pure algal medium was used for background correction.

Results showed a mean carbon content of 0.048 ± 0.009 mg C for 10^4 algae mL^{-1}, so that supplied amounts of food were about half of the values recommended by the OECD guideline (OECD, 2008).

S7.2 Overview of statistical analyses

Table S7.1a: Overview on analyzed datasets and corresponding statistical analysis for silver amounts in the mass-balance experiment.

Depending variable	Independent variables	Model	Signifcant effects
Amount of silver in test medium*	AgNP	Kruskal-Wallis rank sum test	None (0.729)
Amount of silver in test medium*	concentration	Kruskal-Wallis rank sum test	Yes (0.001)
Amount of silver in test medium*	time	Kruskal-Wallis rank sum test	Yes (0.004)
Amount of silver in test medium at 10 µg Ag L^{-1}*	AgNP	Kruskal-Wallis rank sum test	None (0.522)
Amount of silver in test medium at 10 µg Ag L^{-1}*	time	Kruskal-Wallis rank sum test	Yes (0.010)
Amount of silver in test medium at 30 µg Ag L^{-1}	AgNP; time	Linear Model (time)	Time (<0.001)
Amount of silver at beaker	AgNP; concentration ; time	Linear Model [(AgNP+concentration+time)^2-AgNP:time]	AgNP (0.007) Concentration (<0.001) AgNP: concentration (<0.001) Concentration: Time (0.005)
Amount of silver at beaker at 10 µg Ag L^{-1}	AgNP, time	Linear Model (AgNP + Time)	AgNP (0.006) time (0.002)
Amount of silver at beaker at 30 µg Ag L^{-1}	AgNP, time	Linear Model (AgNP + Time)	AgNP (<0.001) time (<0.001)
Amount of silver in *Daphnia*	AgNP; concentration ; time	Linear Model with square root transformation [(AgNP + concentration + time)^2 - AgNP:time]	AgNP: concentration (0.012) concentration:time (<0.001)
Amount of silver in *Daphnia* at 10 µg Ag L^{-1}	AgNP, time	Linear Model (AgNP * time)	time (0.005)
Amount of silver in *Daphnia* at 30 µg Ag L^{-1}	AgNP, time	Linear Model (AgNP + time)	AgNP (0.008) time (<0.001)

* indicate variables that did not show homogeneity of variance after transformation and could only be analyzed by non-parametric tests, so that interactions could not be tested.

Table S7.1b: Overview on analyzed datasets and corresponding statistical analysis for silver relative to initally added amounts in the mass-balance experiment.

Depending variable	Independent variables	Model	Signifcant effects
Proportion of silver in test medium*	AgNP	Kruskal-Wallis rank sum test	No (0.149)
Proportion of silver in test medium*	concentration	Kruskal-Wallis rank sum test	No (0.686)
Proportion of silver in test medium*	time	Kruskal-Wallis rank sum test	Yes (<0.001)
Proportion of silver in test medium at 10 µg Ag L^{-1}*	AgNP	Kruskal-Wallis rank sum test	No (0.423)
Proportion of silver in test medium at 10 µg Ag L^{-1}*	time	Kruskal-Wallis rank sum test	Yes (0.025)
Proportion of silver in test medium at 30 µg Ag L^{-1}	AgNP; time	Linear Model (AgNP + time)	AgNP (0.009) time (<0.001)
Proportion of silver at beaker*	AgNP	Kruskal-Wallis rank sum test	Yes (0.016)
Proportion of silver at beaker*	concentration	Kruskal-Wallis rank sum test	No (0.0667)
Proportion of silver at beaker*	Time	Kruskal-Wallis rank sum test	Yes (<0.001)
Proportion of silver at beaker at 10 µg Ag L^{-1}	AgNP; time	Linear Model (time)	time (<0.001)
Proportion of silver at beaker at 30 µg Ag L^{-1}	AgNP; time	Linear Model (AgNP + time)	AgNP (<0.001) time (<0.001)
Proportion of silver in *Daphnia*	AgNP; concentration ; time	Linear Model with square root transformation (AgNP * concentration * time)	Concentration (0.048) AgNP: time (0.030) Concentration: time (0.019)
Proportion of silver in *Daphnia* at 10 µg Ag L^{-1}	AgNP; time	Linear Model (AgNP * time)	AgNP (0.033) time (0.005) AgNP:time (0.041)
Proportion of silver in *Daphnia* at 30 µg Ag L^{-1}	AgNP; time	Linear Model (time)	time (<0.001)

* indicate variables that did not show homogeneity of variance after transformation and could only be analyzed by non-parametric tests, so that interactions could not be tested.

Table S7.2: Overview on analyzed datasets and corresponding statistical analysis in the uptake and depuration experiment.

Depending variable	Independent variables	Model	Signifcant effect
Amount of silver per Daphnia after 1 h of AgNP exposure	AgNP	Welch Two Sample t-test	none
Amount of silver per Daphnia after 2 h of AgNP exposure	AgNP	Welch Two Sample t-test	none
Amount of silver per Daphnia after 6 h of AgNP exposure	AgNP	Welch Two Sample t-test	none
Amount of silver per Daphnia after 24 h of AgNP exposure	AgNP	Welch Two Sample t-test	AgNP (0.049)
Amount of silver per Daphnia after 1 h of AgNP depuration	AgNP	Welch Two Sample t-test	none
Amount of silver per Daphnia after 2 h of AgNP depuration	AgNP	Wilcoxon test	none
Amount of silver per Daphnia after 6 h of AgNP depuration	AgNP	Welch Two Sample t-test	none
Amount of silver per Daphnia after 24 h of AgNP depuration	AgNP	Welch Two Sample t-test	none
Proportion of silver per Daphnia in relation to aquatic silver concentration	AgNP; Time	Linear Model (AgNP*Time)	Time (<0.001)

Table S7.3: Overview on analyzed datasets and corresponding statistical analysis in the chronic *Daphnia* test.

Depending variable	Independent variables	Model	Signifcant effects
Cumulative number of neonates	AgNP; concentration	General additive model (ti($Conc_{citAgNP}$,$Conc_{detAgNP}$); family=gaussian; link= identity	Complete term (<0.001) >> two curves with the same slope
Cumulative number of aborted neonates*	AgNP	Kruskal-Wallis rank sum test	none
Cumulative number of aborted neonates*	concentration	Kruskal-Wallis rank sum test	Concentration (0.004)
Cumulative number of reproductions	AgNP; concentration	General additive model (ti($Conc_{citAgNP}$) +ti($Conc_{detAgNP}$)); family=gaussian, link=log	Both terms (<0.001 for each) >> two curves with different slopes
Number of neonates at first reproduction	AgNP; concentration	General additive model (ti($Conc_{citAgNP}$,$Conc_{detAgNP}$); family=gaussian; link= identity	Complete term (0.0285) >> two curves with the same slope
Number of aborted neonates at first reproduction	AgNP; concentration	General linear model (AgNP); family=binomial, link=logit	None
Cumulative number of moults	AgNP; concentration	General additive model (ti(Conc)) family=gaussian, link=log	None
Body length	AgNP; concentration	General additive model (ti($Conc_{citAgNP}$,$Conc_{detAgNP}$); family=gaussian; link= identity	None
Wet weight	AgNP; concentration	Linear model (Concentration)	None
Silver body burden	AgNP; concentration	Linear model (AgNP*Concentration) with log-transformation	Concentration (<0.001)
Silver body burden as proportion	AgNP	Anova	Yes (0.021)

* indicate terms where no interaction could be tested due to missing the criteria for linear models. Mortality could not be analyzed in the complete dataset, as no model met the criteria for linear models.

Table S7.3 continued:

Depending variable	Independent variables	Model	Signifcant effects
Mean number of neonates per reproduction	AgNP; concentration	General additive model (ti($Conc_{citAgNP}$,$Conc_{detAgNP}$); family=gaussian; link= identity	Complete term (<0.001) >> two curves with the same slope
Day at first reproduction*	AgNP	Wilcoxon test	none
Day at first reproduction*	concentration	Kruskal-Wallis rank sum test	none

* indicate terms where no interaction could be tested due to missing the criteria for linear models. Mortality could not be analyzed in the complete dataset, as no model met the criteria for linear models.

Table S4: Overview on analyzed datasets and corresponding statistical analysis used for NOEC and LOEC determination. The independent variable was concentration of silver. Endpoints without response to silver exposure where not used for NOEC/LOEC analysis (see Table S3).

AgNP	Depending variable	Model	Signifcant differences
citAgNP	Cumulative number of neonates	Anova with post-hoc analysis	6.4 vs. control 19.2 vs. 3.2;6.4;9.6
	Cumulative number of aborted neonates	Kruskal-Wallis test with one-tailed post-hoc analysis (vs control)	6.4 and 9.6 vs. control
	Cumulative number of reproductions	Anova with post-hoc analysis	19.2 vs. control and all other concentrations
	Number of neonates at first reproduction	Kruskal-Wallis test with one-tailed post-hoc analysis (vs control)	None
	Mean number of neonates per reproduction	Kruskal-Wallis test with one-tailed post-hoc analysis (vs control)	6.4 vs. control

Table S4 continued:

AgNP	Depending variable	Model	Signifcant differences
citAgNP	Silver body burden	Kruskal-Wallis test with one-tailed post-hoc analysis (vs. control)	6.4;9.6; 19.2 vs. control
	Mortality	General linear model; family=binomial, link=logit; stepwise deletion of concentrations	19.2 and 38.5 vs. all others
detAgNP	Cumulative number of neonates	Anova with post-hoc analysis	none
	Cumulative number of aborted neonates	Kruskal-Wallis test with one-tailed post-hoc analysis (vs. control)	None
	Cumulative number of reproductions	Kruskal-Wallis test with one-tailed post-hoc analysis (vs. control)	none
	Mean number of neonates per reproduction	Anova with post-hoc analysis	none
	Number of neonates at first reproduction	Anova with post-hoc analysis	none
	Silver body burden	Kruskal-Wallis test with one-tailed post-hoc analysis (vs. control)	4.6; 6.9; 13.8; 27.5 vs. control
	Mortality	General linear model; family=binomial, link=logit; stepwise deletion of concentrations	none

S7.3 Silver body burden during the uptake and depuration experiment

Table S7.5: Amount of silver per *Daphnia* in relation to sampling time and used AgNP for both uptake and depuration phase.

Nanoparticle	Time	Body Burden during uptake [ng Ag Daphnia^{-1}]	Body Burden during depuration [ng Ag Daphnia^{-1}]
citAgNP	**1h**	0.057 ± 0.057	0.320 ± 0.057
	2h	0.098 ± 0.070	0.087 ± 0.015
	6h	0.128 ± 0.081	0.031 ± 0.017
	24h	0.693 ± 0.095	n.d.
detAgNP	**1h**	0.071 ± 0.057	0.147 ± 0.147
	2h	0.067 ± 0.052	0.175 ± 0.175
	6h	0.091 ± 0.077	0.020 ± 0.020
	24h	0.346 ± 0.075	n.d.

All values are given as mean values with standard error for each sampling (n=3). The detection limit of the device was 0.03 ng Ag Daphnia^{-1}.

S7.4. Additional figures of the mass balance analysis

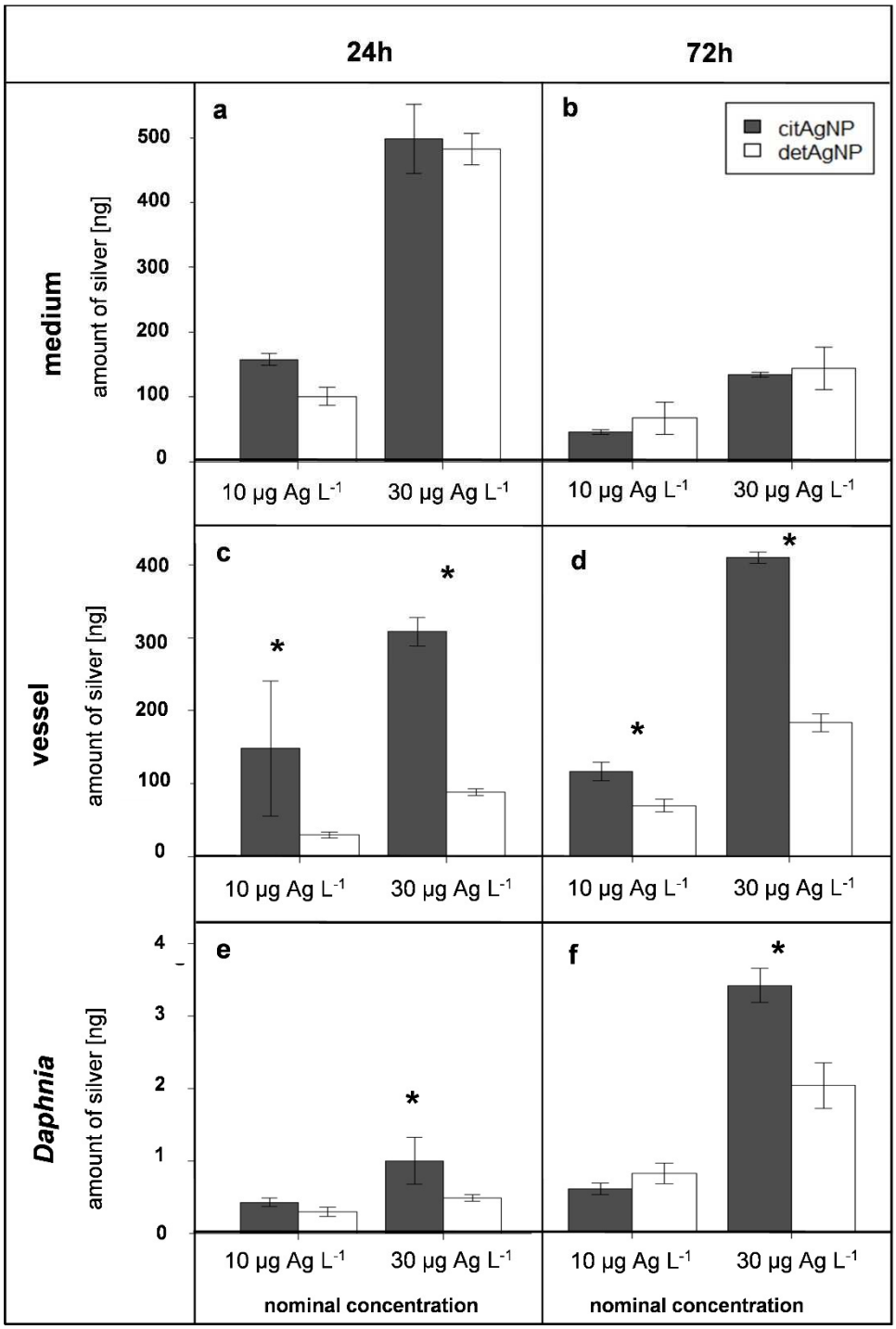

Figure S7.1: Amount of silver in all compartments of the test system for both nominal concentrations after 24 h (left) and after 72 h (right). Each measurement is shown as mean value with standard errors (n=3).
a) proportional amount of silver in the test medium after 24 h, **b**) proportional amount of silver in the test medium after 72 h; **c**) proportional amount of silver on the beaker after 24 h, **d**) proportional amount of silver on the beaker after 72 h; **e**) proportional amount of silver in the test organism after 24 h, **f**) proportional amount of silver in the test organism after 72 h.

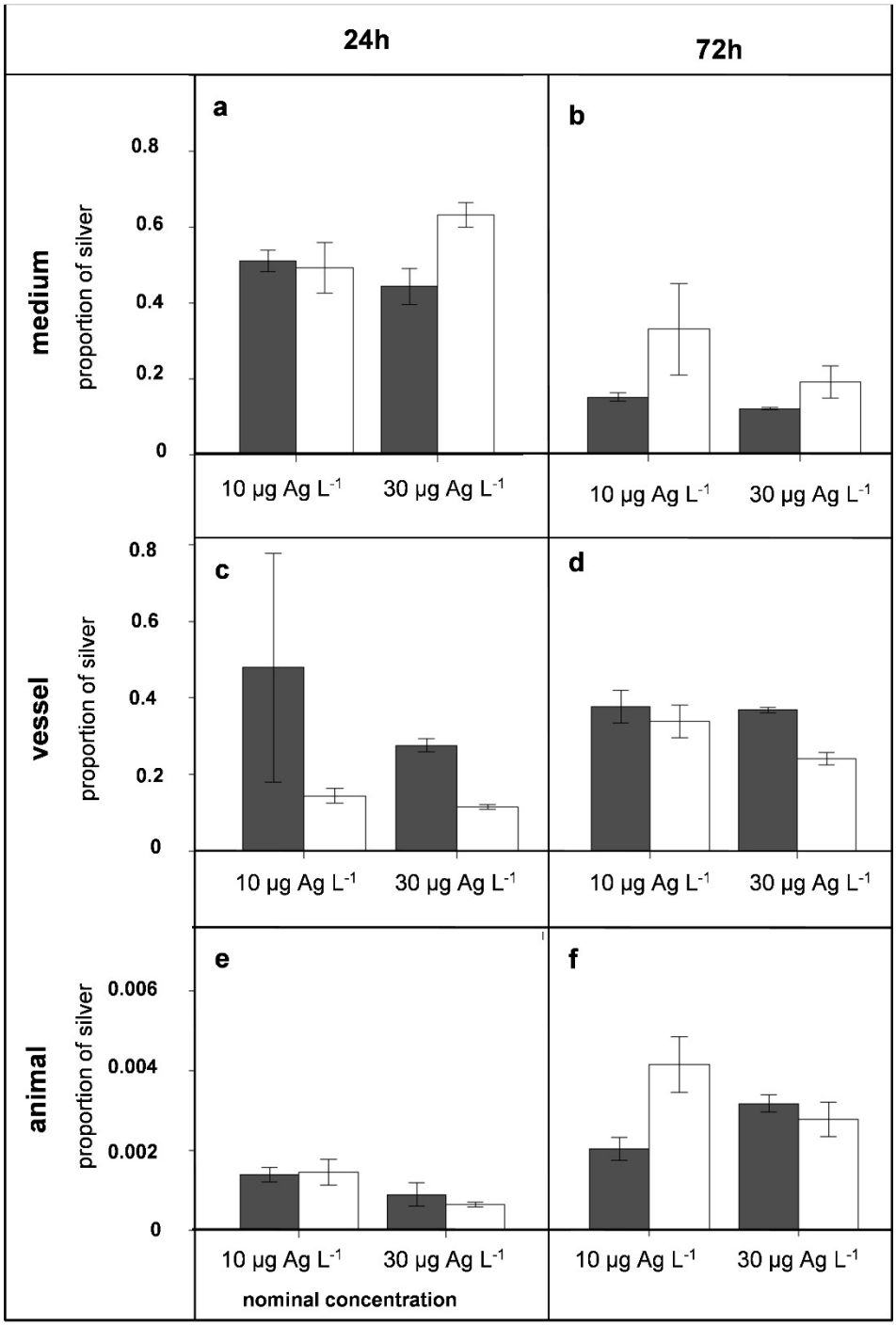

Figure S7.2: Relative amount of silver in all compartments of the test system for both nominal concentrations after 24 h (left) and after 72 h (right). Each measurement is shown as mean value with standard errors (n=3).
a) proportional amount of silver in the test medium after 24 h, **b**) proportional amount of silver in the test medium after 72 h; **c**) proportional amount of silver on the beaker after 24 h, **d**) proportional amount of silver on the beaker after 72 h; **e**) proportional amount of silver in the test organism after 24 h, **f**) proportional amount of silver in the test organism after 72 h.

S7.5 Mortality in the chronic *Daphnia* test

Table S7.6: Relative mortality (n=10) at the end of the test in relation to the used AgNP and the silver concentration in the test medium.

Nanoparticle	**Silver Concentration [µg Ag L^{-1}]**	**Mortality [%]**
citAgNP	0	20
	3.21	10
	6.41	0
	9.61	10
	19.23	60
	38.45	100
detAgNP	0	20
	2.29	0
	4.59	10
	6.88	10
	13.76	0
	27.52	60

8 Nutrient deficiency increases sensitivity of green algae towards silver nanoparticles

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Submitted manuscript

Contributions of Yvonne Sakka:

- Supervision of the master thesis of Asif Chowdhury on which this manuscript is based on
- Support during wiriting and finalizing the manuscript
- Finalizing the figures and tables
- Revision of the manuscript

Asif Chowdhury performed the experiments of this work, made the statistical analyis and figures, and wrote the manuscript with the help of Yvonne Sakka and Juliane Filser.

Nutrient deficiency increases sensitivity of green algae towards silver nanoparticles

Abstract
According to the OECD guideline for algae growth inhibition tests, the amount of nitrogen and phosphorus used are set to levels much higher than the corresponding values found in eutrophic lakes worldwide. In this study, the effect of a reduction in these parameters to eutrophic and mesotrophic conditions on silver nanoparticle (AgNP) toxicity was tested. For this purpose, the amount of both nitrogen and phosphorus was lowered to half and to one-fourth of that suggested in the OECD guideline. To increase generality of the obtained results, two freshwater micro-algae *Raphidocelis subcapitata* and *Desmodesmus supspicatus* were tested. The EC_{50} value in standard OECD medium for the growth of *R. subcapitata* was 72.65 ± 1.48 µg L^{-1} and 78.02 ± 1.38 µg L^{-1} for *D. subspicatus.* The reduction in nutrients caused a decrease in EC_{50} values for both algae species, with the effect being more pronounced for nitrogen than for phosphorous (48.88 ± 1.79 and 54.77 ± 2.22 µg Ag L^{-1} at ¼ P for *R. subcapitata* and *D. subspicatus*, respectively, compared to 27.30 ± 1.29 and 53.19 ± 1.71 µg Ag L^{-1} at ¼ N). However, the reduction in nutrient levels also increased dissolution of AgNP, causing an interactive effect of nutrient reduction and ionic silver toxicity. Our results thus indicate higher risks of AgNP in nutrient deficient environments resulting from both increased dissolution levels and reduced resource availability.

Keywords
Algae, silver nanoparticles, nitrogen limitation, phosphorous limitation

1. Introduction

During the last few years, AgNP have received intense research attention (Tran et al., 2013; Fabrega et al., 2011; Sharma et al., 2014) because of their potential health risk to different organisms (Luoma, 2008; Navarro et al., 2008a). AgNP are considered mostly harmless to humans unlike other organisms (Gurunathan et al., 2015). On the other hand, AgNP are one of the most investigated toxic metals found in natural waters systems mainly for phytoplankton and microorganisms (Książyk et al., 2015).

The environmental impact of AgNP, however, is greatly dependent on their aggregation behavior and mobility in the environment (Li et al., 2010). AgNP-containing products can release dissolved silver which is highly toxic, likely to be bioaccumulated and thus hazardous for the environment (Benn and Westerhoff, 2008; Geranio et al., 2009; Gottschalk et al., 2009; McTeer et al., 2014).

Several studies show that the dissolution of AgNP to Ag^+ ion is the main reason causing toxicity to test organisms (Asharani et al., 2008; Gil-Allué et al., 2015; Navarro et al., 2008b). In contrast, Fabrega et al. (2009) presented evidence of AgNP being the cause for toxicity on bacterial growth. Also, Griffitt et al. (2008) found that toxicity of AgNP to *Danio rerio* and *Daphnia pulex* was not only exerted by ionic, but also nanoparticular silver. The sharing between ionic and nanoparticulate silver toxicity thus seem to depend on the dissolution rate under the given test conditions (Behra et al., 2013; Kittler et al., 2010).

Marine and estuarine phytoplankton is often nitrogen (N)-limited whereas freshwater phytoplankton suffer from phosphorus (P) limitation (Hecky and Kilham, 1988). The mean total N content in eutrophic lakes is around 1.875 mg L^{-1} and mean total P is 0.0844 mg L^{-1} (Ongley, 1996; Vollenweider, 1979). According to the OECD guideline No.201 (OECD,2011), the growth inhibition test with algae, the amount of N and P in the growth medium are more than twice and three times as high (3.927 mg L^{-1} for N and 0.285 mg L^{-1} for P). As the amount of N and P limit algal populations in the environment (Jones & Lee, 1982), the OECD algae medium allows the development of much higher algae densities than those found in the environment.

Investigating whether using more ambient nutrient levels affect AgNP toxicity is thus an important question. Earliest studies addressing the effect of reduced P levels had inconsistent results: in some cases, AgNP toxicity was higher when P supply was reduced (Serra et al., 2010) due to the role of intracellular phosphate bodies in metal detoxification (Twiss and Nalewajko, 1992; Wang and Dei, 2006). In another study high P availability favored AgNP toxicity (Norman et al., 2015) may be due to the formation of Ag-P complexes which can be assimilated by autotrophs (Xiu et al., 2011). As there is limited knowledge on these contradictory issues, there is also a need to assess the impact of phosphorus reduction on AgNP toxicity for green algae species.

Hence, this study aims to test the sensitivity of algae towards silver nanoparticles with OECD test medium and lower nutrient concentrations under standard test conditions to improve comparability and to exclude any other confounding factor aside nutrient levels. Two unicellular freshwater microalgae *Desmodesmus subspicatus* and *Raphidocelis subcapitata* were chosen due to their status as standard test organisms for the algae growth inhibition test (OECD, 2011) and the response to changes in nutrient supply was compared. As test substance, the OECD reference material NM-300K was used.

The main objectives of our study were (1) to test AgNP toxicity to *D. subspicatus* and *R. subcapitata* using standard test conditions; (2) to investigate the toxicity of AgNP in different nutrient conditions for both species; (3) to compare the sensitivity of the two algae species at different P and N concentrations with and without the application of AgNP.

2. Materials & Methods

Characterization of AgNP

NM-300K (detAgNP, ras materials GmbH, Regensburg, Germany), OECD reference material for AgNP, were of a nominal size of 20 nm and delivered as 10.16% Ag wt dispersion containing 4% TWEEN 20 and TAGAT TO each. The dispersion was diluted with MilliQ water (18.2 MΩ cm^{-1} in resistivity) was produced from Millipore MilliQ Plus water purification system (Massachusetts, USA) to a silver concentration of 2% Ag wt and sonicated afterwards for 15 min in a Sonorex water bath (BANDELIN electronic GmbH, Berlin, Germany). This sonicated dispersion was then diluted 1000 times with OECD medium to make a stock dispersion of 20 µg Ag mL^{-1}. Stock dispersions for all nutrient reduced media were prepared accordingly from the sonicated dispersion. These stock dispersions were then stored in the dark at 4 °C. To prepare test dispersions, stock dispersions were diluted using the corresponding media to represent test concentrations of 25, 50 and 100 µg Ag L^{-1} in 20 mL solution.
AgNP dispersions with a concentration of 10 µg Ag mL^{-1} were characterized for zeta potential and hydrodynamic diameter by Dynamic Light Scattering (DLS; Malvern Zeta Sizer Nano ZS, Malvern Instruments, Worcestershire, UK) in all five media. All measurements were run at 25 ° C. The absorption spectra of AgNP in dispersion were measured in the range of 350-800 nm (L-4250 *UV/Vis*-Detector, Hitachi Ltd. Tokyo, Japan). The shape was controlled by Transmission Electron Microscopy (TEM; Titan 80-300 ST microscope, FEI™, Eindhoven, Netherlands) images. For this purpose, one drop of the 2% wt dispersion (without dilution in algae media) was placed onto a copper grid and let dry overnight.

Algae Growth Media

Five different growth media on the basis of green algae OECD medium were prepared to cover the range of hypertrophic to mesotrophic conditions. The original medium was used as the reference (standard). For the other four media, the amount of either nitrogen or phosphorus in the medium was lowered from half (50%) to one-fourth (25 %) of that of the OECD guideline, resulting in the following media: 50% N, 25% N, 50% P and 25% P medium. To overcome differences in ionic strength between media, sodium chloride (NaCl) was added in relation to the loss of ions due to nutrient reduction. Sodium chloride was chosen as replacement, as both, sodium and chloride are the main components of the standard medium (about 0.6 mM) without being related to algal growth. The pH was between 7.7 and 7.9 at the beginning of the experiment in all media (Table 8.1).

Table 8.1 pH values in all the media at the beginning of the experiment.

Medium	OECD	50% N	25% N	50% P	25% P
pH	7.85	7.76	7.81	7.80	7.72

Test organisms

The unicellular green algae *Raphidocelis subcapitata* (formerly known as *Pseudokirchneriella subcapitata*; strain no. 61.81, Algae culture collection SAG, Göttingen, Germany) and *Desmodesmus subspicatus* (strain no. 86.81 Algae culture collection SAG, Göttingen, Germany) were used in this study. Permanent cultures of both species were kept at daylight

and room temperature. Prior to starting the experiments, algae stock cultures were established by transferring an inoculum from the permanent culture into freshly prepared algae medium. These algae stock cultures were maintained on a shaker (MTS 2/4 digital, IKA-Werke GmBH & Co.KG, Staufen, Germany) with 150 rpm at 24 ± 0.5°C and a 14:10 h light-dark cycle in a climate chamber (AQUALYTIC, Dortmund, Germany) for 72 h. Illumination was in the range of 4800 – 5200 lux (Gossen Panlux ElectronicLuxmeter, GOSSEN Foto- und Lichtmesstechnik GmbH, Nürnberg, Germany). As there is a surplus store of phosphorus in algae (Eixler et al., 2006; Fitzgerald and Nelson, 1975), this culturing step was repeated for additional 72 h for all P reduced media.

Algae growth inhibition tests

All tests were run according to the OECD guideline 201 (OECD, 2011) with sterilized 50 mL CELLSTAR cell culture flasks (VWR International GmbH, Hannover, Germany).

At the end of the algae stock culturing time (72 h for OECD and N reduced media, 144 h for P reduced media), algal densities were determined by Neubauer cell counting chamber (Celeromics, Valencia, Spain) and diluted to 10^4 cells mL^{-1} for all tests. Each medium was tested using the test conditions for culturing with 3 replicates. Test concentrations for both algae species were 0, 25, 50 and 100 µg Ag L^{-1} for OECD, 50% P and 25% P while for both N reductions, the silver concentrations were 0, 10, 25 and 100 µg Ag L^{-1}. Samples for determining the algal density were taken at every 24 h. All samples were preserved by adding 1 µL of 1% Lugol's Iodine solution to per 99 µl of sample. The preserved samples were then counted using the Neubauer cell counting chamber and a stereo zoom microscope (Olympus SZX12 with Olympus DF Plapo 40 × PF microscope lens).

Determination of total and dissolved silver

Actual silver concentrations and ionic silver concentrations were measured at the highest test concentration used (100 µg Ag L^{-1}) in *R. subcapitata* treatments only to reduce the number of samples. Samples were taken after 72 h from the medium, digested with HNO_3 and HCl, heated and dried overnight and resuspended with diluted Aqua regia (11 mL HNO_3 and 33 mL HCl per 100 mL). Ionic silver samples were also taken from the medium and ultracentrifuged (30 min, 10,000 g, MiniSpin, Eppendorf, Hamburg, Germany) using ultracentrifugation cups (Vivaspin 500, Sigma-Aldrich Chemie GmbH, Munich, Germany). Samples from the supernatant were prepared as described (Cornelis et al., 2010) for medium samples and measured using a graphite furnace atomic adsorption spectrometer (GF 90 and Solaar 989QZ, Unicam, Cambridge, UK).

Data analysis

The average specific growth rate for the whole experiment (72 h) and for every 24 h section were calculated according to the OECD guideline 201 (OECD, 2011). For statistical analysis, the software R, version 3.3 (R Core Team, 2016) was used. For determination of EC_{50} values, the drfit package of R was used which was performed with linlogit or probit model. To compare the differences in media composition one way ANOVA was performed alongside with paired *t*-test. Levene's test was also applied to test homogeneity of variance before running the ANOVA.

3. Results.

Characterization of Ag nanoparticles

The shape of detAgNP was mostly spherical or close to spherical, but also triangular and cubical shapes were detected (Figure 8.1). The nanoparticles were well dispersed and in the size range of 2 to 20 nm in diameter.

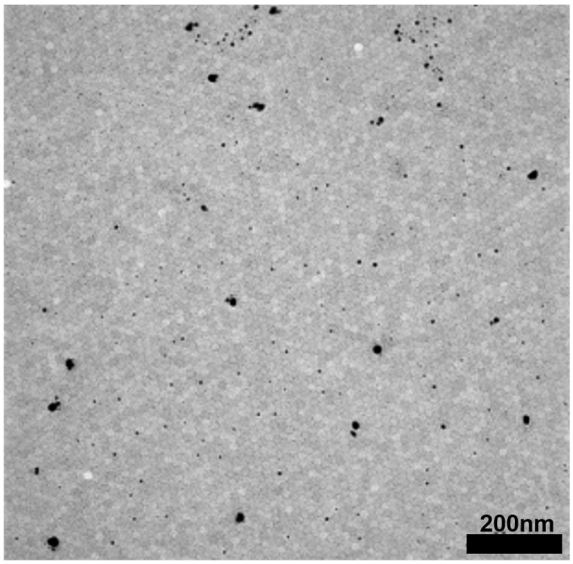

Figure 8.1 TEM picture of detAgNP in dispersion (diluted with Milli-Q water).

The z-average hydrodynamic diameter (HDD) ranged from 78 to 114 nm in the beginning of the test and was between 102-145 nm after 72 h (Table 8.2). HDDs in were comparable in all media with slightly higher results in P reduced media in the beginning as well as the end of the experimental period (Table 8.2). All HDDs increased over time without any media showing exceptional increase. The polydispersity indices for all media remained between 0.2 and 0.3 during the 3 days of aging. At day 0, zeta-potentials were significantly higher in P reduced media compared to the standard medium (about-13 mV vs. about -16.5 mV, respectively; $p<0.001$, ANOVA, Table 8.2). Zeta-potentials significantly decreased within the exposure period in the P reduced media ($p<0.001$, ANOVA for 50% P and $p<0.001$, ANOVA for 25% P; Table 8.2). And at the end of the test, zeta-potentials were in a comparable range in all media at the end of the test ($p=0.05$, ANOVA Table 8.2,).

Maximum absorption ranged between 405–415 nm for all media from day 0 to day 3 (Table 8.2). The peak extinction decreased in all media between 10% to 20% from day 0 to day 3 and peaks for day 0 appeared slightly narrower compared to day 3 (Figure 8.2).

Table 8.2 Results of the particle characterization at the beginning and the end of the test in all test media used. Particle size is given as average hydrodynamic diameter (HDD) with corresponding polydispersity index, surface portential as zeta-potential with corresponding conductivity, and colloidal stability is indicated by adsorption peak wavelength (λ) and corresponding absorbance (peak Absorbance, pA). All values are given as mean values with standard errors (n=6).

Media	Time	HDD [nm]	Poly-dispersity Index (PDI)	Zeta-potential [mV]	Conductivity [mS/cm] →	λ [nm] →	pA
OECD	0 h	78.0 ± 9.0	0.27 ± 0.08	-15.8 ± 0.6	0.169 ± 0.002	413	1.06
	72 h	121.5 ± 10.5	0.21 ± 0.02	-16.4 ± 0.5	0.177 ± 0.001	411	0.89
50% N	0 h	79.2 ± 5.9	0.21 ± 0.07	-17.0 ± 0.3	0.171 ± 0.001	410	0.68
	72 h	102.2 ± 6.8	0.25 ± 0.04	-16.7 ± 0.6	0.175 ± 0.001	410	0.56
25% N	0 h	89.5 ± 9.1	0.20 ± 0.05	-17.7 ± 0.7	0.169 ± 0.001	414	0.82
	72 h	133.5 ± 10.2	0.24 ± 0.01	-16.3 ± 0.3	0.176 ± 0.001	414	0.81
50% P	0 h	100.3 ± 11.8	0.22 ± 0.04	-13.6 ± 0.5	0.169 ± 0.001	409	0.57
	72 h	145.0 ± 10.7	0.27 ± 0.02	-16.7 ± 0.3	0.172 ± 0.002	410	0.46
25% P	0 h	113.7 ± 6.8	0.18 ± 0.01	-13.2 ± 0.4	0.171 ± 0.001	410	0.68
	72 h	140.8 ± 13.6	0.28 ± 0.03	-17.1 ± 0.8	0.173 ± 0.002	410	0.63

Response of the two algae species in different media

Control growth

For both algae species, the average specific growth rate was highest in OECD medium with 1.78 ± 0.48 for *D. subspicatus* and 1.41 ± 0.39 for *R. subcapitata* (Supporting Information, Figure S8.1). The growth rate decreased in all nutrient reduced media in the following order: 50% P, 25% P, 50% N, and 25% N. Minimal growth rates were about 1.3 for *D. subspicatus* and around 1.1 for *R. subcapitata.* Growth rates were always higher for *D, subspicatus* than for *R. subcapitata* irrespective of media composition.

AgNP toxicity

The EC_{50} value in the standard medium (OCED) was 78 ± 1 µg Ag L^{-1} for *D. subspicatus* and 73 ± 1 µg Ag L^{-1} for *R. subcapitata* after 72 h (Table 8.3).

Reduction of phosphorous and nitrogen concentrations caused an increase in detAgNP toxicity for both algae species (Table 8.3). detAgNP toxicity was highest in 25% N medium for *R. subcapitata*, while 25% P and both nitrogen reductions caused similar EC_{50} levels in *D. subspicatus* (Table 8.3).

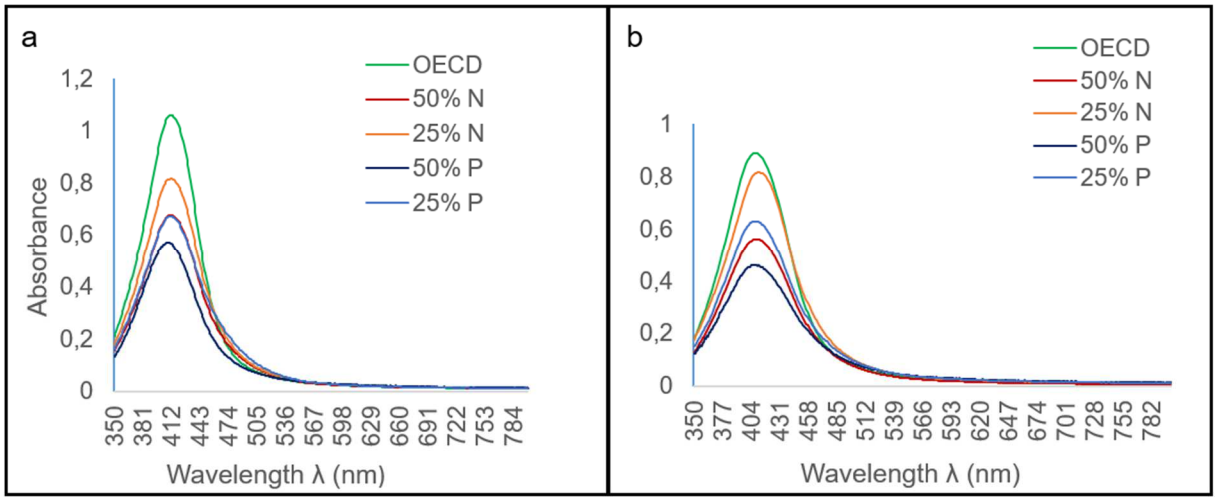

Figure 8.2. Absorption spectra of silver nanoparticles in relation to the used test medium. All absortion values are mean values (n=2). a) absorption spectra at day 0, b) absorption spectra after 72 h.

These minimal EC_{50} values ranged between 27 ± 1 and about 56 µg Ag L^{-1} for *R. subcapitata* and *D. subspicatus*, respectively. The ranking of toxicity according to EC_{50} values was OECD < 50 % P < 25 % P ≈ 50 % N ≈ 25% N for *D. subspicatus* and OECD < 50 % P < 25 % P < 50 % N < 25 % N for *R. subcapitata*.
Overall, *R. subcapitata* was more sensitive to AgNP exposure than *D. subspicatus*. This difference was smallest in 50 % P and OECD medium and largest in 25% N medium.

Table 8.3: AgNP toxicity in all media and for both test species shown as 72 h EC_{50} with 95% confidence interval. All EC_{50} values are given as µg Ag L^{-1}.

Medium	*D. subspicatus*		*R. subcapitata*	
OECD	78.08	± 1.38	72.65	± 1.48
50% N	58.04	± 1.07	39.53	± 1.73
25% N	53.19	± 1.71	27.30	± 1.29
50% P	73.20	± 0.96	70.98	± 1.30
25% P	54.77	± 2.22	48.88	± 1.79

Total and ionic silver

The total silver concentrations at the end of the experiments were clearly lower than the nominal concentration (100 µg Ag L^{-1}) with values ranging from 26 µg Ag L^{-1} in 25 % N medium to 36 µg Ag L^{-1} in 50 % N medium. This decrease was highest in both 25 % nutrient media, but this difference was not significant ($p > 0.05$, test = ANOVA, Figure 8.3a). Ionic silver concentrations were separated into two groups: in OECD and 50 % N medium, ionic silver concentrations were between 7 and 8 µg Ag L^{-1} and significantly lower compared to all other

media ($p < 0.001$, test = ANOVA; Figure 8.3b). In these media, the ionic silver concentration ranged between 13 and 16 µg Ag L^{-1}.

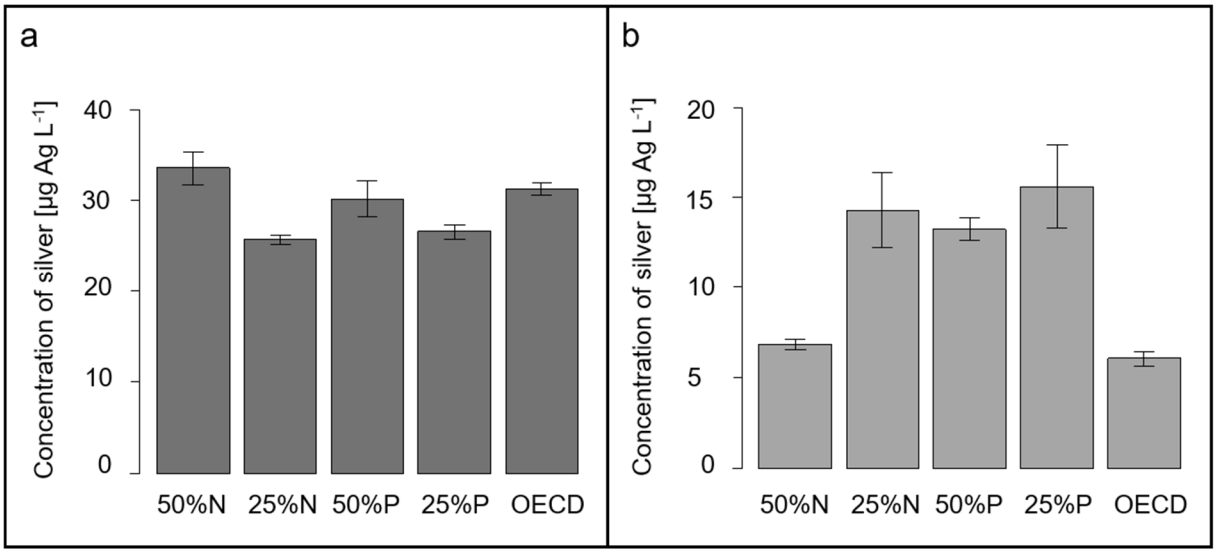

Figure 8.3: Results of the silver quantification in experiments with *R. subcapitata* at a nominal concentration of 100 µg Ag L^{-1} at the end of the test period in relation to the medium used for the test. All values are given as mean ± se (n=3). a) results of total silver in the test medium, b) results of ionic silver in the test medium.

4. Discussion

AgNP behaviour

Total detected silver in all five media were around 30% of the applied amount in test media indicating high losses of silver. Depending on the test vessel material and the stabilizer of the AgNP, losses due to sorption onto the plastic test vessels can reach values between 60% and 97% depending also on the concentration of AgNP in the medium (Malysheva et al., 2016; Sekine et al., 2015). This result emphasizes the need for ongoing analytics in algae tests. We still used nominal concentrations for the sake of simplicity, as the total silver concentrations were comparable in all test media. According to the morphological study done by transmission electron microscopy (TEM), the nanoparticles were well dispersed and spherical, with core diameters ranging from 2 to 20 nm. However, the determination of sizes in the media indicates changes in colloidal stability and dissolution during the test.

The general behaviour of AgNP was similar in all media: Increasing average HDDs and rather stable zeta-potentials and adsorption peak wavelengths. Even though the constant peak wavelengths suggest high colloidal stability, the increasing HDD and lowering of absorbance peak length from day 0 to day 3 indicate aggregation and dissolution of particles (Poda et al. 2011). However, the PDI ranged below 0.3 in all samples, which indicates rather homogeneous particle size distributions (Tomaszewska et al., 2013).

The results of the silver quantification also show that different fractions of ionic silver were present in the different test media. As the same vial of AgNP dispersion was used to prepare all test dispersions, these differences in ionic silver concentration indicate dissolution of AgNP at least in the two media containing the highest ionic silver fraction (25% N and 25% P). Differences in ionic strength might change the NP behavior (Yin et al., 2014), as sodium chloride was added as replacement in nutrient reduced media. However, in our study, the difference in NaCl molarity was 0.2 M (between the 25% N and the OECD medium), which is a small difference when compared to other media, such as Daphnia medium with a chloride content of about 4 M. Still, as both ions, sodium and chloride, are among the main ions in OECD algae medium and have total molarities of about 0.6 M, a difference of 0.2 may

become important. Dissolution of AgNP has also been reported in other studies using algal growth medium (Fabrega et al., 2009). In addition, a few studies have estimated that the solubility of AgNP can be augmented when they are in contact with microorganisms (Lok et al., 2007; Fabrega et al., 2009; Tuominen et al., 2013) or algae (Navarro et al., 2008b). To investigate the reasons for higher levels of dissolution, additional analytics investigating the dissolution of the used AgNP in the different media over time would be needed.
In the present study, however, the determination of AgNP behaviour should enable to separate effects rising from differences in nutrient supply from effects caused by differences in AgNP behaviour.

Control growth
All controls of *D. subspicatus* met the validity criteria for the algae growth inhibition test, except for the 50 % N medium. In this case, the daily growth rate from day 2 to 3 showed a variation > 35 %. For *R. subcapitata*, a similar result was obtained, but in this case, the validation criteria was violated by the 25% N medium also on day 2 to day 3. The coefficient of variation was smallest in OECD medium for both species, which is supporting the optimization for growth by the guideline. However, also the guideline suggests the use of six instead of three replicates for the control to reduce variation, and this recommendation is supported by the results obtained in this study. It was not done here due to the high workload related to counting algal numbers in the controls. However, as this increased variation only occurred for a limited time period and the result of the tests were in line with the other results for the corresponding algal species (Figure 8.3), they were included in the comparison of nutrient effects. Even though control growth met the growth rate criteria in all media, due to nutrient reduction total numbers of algae were lower in all reduced media (data not shown).

AgNP toxicity
AgNP toxicity increased with increasing nutrient reduction (Table 8.3). However, also dissolution of AgNP needs to be considered here. Navarro et al., (2008b) showed that AgNP contribute to the toxicity of green algae *Chlamydomonas reinhardtii* as a source of dissolved Ag ions. However, ionic silver concentrations were considerably lower than those required to cause the observed effects on algae in other studies (Fabrega et al., 2009; Griffitt et al., 2009). In the present study, dissolved silver concentrations were highest in 25% N and 25% P media. However, the toxicity of AgNP in 25% N medium was clearly higher than the one observed in 25% P medium for *R. subcapitata*. In addition, the AgNP toxicity was also higher in 50% N treatments than in 50% P medium for both algae species without an increase in dissolved silver concentrations. While the response of AgNP toxicity to reductions in phosphate levels can be related to AgNP dissolution, the increasing AgNP toxicity at reduced levels of nitrogen indicate that the sensitivity of algae is higher with N deficiency compared to P deficiency. According to Hecky and Kilham (1988), the internal cellular nutrient concentrations, instead of surrounding concentrations, determine marine and freshwater phytoplankton growth rates, especially in natural situations. So there might be *a* surplus stored phosphorus (luxury consumption) in algae as postulated by *Fitzge*rald and Nelson (1975*), Norman et al. (2015) and* Eixler et al. (2006*).* Even with *a* second propagation of the inoculum culture, the potential surplus phosphorus storage cannot be ignored. So, 50% reduction of P had a similar (sometimes even higher) growth rate as OECD medium in control (Figure 8.3). Consequently, P reduction had less impact on growth compared to N reduction without AgNP application.
Exposure of algal culture to AgNP has been shown to induce defense responses against abiotic and biotic stresses through the generation of various intermediates (H_2O_2, OH^-, and O^{2-} molecules), which are collectively called intracellular reactive oxygen species (ROS) (Oukarroum, et al., 2012). High levels of ROS initiation can lead to cell structure damage and may involve lipid peroxidation (Halliwell and Gutteridge, 1999). Oxidative stress can directly damage amino acids, proteins, nucleic acids, porphyrins etc (von Moos and Slaveykova, 2014). For detoxification of ROS, defense systems which include several antioxidant enzymes are used by algae. These antioxidant enzymes are recognized as indicators for the cellular level of oxidative stress (Malecka et al., 2001), which has been suggested as one of the main mechanisms by which AgNP exert their toxic effect on organism (Rinna et al., 2015).

In addition, NP have been shown to interact with various targets in the cell, including DNA and proteins (Newman and Jagoe, 1996; (Kumari et al. 2009). Repaired or fresh protein is required to overcome this modification of cell physiology and deficiency of nitrogen in growth medium, which can indirectly or directly hamper the process of amino acid metabolism and protein formation (Shimizu, 1993). So, aside from storage of phosphorous, the effect of N deficiency on AgNP toxicity may be more pronounced compared to P reduction, due to higher levels of demand for this nutrient.
According to OECD (2011), the most frequently observed growth rate per day in OECD medium for *D. subspicatus* is 1.2 to 1.5 and for *R. subcapitata* it ranges from 1.5 to 1.7 per day. In this study, *R. subcapitata* was within this range but the range was exceeded by the growth rate of around 1.75; even with 50% P reduction the value was 1.7 for *D. subspicatus.* Also the EC_{50} values from Table 8.3 suggest that the growth inhibition by AgNP was more pronounced on *R. subcapitata*. We observed colony formation for *D. subspicatus*, while *R. subcapitata* remained dispersed as single cells during exposure, which is indicating a stress response usually related to presenc of predators for *D. subspicatus* (Verschoor et al., 2004). As single cells possess a higher surface-to-volume ratio than algal colonies, less surface area of *D. subspicatus* is exposed to AgNP of the medium for each single cell of the colony. As a result, *D. subspicatus* might be less sensitive to the exposure of AgNP compare to *R. subcapitata.* The colonies were more visible with increasing AgNP exposure up to EC_{50} range for each medium. Thereafter a decreasing trend was found.

5. Conclusion

The comparison of AgNP toxicity at varying nutrient levels confirmed the strong effect of ion composition on the colloidal stability of AgNP. Still, it can be considered as not typical due to the sterical nature of the stabilization and illustrates the need to investigate ion composition influences on sterically stabilized AgNP in more detail to reveal general patterns.
However, the main aim of this work was to investigate how AgNP toxicity changes with increasing nutrient reduction. The clear increase of AgNP toxicity with decreasing nitrogen supply is in line with the hypothesis of AgNP toxicity inducing ROS and increasing cellular defense levels. The decrease of phosphorous did not cause any nutrient-related change in toxicity, most likely due to remaining phosphorous storages in the cells. This clear difference in nutrient effects illustrates the high importance of the test organism biology for estimating environmental consequences. This is further supported by the differences in sensitivity and in the effect of nutrient reduction on AgNP toxicity of the two test species, suggesting an effect of AgNP on algal community composition in the environment.
Despite both algae being able to compensate medium reduced phosphorous levels, the additional reduction of phosphorous caused higher release of silver ions, which then also caused higher levels of toxicity. In terms of environmental risk, it may be not interesting whether increased toxicity is caused by the release of silver ions or by insufficient nutrient supply. Overall, the results presented here indicate elevated environmental risk for green algae in nutrient limited freshwater systems.

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Supporting Information

Content:

S8.1: Overview on control growth as daily growth rate and mean growth rate

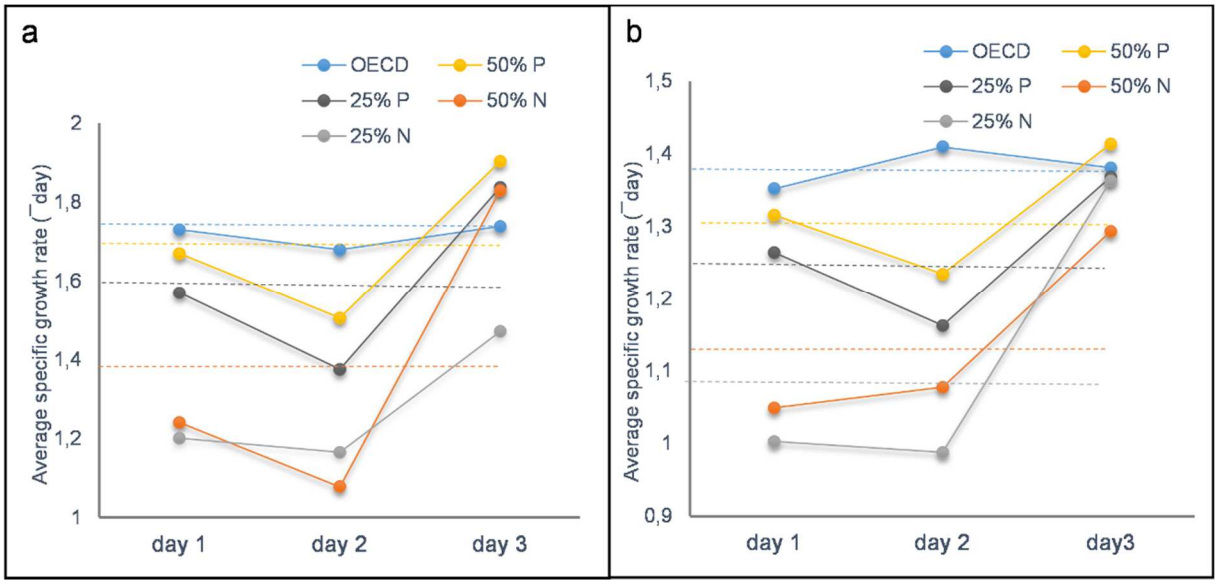

Figure S8.1 Daily growth rate in control treatments in all test media throughout the test period for both algae species. Daily growth rates are given as mean values (n=3). Mean growth rates (Day 0 to 3) are provided for comparison. Solid lines: daily growth rate, dashed lines: mean growth rates, blue: OECD medium, yellow: 50% N medium, orange: 25% N medium, light grey: 50% P medium, dark grey: 25% P medium. a) growth rates in controls of *D. subspicatus*, b) growth rates in controls of *R. subcapitata*.

9 Food reduction adds stress to chronic silver nanoparticle toxicity for *Daphnia magna*

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Submitted manuscript

Contributions of Yvonne Sakka:

- Participation in the chronic test
- Quantification of silver
- Performance of statistical data analysis
- Preparation of all figures and tables
- Writing and final improvement of the manuscript

Alexander Völkel conducted the chronic test as a part of his bachelor thesis. Juliane Filser revised the manuscript.

Food reduction adds stress to chronic silver nanoparticle toxicity for *Daphnia magna*

Abstract
Chronic toxicity of silver nanoparticles (AgNP) to *Daphnia magna* has received increasing attention in research, also with regard to influencing factors such as coating or feeding regime. However, only the effect of increasing food quantity has been tested up to now. In the present study, we investigated whether a decrease in food quantity causes increasing toxicity as suggested by the results in the above-mentioned study. For this purpose, a chronic test using the reference material NM-300K at increasing concentrations up to 6.6 µg Ag L^{-1} and three different food levels.
A moderate decrease in food quantity (75 % of standard food level) nearly no difference in toxicity compared to the standard food level was observed, while the strong reduction (50% of standard food) caused a clear increase in toxicity. However, the extent of this increase varied with the investigated endpoint of the chronic test and some endpoints did not vary between food levels at all. Number of clutches, and onset of reproduction showed the clearest response in terms of decreasing LOECs for reproductive endpoints (from >6.6 to 4.6 µg Ag L^{-1}, and from 6.6 to 5.6 µg Ag L^{-1}, respectively). And mortality in terms of relative increase: at the highest AgNP concentration, mortality increased from 40 % in the standard food treatment to 90 % at 50% food level. Even though this may rise concern about risk assessment of AgNP using standard food conditions, the high similarity in concentration dependence between most endpoints indicates that the chronic *Daphnia* test is protective despite the use of unnaturally high food supply.

Keywords
food quantity, silver nanoparticles, *Daphnia*, chronic

1. Introduction

Silver nanoparticles (AgNP) are used in various consumer products which are mostly related to sports and health (The Nanodatabase, 2016). For many of those products, for example textiles, washing is an inevitable task during the product life cycle and may cause the release of silver as ions as well as nanoparticles. Some studies have addressed the amount and type of the released silver from textiles into the wastewater system, as well as their fate during the wastewater treatment process (Benn and Westerhoff, 2008; Geranio et al., 2009; Gottschalk et al., 2013; Kaegi et al., 2011). In most cases, releases have been reported to be at very low levels under standard washing conditions and include varying amounts of particles in the range of "nano"(= smaller than 100 nm) (Benn and Westerhoff, 2008; Geranio et al., 2009). The amounts and sizes of the released AgNP as well as their fate are highly influenced by the identity of the washed product and the washing conditions chosen (Benn and Westerhoff, 2008; Wigger et al., 2015). However, a constant release of low doses of AgNP can be assumed for a simplified experimental design.

Despite these results, most experimental studies on AgNP ecotoxicity have used acute tests with high doses compared to estimated environmental conditions (Fabrega et al., 2011), and results of chronic toxicity studies or mesocosm experiments are just emerging (Sharma et al., 2014). For the commonly used aquatic test organism *Daphnia magna*, concentrations causing negative effects during chronic exposure are in the range of several to more than one hundred µg Ag L^{-1} (Blinova et al., 2013; Mackevica et al., 2015; Sakamoto et al., 2015; Sakka et al., under review; Zhao and Wang, 2011). Predicted environmental concentrations are mainly in the range of ng L^{-1} (Gottschalk et al., 2013). Thus, long-term exposure of AgNP may only be critical in case of additional factors which increase effects of AgNP.

For short-term experiments with AgNP, it has been shown that exposure conditions strongly affect AgNP behavior and toxicity (reviewed by Levard et al., 2012; Sharma et al., 2014). An important difference between acute and chronic toxicity tests with *D. magna* is the presence of food. Food in terms of algae has been shown to reduce acute toxicity of AgNP (Blinova et al., 2013; Sakamoto et al., 2015), but also to represent one of the main uptake routes of AgNP (Zhao and Wang, 2011). In addition, Lam and Wang (2006) showed that dietary assimilation of silver was much higher at low than at high food concentrations, which may be also the case for AgNP.

Beside affecting the toxicity of AgNP, algae as food directly affect the population growth of daphnids; a relation which has been intensely studied and modelled (e.g. Boersma, 1997; Giebelhausen and Lampert, 2001; Gliwicz et al., 1981; Guisande and Gliwicz, 1992; Lampert et al., 1986; McCauley et al., 1990; Rinke and Vijverberg, 2005). Increasing food quantity was well-related to increased individual size as well as population growth in the laboratory (Boersma and Vijverberg, 1994a; Lynch, Michael, 1989; Vijverberg, 1976), and in the field (Boersma and Vijverberg, 1994b; Lampert, 1978; Müller-Navarra and Lampert, 1996). This relation has also been confirmed by modelling of data (Enserink et al., 1993; Rinke and Petzoldt, 2003; Vigano, 1993). Algae in tests with *D. magna* may thus have two different functions: serving as energy resource (food) and as "carrier" of AgNP into the test organism.

To our knowledge, only one study addressed the effect of food availability on AgNP toxicity (Mackevica et al., 2015). In this study, 3-fold elevation of food quantity reduced the toxicity of citrate coated AgNP in terms of mortality, growth and reproduction compared to the standard food level, indicating interactions between food quantity and AgNP toxicity. The authors suggest that either released silver ions (Ag^+) are reduced by complexation with exudates of the algae used as food, or that additional food reduced the uptake of AgNP through a dilution effect. In the present study, we addressed whether we can find a similar pattern for the relation between food quantity and AgNP toxicity using differently stabilized AgNP and food reduction

instead of increase. The later is also of interest in the context of environmental hazard of AgNP: if food decrease increases AgNP toxicity, it may be possible that LOECs reach estimated environmental concentrations.
To investigate the relation between AgNP exposure and food decrease, a full-factorial design with three different food levels and five AgNP concentrations was used. Based on the assumption of food being an important energy resource and the reduced uptake of silver at higher food quantities, we expected higher growth, as well as reproduction when more food is provided. In addition, we expected the lowest mortality to occur at standard food conditions, and based on other chronic results (Blinova et al., 2013; Mackevica et al., 2015; Sakamoto et al., 2015; Sakka et al., under review; Zhao and Wang, 2011) mortality to be the most sensitive endpoint at all food conditions.

2. Materials & Methods

Test Chemicals

Silver Nanoparticles (detAgNP) were purchased from ras materials GmbH (Regensburg, Germany; standard material NM-300K). detAgNP were of spherical shape and a mean size of 20 nm according to the producer information. They were delivered as 10.16% wt silver in a dispersion containing 4% TWEEN and TAGAT TO each. Prior to use, the delivered dispersion was diluted to a silver content of 2% wt with Milli-Q-water (Millipore, Merck KgaA, Darmstadt, Germany), sonicated for 15 min in a water bath (Sonorex, RE100H, Bandelin electronic GmbH & Co KG, Berlin, Germany), and used to prepare a stock dispersion in the test medium, Elendt M7, with a nominal silver concentration of 10 mg Ag L^{-1}. The medium stock dispersion was stored in the dark at 4 °C until use.
Stability and mean particle size during the chronic test were measured in a pilot test with two beakers of stock dispersion and the same temperature, test volume and illumination, but without algae or *Daphnia*. Measurements were done every 24 h by absorption analysis (Cadas200 Spektralphotometer, HACH LANGE GmbH, Berlin, Germany) and dynamic light scattering (DLS) and (Delsa NanoC, Beckmann Coulter Inc., Brea, USA) with 10 repetitions in case of DLS and 6 in case of zeta-potential measurements. Results of both beakers were pooled after measurement to reduce scatter between samples. One sample of the stock dispersion was measured again prior to the start of the test to assure similar starting conditions.

Test Organisms

The green algae *Raphidocelis subcapitata* (strain no. 61.81, from the Culture Collection of Algae SAG, Göttingen, Germany) was used for feeding. Algae were cultured using a green algae medium (see Supporting Information, SI Table 1) and permanent light at 20 °C. Based on the assumption that one algae corresponds to an average of 1.59 * 10^{-8} mg C, *D. magna* cultures were fed 0.15 mg C *Daphnia*$^{-1}$ d^{-1} with every media exchange twice a week.
D. magna originated from Bayer GmbH (Monheim, Germany) and were cultured in our laboratory for several years after obtaining them from the Center for Environmental Research (UFZ; Leipzig, Germany). *D. magna* were cultured at 20 ± 1 °C using a 16:8 h light-dark cycle and a density of 20 animals L^{-1}.

Chronic *Daphnia* Test

The chronic *Daphnia* test was conducted according to OECD guideline 211 (Organisation for Economic Co-operation and Development, 1998), using 10 replicates per concentration, 20 ±

1 °C, and a 16:8 h light-dark cycle. A linear equation was used to calculate the optimal amount of food throughout the test (mg C = 0.0057 * d + 0.008; with d = median day of the media exchange period). Food was added with every media exchange, every 2 to 3 days. To assess the effect of food reduction, three quarters and half of the calculated optimal amount of food were used. The three different food regimes (100 %, 75 %, 50%) were applied in a full-factorial design with four concentrations (5, 9, 11, 13 µg Ag L^{-1}) and a control. Survival and reproduction was controlled daily, number of molts with every media exchange. At the end of the test, animals were fixed with formaldehyde at a final concentration of 1.5 % (v/v). Body length was measured using picture analysis and a stereomicroscope (Olympus SZX-ILLB200, Optical Co. LTD, Ritto, Japan) connected camera (MCA-1000, Müller Optronic GmbH, Erfurt, Germany). Analyzed endpoints were survival, reproduction (including cumulative number of neonates, day and number of neonates at first reproduction, cumulative number of clutches, and mean number of neonates per clutch), cumulative number of molts and body length.
To obtain actual values for carbon content of the algae used for feeding during the chronic test, carbon content was measured prior to feeding with every media exchange: First, the algae were centrifuged for 10 min at 4480 rpm (3-18K, Sigma, Osterode, Germany) and re-suspended in carbon-free algae medium (SI Table 1). The re-suspended algae were counted with a cell counting chamber (Neubauer, Marienfeld GmbH & Co KG, Lauda-Königshofen, Germany) and their carbon content was analyzed by laser driven CO_2 detection (C-mat 5500, Ströhlein Instruments, Germany) after combustion (I-05/RP and D-03GTE, Ströhlein Instruments, Germany). The corresponding carbon content per alga was then calculated and the algae culture stored in the dark at 4 °C for use the following day.
Actual silver concentrations in the test dispersions were measured at the beginning and the end of every media exchange. For this, 100 µL of test dispersion were acidified with 1% HNO_3 and stored in the dark. The highest concentration of each food regime was measured using graphite furnace atomic adsorption spectrometry (GF 90 and Solaar 989QZ, Unicam, Cambridge, UK). After measurement, time weighted mean was calculated for each food level in the measured concentration as described in the guideline (OECD, 1998). The proportional relation between time weighted mean and nominal concentration was used to calculate actual concentrations of all other concentrations.
For estimating differences in silver uptake, 3 animals of each food level were also measured by graphite furnace atomic adsorption spectrometry and silver body burden in relation to weight after fixation was calculated. This analysis was only done at 11 µg Ag L^{-1} due to the detection limit of the device (about 0.03 µg Ag g^{-1}) and the limited number of surviving animals at higher concentrations.

Data Analysis

Each endpoint of the chronic test was analyzed separately using R version 3.1.2 (The R project, 2016), In no case homogeneity of variance could be observed or reached after transformation when both silver concentration and food level were used, so the data were split according to food levels and to silver concentration and analyzed separately. Details on transformations and used models are listed in the Supporting Information (SI, Tables S9.1-S9.7). For all endpoints and all datasets, Levene's test was used to analyze homogeneity of variance and if this criterion was met, linear models were applied to datasets using silver concentration or food level as only explanatory variable, and one-factorial ANOVAs with post-hoc analysis (Tukey's test) were used to determine of LOEC and NOEC values or reveal significant differences between food levels. In case homogeneity of variance was not given, transformations were applied to the data (square-root or boxcox) and the test was repeated. If the data did still not show homogeneity of variance, Kruskal-Wallis tests with post-hoc analysis

(multiple comparison of means for Kruskal-Wallis tests, package pgirmess) were made. All linear models and ANOVAs were checked for normality of errors using a Shapiro-Wilk test.

3. Results

Particle characterization

Results of the particle characterization of all measurements are summarized in Table 9.1. Cumulative hydrodynamic diameter (cumulative HDD) and peak HDD increased throughout the pilot test (150 ± 27 nm to 469 ± 27 nm and: 55 ± 2 nm to 69 ± 3 nm, respectively), while the polydispersity index (PDI) was similar in all three measurements (about 0.2). The surface potential was between -16 and -11 mV with a conductivity of 0.74 and 0.85 mS cm^{-1}.

Table 9.1 Results of the particle characterization during the pilot test and before the start of the chronic test in Elendt M7 medium at a concentration of 10 mg Ag L^{-1}. All values are given as mean values with standard errors ([a] n=20, [b] n=9, [c] n=12, [d] n=3)

Exp.	Day	Cumulative HDD [nm]	Peak HDD [nm]	Polydispersity Index (PDI)	Zeta-potential [mV]	Conductivity [mS cm^{-1}]
Pilot test	0	150 ± 27[a]	55 ± 2[a]	0.21 ± 0.02[a]	-11.1 ± 0.6[c]	0.737 ± 0.002[c]
	1	301 ± 35[a]	64 ± 4[a]	0.16 ± 0.01[a]	-16.1 ± 0.6[c]	0.784 ± 0.001[c]
	3	469 ± 27[a]	69 ± 3[a]	0.22 ± 0.01[a]	-12.4 ± 0.4[c]	0.852 ± 0.002[c]
Chronic test	0	381 ± 66[b]	44 ± 2[b]	NA	-16.4 ± 0.4[d]	0.718 ± 0.001[d]

The results for the stock dispersion used for the chronic test were similar to the results of the pilot test: The peak HDD was slightly lower in the chronic test stock dispersion than in the pilot test on day 0 while the cumulative HDD was higher than in the pilot test, but in the range of all later measurements of cumulative HDD values in the pilot test (Table 9.1). The surface potential and the conductivity were also similar to the range measured during the pilot test (16.4 ± 0.4 mV at 0.718 ± 0.001 mS cm^{-1}).

Chronic *Daphnia* test

Analytical results

The measurement of actual silver concentrations before and after each media exchange in the highest nominal silver concentration resulted in similar time-weighted mean values for all food treatments, with differences in the range of calculation and measurement uncertainty. For reasons of simplicity, one pooled time-weighted mean value was used (6.6 µg Ag L^{-1} at the nominal concentration of 13 µg Ag L^{-1}). The relative decrease between nominal and time-weighted mean was about 51 %. This value was used for the calculation of all other time-weighted mean concentrations, resulting in the following: 0, 2.5; 4.6; 5.6; 6.6 µg Ag L^{-1}, corresponding to 0; 5; 9; 11; 13 µg Ag L^{-1}, respectively. The calculated concentrations were used for all data analyses in the current study.

The measurement of silver body burden of a subsample of fixed animals at 5.6 µg Ag L^{-1} showed a decreasing trend with increasing food quantities, but differences between mean silver body burden in all food treatments were not significant (p=0.088, ANOVA). The mean silver body burdens were as follows: 100 % food = 0.8 ± 0.2 µg Ag g^{-1}; 75 % food = 2.7 ± 0.2 µg Ag g^{-1}; 50 % = 2.3 ± 0.9 µg Ag g^{-1}.

Single treatment effects

Food level effects in the control

The reduction of food reduced the reproduction in terms of cumulative number of neonates (p<0.001, Kruskal-Wallis test; Figure 9.1, Table 9.2) and mean number of neonates per clutch (p<0.001, Kruskal-Wallis test; Table 9.2). For both endpoints, the reduction at the 75 % food level did not differ significantly compared to the other food levels (Table 9.2). Number of clutches and onset of reproduction did not respond to changes in the food regime (p=0.352 and p=0.945, respectively; Kruskal-Wallis tests for both endpoints; Table 9.2).

The body length at the end of the test was also significantly smaller in the 50 % food treatment compared to the other two food regimes (6.0 ± 0.1 mm vs. 6.7 ± 0.1 mm at 75 % food and 6.8 ± 0.1 mm at 100 % food; Table 9.2). Number of molts and mortality were similar in all food regimes (8.7 ± 0.2 and 0, respectively).

AgNP toxicity at standard food level

In the standard food level treatment, detAgNP significantly reduced the reproduction in terms of cumulative number of neonates (p=0.025, linear model), total number of clutches (p=0.025, Kruskal-Wallis test), and delayed the onset of reproduction (p<0.001, Kruskal-Wallis test, Figure 9.1). However, significant differences between single concentrations could only been found for the start of the reproduction (Table 9.2). Mean number of neonates per clutch did not respond to detAgNP exposure (p=0.752, Kruskal-Wallis test).

Moulting was also significantly reduced by detAgNP exposure (p=0.007, linear model; Figure 9.2b), but no significant difference could be detected between single concentrations. Body length at the end of the chronic test was significantly higher in the lowest AgNP concentration (2.5 µg Ag L^{-1}) than in all higher concentrations (p<0.05, Kruskal-Wallis post-hoc analysis; Table 9.2, Figure 9.2a), while mortality increased with increasing detAgNP concentration up to 40 % in the highest AgNP concentration (Table 9.3) what was significantly different from all other concentrations (Table 9.2).

Table 9.2 Overview of detAgNP effects for all investigated endpoints in the chronic test. Food effects are presented in the supporting information (SI, Table S9.8). detAgNP effects are given

as NOEC and LOEC compared to control as well as compared to 2.5 µg Ag L^{-1}. All concentrations are given as µg Ag L^{-1}. The level used for significance was $p < 0.05$ and all results rose from post-hoc analysis of ANOVA or Kruskal-Wallis tests (SI, Table S9.1-S9.6).

Endpoint	**Food regime**	**NOEC**	**LOEC**	**NOEC**	**LOEC**
		compared to control		**compared to 2.5 µg Ag L^{-1}**	
Cumulative Number of Neonates	100%	6.6	> c_{max}		
	75%	6.6	> c_{max}	4.6	5.6
	50%	6.6	> c_{max}		
Cumulative Number of Clutches	100%	6.6	> c_{max}		
	75%	6.6	> c_{max}	5.6	6.6
	50%	2.5	4.6		
Mean Neonates per Clutch	100%	6.6	> c_{max}		
	75%	6.6	> c_{max}		
	50%	6.6	> c_{max}		
Onset of Reproduction [days]	100%	5.6	6.6		
	75%	4.6	5.6		
	50%	4.6	5.6		
Size [mm]	100%	6.6	> c_{max}	4.6	5.6
	75%	6.6	> c_{max}		
	50%	6.6	> c_{max}		
Cumulative Number of Molts	100%	6.6	> c_{max}		
	75%	6.6	> c_{max}		
	50%	6.6	> c_{max}		
Mortality	100%	5.6	6.6		
	75%	NA	NA		
	50%	5.6	6.6		

NA indicates treatments where no suitable model could be found.

Interactions between food reduction and AgNP toxicity

The effect of food quantity on body length was no longer detectable at the highest silver concentration (SI, Table S9.7), but in most cases, differences between food regimes remained also with increasing AgNP concentration. Despite this, number of clutches and the onset of reproduction showed decreasing LOECs when each food level was investigated separately (Table 9.2). This decrease was most pronounced for cumulative number of clutches (Figure 9.1b, Table 9.2), but these values increased slightly at higher AgNP concentrations. Although mortality increased to almost 100 at the highest AgNP concentration when food levels decreased (Tables 9.2+9.3), LOEC values did not differ between food treatments (Table 9.2). None of the other endpoints did show any interactions between detAgNP exposure and food regime.

Sensitivity of different endpoints

To compare which endpoints were most sensitive to detAgNP exposure, LOEC values and relative difference to control (% control) were used. At standard food, onset of reproduction and mortality were the only endpoints with LOECs in the tested range of AgNP concentrations. The relative decrease was 40% for mortality and about 17% for onset of reproduction. The highest relative decrease for reproduction was for cumulative number of neonates with a value of about 21%.

With decreasing amount of food, cumulative number of clutches also showed LOEC values in the tested range of concentrations (Table 9.2).

Overall, the results of the 75% food level did not differ significantly from the values measured in the standard food level at comparable AgNP exposure, but the response of single endpoints varied. Here, only onset of reproduction had a true LOEC value, while cumulative number of neonates and number of clutches showed decreases only compared to 2.5 µg Ag L^{-1} where slightly higher values were observed than in the control (Table 9.2, Figure 9.1). A LOEC for mortality could not be calculated here, as no model could be fitted to the data. The relative decrease was 40% for mortality and about 33% for onset of reproduction which was the most sensitive reproductive endpoint also in terms of relative decrease.

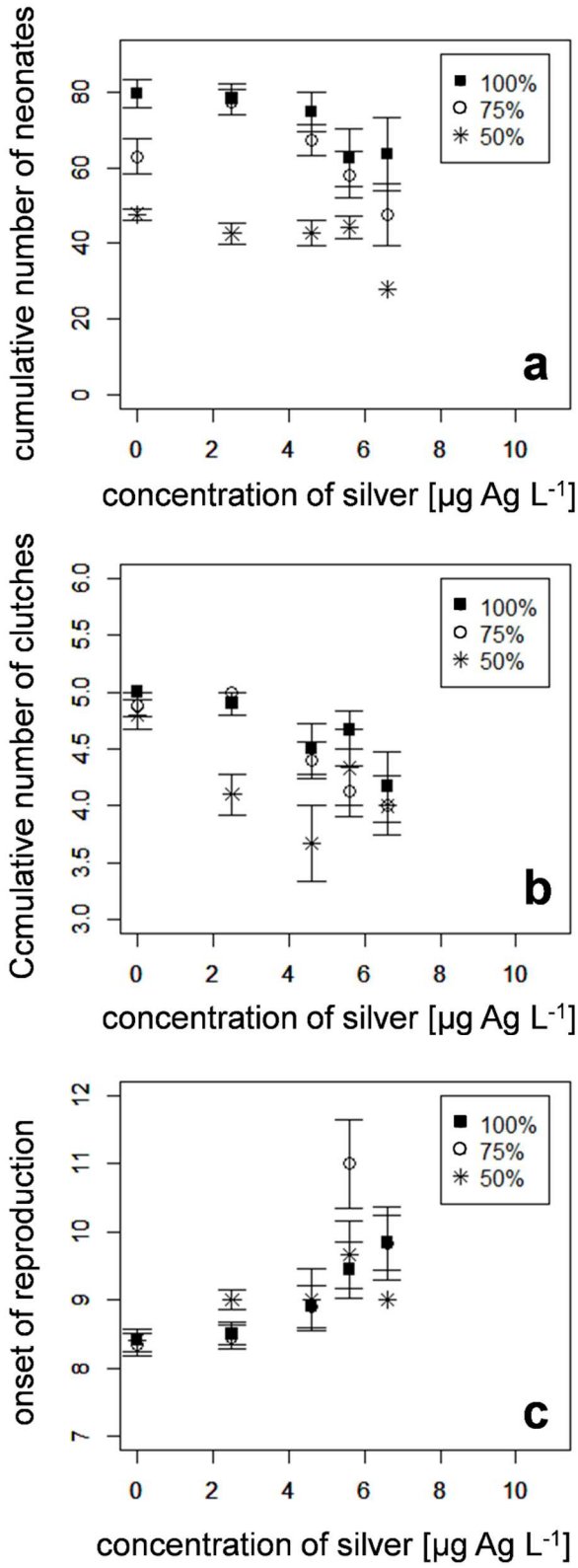

Figure 9.1 Reproductive effects of detAgNP exposure in relation to silver concentration and food level. a) cumulative number of neonates, b) cumulative number of clutches, c) onset of reproduction in days. All values are given as mean values and standard errors (n: according to survival, Table 3).

The 50% food level showed an increasing mortality compared to the standard food treatment, but a decreasing effect on number of neonates. Here, the most sensitive endpoint according to LOEC values was cumulative number of clutches, followed by onset of reproduction and mortality (Table 9.2). However, the relative decrease was highest for mortality (90%), followed by cumulative number of neonates (about 41%). Onset of reproduction and cumulative number of clutches had only low relative decreases compared to the control (about 17%).

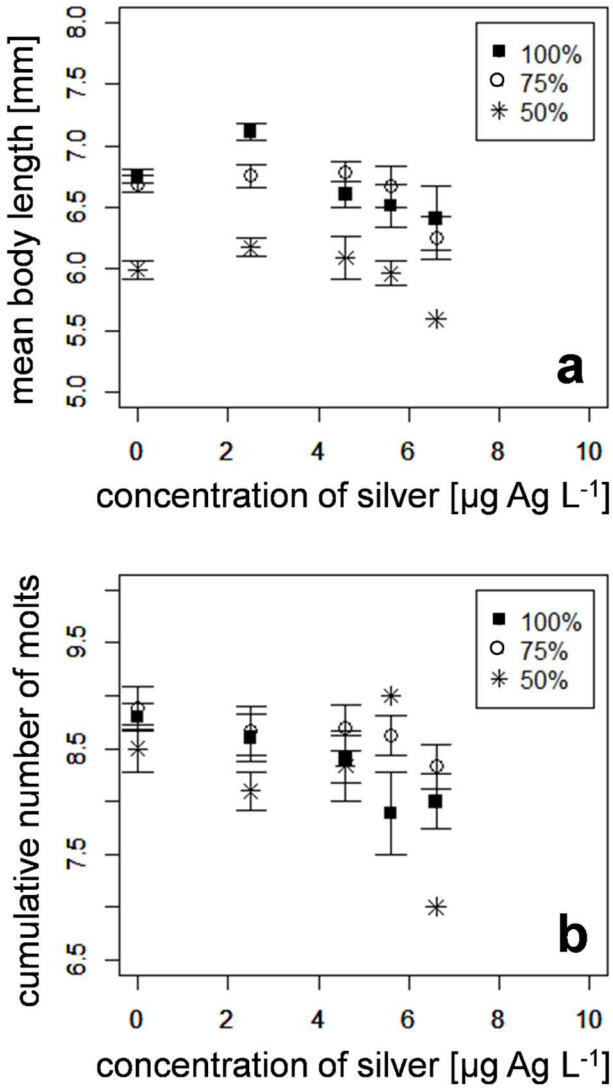

Figure 9.2 Body length at the end of the test and cumulative number of molts in relation to silver concentration and food level. a) body length, b) cumulative number of molts. All values are given as mean values and standard variation (n: according to survival, Table 3).

4. Discussion

As can be expected, food reduction caused a decrease in growth and reproduction in terms of numbers of neonates.

These results are in line with published literature on food quantity effects in several *Daphnia* species (Giebelhausen and Lampert, 2001; Guisande and Gliwicz, 1992; Lampert, 1978; Müller-Navarra and Lampert, 1996; Vijverberg, 1976). In these studies, the intensity of the observed effect varied with the algae species used as food, i.e. food quality, (Boersma and Vijverberg, 1995), as well as with the *Daphnia* species (Guisande and Gliwicz, 1992), while the response on reproduction remained similar. This is of special interest, as low levels of food caused a delay of reproduction (Boersma and Vijverberg, 1994a; Giebelhausen and Lampert, 2001; Guisande and Gliwicz, 1992; McCauley et al., 1990a) that was not detected in the present study. However, for *D. magna* as test species this effect was only detectable for the lowest food level used in the experiment (0.1 mg C L^{-1}) (Giebelhausen and Lampert, 2001). As 5 animals were placed into the same test vessel in the cited study, it is possible that the carbon quantity available was even below 0.1 mg C L^{-1}, so that the carbon concentration in our study was above the threshold for delay in reproduction.

Table 9.3 Mortality at the end of the chronic Daphnia test in relation to detAgNP concentration and food level.

Food Level	Silver Concentration [µg Ag L^{-1}]	Number of Dead Individuals
100%	Control	0
	2.5	0
	4.6	0
	5.6	1
	6.6	4
75%	Control	0
	2.5	1
	4.6	0
	5.6	2
	6.6	4
50%	Control	0
	2.5	0
	4.6	4
	5.6	4
	6.6	9

Another possible explanation is a less fixed relation between size and reproduction, so that *D. magna* do not start reproduction at a fixed size, but adapt size at first reproduction to the amount of food available. This would cause similar ages at first reproduction despite reduced

growth in the different food treatments. Size at first reproduction was lower for *D. magna* when cultured at low food conditions (Giebelhausen and Lampert, 2001). To test this hypothesis, a measurement of sizes at first reproduction would have been necessary. This was omitted in the present study due to the additional stress related to such measurements.

Using standard food conditions, chronic detAgNP exposure caused an increase in mortality, and two reproductive effects: delay in reproduction and reduction of number of neonates. Increased mortality, as well as reduction in neonate numbers has been reported for several kinds of AgNP (Blinova et al., 2013; Mackevica et al., 2015; Zhao and Wang, 2011) and has been observed for det AgNP in a previous study (Sakka et al., 2016). For mortality, the LOEC in the present study is clearly lower than those of the other studies: 13 µg Ag L^{-1} compared to 20 µg Ag L^{-1} for citrate coated AgNP (Mackevica et al., 2015), 29 µg Ag L^{-1} for PVP coated AgNP (Blinova et al., 2013), and 79 µg Ag L^{-1} or > 50 µg Ag L^{-1} for collargol coated AgNP (Blinova et al., 2013; Zhao and Wang, 2011). However, in a chronic test with detAgNP in a different laboratory, the LOEC was higher than 60 µg Ag L^{-1} (Sakka et al., 2016). This difference is still consistent when LOECs are based on time-weighted mean values (6.6 vs. > 27 µg Ag L^{-1}, respectively), and may at least partly be explained by differences in colloidal stability in the test medium. Even though Elendt M7 was used in both studies, the pH and the water used for medium preparation differed - due to the different locations and the different requirements of the *Daphnia* clones. In the present study (pH between 6.8 and 7.8), detAgNP increased in size during the pilot experiment, up to about 470 nm. In the second experiment (pH between 8.0 and 9.0), diameter remained stable at about 100 nm under comparable conditions. The same study compared two differently stabilized AgNP and showed that the AgNP with lower colloidal stability had a higher toxicity (Sakka et al., 2016). Even though clonal variation may also have added to the observed difference in LOEC values, the comparison emphasizes the strong influence of test conditions on the observed toxicity of AgNP, and underlines the importance of detailed particle characterization.

A reduction in numbers of neonates has also been observed in all other chronic studies with AgNP (Blinova et al., 2013; Mackevica et al., 2015; Zhao and Wang, 2011), while delay in reproduction has only been reported for citrate coated AgNP (Mackevica et al., 2015). Systematic investigations on chronic toxicity and certain AgNP properties are needed to relate this difference to differences in AgNP properties, similar to Blinova et al. (2013). Unfortunately, this study does not include start of reproduction as measurement of toxicity. In our previous study using detAgNP and a citrate coated AgNP, no delay in reproduction could be observed (Sakka et al., 2016), again suggesting a strong effect of test conditions on the detected result. Aside from inhibitory effects on reproduction, citrate and collargol coated AgNP have been reported to cause a reduction in size during chronic exposure (Mackevica et al., 2015; Zhao and Wang, 2011). In the present study, size reduction was observed, though not significant. As body length and molting are closely related in all crustaceans, the reduction of molts (significant in the standard as well as in the 50% food treatment) supports an effect of AgNP on size. However, this effect was not as pronounced as the effects on mortality and reproduction, while growth was one of the most sensitive endpoints in the other studies.

In the present study, the most sensitive endpoints were mortality and start of reproduction, if LOEC values are considered. However, the increase in mortality was much higher than the delay in reproduction, so that mortality can be considered to be the most sensitive endpoint at standard food level, followed by start of reproduction and number of neonates, which was the third endpoint for which a LOEC could be determined. This is similar to results for PVP and collargol coated AgNP in Blinova et al. (2013), but collargol coated AgNP have also been reported to act mainly on growth (Zhao and Wang, 2011). This difference again illustrates that test conditions strongly affect the observed toxicity and more systematic studies on the relation

between test conditions, including the used *Daphnia* clone, and observed AgNP toxicity are recommended.

When the amount of food was reduced, mortality remained one of the most sensitive endpoints and showed a clar increase in toxicity while the increase of cumulative number of neonates was less pronounced. In addition, the number of clutches became increasingly sensitive and showed the highest sensitivity (lowest LOEC) of all endpoints at the 50% food level. In the other study addressing the effect of food on AgNP toxicity, an increase in food quantity reduced the effect on reproduction and survival (Mackevica et al., 2015), supporting an interaction between AgNP effects and food supply. This is of special interest, as uptake by ingestion is an important pathway in AgNP accumulation (Zhao and Wang, 2010) and may be important for the intensity of toxic effects (Sakka et al., 2016). The observed reduction of detAgNP toxicity at higher food concentrations can thus be explained either by reduction of detAgNP uptake due to a “dilution effect“ of the additional algae present in the medium or by the additional energy provided by higher food quantities that allow higher levels of self-maintenance.

The measurement of silver body burden in the present study suggests a reduced uptake of silver at higher food quantities, but the values measured at both reduced food quantities were comparatively similar while the toxicity is largely different. So, despite the apparent dilution effect by algae not attached to AgNP, the toxicity is affected by the available energy in terms of carbon.

It is known that variations in food supply affect the toxicity, but the direction (reduction/increase) often varies with the toxicant (Pieters et al., 2006). DEBtox modelling has been used to analyze the relation between quantity of food and toxicity (Pieters et al., 2006) and allows a general description of the toxicant’s mode of action according to its effect on the model parameters (Kooijman and Metz, 1984; Pieters et al., 2006). It may be a helpful tool also for further analyzing the results of the present study to identify possible mechanisms linking AgNP uptake via algae ingestion and AgNP toxicity.

The increase of detAgNP toxicity at low food levels, however, shows that natural populations are most sensitive to AgNP when algae concentrations in lakes are low. A strong reduction in phytoplankton numbers is mainly observed during the spring clear water phase when zooplankton density is high (Lampert, et al., 1986). Introduction of AgNP during this phase of the year may increase zooplankton mortality to a large extent and elongate time to recover. However, the relation between algae and zooplankton populations is affected by numerous other factors such as temperature and presence/absence of predators, and even species-related differences in growth parameters (McCauley and Murdoch, 1987; Rinke and Vijverberg, 2005). Thus, modelling the obtained results using a number of environmental parameters is required to get a clear picture on the environmental effects of AgNP. To our knowledge, only one model addresses the effect of AgNP in the environment. In this case, a modified predator-prey model was used, indicating a reduction of average population densities for both trophic levels, but neglecting the direct and uptake-related effects of AgNP on the zooplankton community (Rana et al., 2015). Due to the comparably high sensitivity of *Daphnia* towards AgNP (Fabrega et al., 2011) and their important role in limnic zooplankton grazer communities (Lampert et al., 1986), the direct effects of AgNP on zooplankton are likely to affect natural populations in a similar manner as AgNP effects on algae.

5. Conclusion

Toxicity in the present study was detectable at comparably low levels and further increased when food was provided in low quantities. Even though endpoints differed in their response to food reduction, reproduction was further reduced by AgNP exposure at low food levels. In addition, mortality increased strongly at low food levels further adding to overall toxicity. Beside the higher levels of toxicity, NOEC and LOEC values decreased for two other endpoints (start of reproduction and number of clutches). However, these endpoints have less intense effects on population growth. Thus, for risk assessment, standard food levels can be considered to be protective, while fate models may require more information on the relation between food level, uptake and toxic effect to assess the consequence of increased toxic levels.

In addition, our study suggests that environmental concentrations slightly above the expected range of ng L^{-1} can pose a risk to *Daphnia* populations in the environment.

Acknowledgements

detAgNP (OECD material NM-300K) were provided by the UMSICHT project (BMBF 03X0091).

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Supporting Information

Table of contents

Overview on statistical analysis and results

Table S9.1 Overview on the transformations and tests used to detect effects of changes in the food regime, as well as the result of the post-hoc analysis. Multiple comparisons with Bonferroni correction were used during post-hoc analysis and significantly different results are given as different letters.

Endpoint	Silver concentration [µg Ag L^{-1}]	transformation	Used model	result
Cumulative number of neonates	control	none	Kruskal-Wallis test with post-hoc analysis	100% : a 75%: ab 50%: b
Cumulative number of clutches		none	Kruskal-Wallis test with post-hoc analysis	100%: a 75%: a 50%: a
Mean number of neonates per clutch		none	Kruskal-Wallis test with post-hoc analysis	100%: a 75%: ab 50%: b
Onset of reproduction		none	Kruskal-Wallis test with post-hoc analysis	100%: a 75%: a 50%: a
Cumulative number of molts		none	Kruskal-Wallis test with post-hoc analysis	100%: a 75%: a 50%: a
Body length		none	Kruskal-Wallis test with post-hoc analysis	100%: a 75%: a 50%: b
mortality		NA	NA	No mortality in all treatments
Cumulative number of neonates	2.5	none	Kruskal-Wallis test with post-hoc analysis	100% : a 75%: a 50%: b
Cumulative number of clutches		none	Kruskal-Wallis test with post-hoc analysis	100%: a 75%: a 50%: b
Mean number of neonates per clutch		none	Kruskal-Wallis test with post-hoc analysis	100%: a 75%: a 50%: b
Onset of reproduction		none	Kruskal-Wallis test with post-hoc analysis	100%: a 75%: a 50%: a
Cumulative number of molts		none	Kruskal-Wallis test with post-hoc analysis	100%: a 75%: a 50%: a
Body length		none	Kruskal-Wallis test with post-hoc analysis	100%: a 75%: ab 50%: b
mortality		NA	NA	No mortality in all treatments

Table S9.1 continued:

Endpoint	Silver concentration [µg Ag L^{-1}]	transformation	Used model	result
Cumulative number of neonates	4.6	none	Kruskal-Wallis test with post-hoc analysis	100% : a 75%: a 50%: b
Cumulative number of clutches		none	Kruskal-Wallis test with post-hoc analysis	100%: a 75%: a 50%: a
Mean number of neonates per clutch		none	Kruskal-Wallis test with post-hoc analysis	100%: a 75%: ab 50%: b
Onset of reproduction		none	Kruskal-Wallis test with post-hoc analysis	100%: a 75%: a 50%: a
Cumulative number of molts		none	Kruskal-Wallis test with post-hoc analysis	100%: a 75%: a 50%: a
Body length		none	Kruskal-Wallis test with post-hoc analysis	100%: ab 75%: b 50%: a
mortality		none	glm(mortality~Food_level, family=binomial(logit))	50%-75%: p=0.99 50%-100%: p=0.99
Cumulative number of neonates	5.6	none	Kruskal-Wallis test with post-hoc analysis	100% : a 75%: a 50%: a
Cumulative number of clutches		none	Kruskal-Wallis test with post-hoc analysis	100%: a 75%: a 50%: a
Mean number of neonates per clutch		none	Kruskal-Wallis test with post-hoc analysis	100%: a 75%: a 50%: a
Onset of reproduction		none	Kruskal-Wallis test with post-hoc analysis	100%: a 75%: a 50%: a
Cumulative number of molts		none	Kruskal-Wallis test with post-hoc analysis	100%: a 75%: a 50%: a
Body length		none	Kruskal-Wallis test with post-hoc analysis	100%: ab 75%: b 50%: a
mortality		none	glm(mortality~Food_level, family=binomial(logit))	50%-75%: p=0.38 50%-100%: p=0.18
Cumulative number of neonates	6.6	none	Kruskal-Wallis test with post-hoc analysis	100% : a 75%: a 50%: a
Cumulative number of clutches		none	Kruskal-Wallis test with post-hoc analysis	100%: a 75%: a 50%: a
Mean number of neonates per clutch		none	Kruskal-Wallis test with post-hoc analysis	100%: a 75%: a 50%: a
Onset of reproduction		none	Kruskal-Wallis test with post-hoc analysis	100%: a 75%: a 50%: a

Table S9.1 continued:

Endpoint	Silver concentration [µg Ag L^{-1}]	transformation	Used model	result
Cumulative number of molts	6.6	none	Kruskal-Wallis test with post-hoc analysis	100%: a 75%: a 50%: a
Body length		none	Kruskal-Wallis test with post-hoc analysis	100%: a 75%: a 50%: a
mortality		none	glm(mortality~Food_level, family=binomial(logit))	50%-75%: p=0.035 50%-100%: p=0.035

Table S9.2 Overview on the transformations and tests used to detect effect of silver concentration, as well as the corresponding p-value. p-values below 0.05 were considered to be significant and are marked as bold in the table.

Endpoint	Food Level	Transformation	model	result
Cumulative number of neonates	100%	none	Linear model (offspring ~ Comp_conc)	p = **0.025**
Cumulative number of clutches		None	Kruskal-Wallis test	p **= 0.025**
Mean number of neonates per clutch		None	Kruskal-Wallis test	p = 0.75
Onset of reproduction		Square-root	Linear model (sqStart ~ Comp_conc)	p = **0.00078**
Cumulative number of molts		none	Linear model (molts ~ Comp_conc.)	p = **0.0069**
Body length		none	Kruskal-Wallis test	p = **0.0034**
Mortality		none	General linear model (Matrix1 ~ comp_conc., family = binomial(logit))	p **= 0.048**
Cumulative number of neonates	75%	Box-Cox	Linear model (bcoff ~ Comp_conc.)	p = 0.081
Cumulative number of clutches		none	Kruskal-Wallis test	p = **0.00087**
Mean number of neonates per clutch		Box-Cox	Linear model (bcCS ~ Comp_conc.)	p = 0.96
Onset of reproduction		none	Kruskal-Wallis test	p = **0.00050**
Cumulative number of molts		none	Kruskal-Wallis test	p = 0.54
Body length		none	Kruskal-Wallis test	p = 0.13
Mortality		none	General linear model (bcCS ~ Comp_conc.)	p = 0.056

Table 9.2 continued:

Endpoint	Food Level	Transformation	model	result
Cumulative number of neonates	50%	none	Linear model (offspring ~ Comp_conc.)	p = 0.08
Cumulative number of clutches		Box-Cox	Linear model (bcC ~ Comp_conc.)	p = **0.015**
Mean number of neonates per clutch		none	Kruskal-Wallis test	p = 0.11
Onset of reproduction		Box-Cox	Linear model (body_length ~ Comp_conc.)	p = 0.011
Cumulative number of molts		none	Kruskal-Wallis test	p = **0.032**
Body length		none	Linear model (body_length ~ Comp_conc.)	p = 0.48
Mortality		None	General linear model (mat5 ~ comp_conc., family = binomial(logit))	p = **0.0022**

Table S9.3 Overview on the the result of the post-hoc analysis at the standard food level (100 %). Multiple comparisons with Bonferroni correction were used during post-hoc analysis and significantly different results are given as different letters. Resulting NOEC and LOEC values are given in addition. 2.5 NOEC and 2.5 LOEC values indicate results where significant differences similar to NOEC/LOEC were observed for the results obtained at 2.5 µg Ag L^{-1} and the indicated higher concentrations.

Endpoint	Silver concentration [µg Ag L^{-1}]	Result of post-hoc analysis	NOEC [µg Ag L^{-1}]	LOEC [µg Ag L^{-1}]	2.5 NOEC [µg Ag L^{-1}]	2.5 NOEC [µg Ag L^{-1}]
Cumulative number of neonates	Control	A	NA	NA	NA	NA
	2.5	A				
	4.6	A				
	5.6	A				
	6.6	A				
Cumulative number of clutches	Control	A	NA	NA	NA	NA
	2.5	A				
	4.6	A				
	5.6	A				
	6.6	A				
Mean number of neonates per clutch	Control	A	NA	NA	NA	NA
	2.5	A				
	4.6	A				
	5.6	A				
	6.6	A				
Onset of reproduction	Control	A	5.6	6.6	5.6	6.6
	2.5	A				
	4.6	AB				
	5.6	AB				
	6.6	B				
Cumulative number of molts	Control	A	NA	NA	NA	NA
	2.5	A				
	4.6	A				
	5.6	A				
	6.6	A				
Body length	Control	AB	NA	NA	2.5	4.6
	2.5	B				
	4.6	A				
	5.6	A				
	6.6	A				

Table S9.4 Overview on the the result of the post-hoc analysis at the standard food level (75 %). Multiple comparisons with Bonferroni correction were used during post-hoc analysis and significantly different results are given as different letters. Resulting NOEC and LOEC values are given in addition. 2.5 NOEC and 2.5 LOEC values indicate results where significant differences similar to NOEC/LOEC were observed for the results obtained at 2.5 µg Ag L^{-1} and the indicated higher concentrations.

Endpoint	Silver concentration [µg Ag L^{-1}]	Result of post-hoc analysis	NOEC [µg Ag L^{-1}]	LOEC [µg Ag L^{-1}]	2.5 NOEC [µg Ag L^{-1}]	2.5 NOEC [µg Ag L^{-1}]
Cumulative number of neonates	Control	AB	NA	NA	4.6	5.6
	2.5	B				
	4.6	AB				
	5.6	A				
	6.6	A				
Cumulative number of clutches	Control	AB	NA	NA	5.6	6.6
	2.5	B				
	4.6	AB				
	5.6	AB				
	6.6	A				
Mean number of neonates per clutch	Control	A	NA	NA	NA	NA
	2.5	A				
	4.6	A				
	5.6	A				
	6.6	A				
Onset of reproduction	Control	A	4.6	5.6	4.6	5.6
	2.5	A				
	4.6	AB				
	5.6	B				
	6.6	AB				
Cumulative number of molts	Control	A	NA	NA	NA	NA
	2.5	A				
	4.6	A				
	5.6	A				
	6.6	A				
Body length	Control	A	NA	NA	NA	NA
	2.5	A				
	4.6	A				
	5.6	A				
	6.6	A				

Table S9.5 Overview on the the result of the post-hoc analysis at the standard food level (50 %). Multiple comparisons with Bonferroni correction were used during post-hoc analysis and significantly different results are given as different letters. Resulting NOEC and LOEC values are given in addition. 2.5 NOEC and 2.5 LOEC values indicate results where significant differences similar to NOEC/LOEC were observed for the results obtained at 2.5 µg Ag L^{-1} and the indicated higher concentrations.

Endpoint	Silver concentration [µg Ag L^{-1}]	Result of post-hoc analysis	NOEC [µg Ag L^{-1}]	LOEC [µg Ag L^{-1}]	2.5 NOEC [µg Ag L^{-1}]	2.5 NOEC [µg Ag L^{-1}]
Cumulative number of neonates	Control	A	NA	NA	NA	NA
	2.5	A				
	4.6	A				
	5.6	A				
	6.6	A				
Cumulative number of clutches	Control	B	NA	NA	NA	NA
	2.5	AB				
	4.6	A				
	5.6	AB				
	6.6	AB				
Mean number of neonates per clutch	Control	A	NA	NA	NA	NA
	2.5	A				
	4.6	A				
	5.6	A				
	6.6	A				
Onset of reproduction	Control	A	4.6	5.6	4.6	5.6
	2.5	AB				
	4.6	AB				
	5.6	B				
	6.6	AB				
Cumulative number of molts	Control	A	NA	NA	NA	NA
	2.5	A				
	4.6	A				
	5.6	A				
	6.6	A				
Body length	Control	A	NA	NA	NA	NA
	2.5	a				
	4.6	A				
	5.6	A				
	6.6	A				

Table S9.6 Overview on the statistical analysis of single silver concentrations on mortality in the different food levels. p-values below 0.05 were considered to be significant and are marked as bold in the table.

Food level	Concentrations included in the model [µg Ag L^{-1}]	Model	Result	Comment
100%	0 to 5.6	General linear model (matrix2 ~ comp_conc., family=binomial(logit))	p = 1.00	All values similar
75%	0 to 5.6	General linear model (mat4 ~ comp_conc., family=binomial(logit))	p = **0.029**	All values below 5.6 similar (0 or 1)
50%	0 to 5.6	General linear model (mat6 ~ comp_conc., family=binomial(logit))	p = **0.033**	
	0 to 4.6	General linear model (mat7 ~ comp_conc., family=binomial(logit))	p = 0.999	All values below 4.6 are zero

Table S9.7 Overview on all statistical results for differences between food levels for each endpoint investigated in the chronic test at each single silver concentration. Food effects are presented as letters indicating significantly different groups with each letter. All concentrations are given as µg Ag L^{-1}. The level used for significance was $p < 0.05$ and all results rose from post-hoc analysis of ANOVA or Kruskal-Wallis tests (SI, Table S9.1-S9.6).

		FOOD EFFECTS				
Endpoint	**Food regime**	**control**	**2.5**	**4.6**	**5.6**	**6.6**
Cumulative Number of neonates	100%	a	a	a	a	a
	75%	ab	ab	ab	ab	ab
	50%	b	b	b	b	b
Cumulative Number of Clutches	100%	a	a	a	a	a
	75%	a	a	a	a	a
	50%	a	b	a	a	a
Mean Neonates per Clutch	100%	a	a	a	a	a
	75%	ab	a	a	ab	ab
	50%	b	b	b	b	b

Table S9.7 continued:

		FOOD EFFECTS				
Endpoint	**Food regime**	**control**	**2.5**	**4.6**	**5.6**	**6.6**
Onset of reproduction [days]	100%	a	a	a	a	a
	75%	a	a	a	a	a
	50%	a	a	a	a	a
Size [mm]	100%	a	a	ab	ab	a
	75%	a	ab	a	a	a
	50%	b	b	b	b	a
Cumulative Number of Molts	100%	a	a	a	a	a
	75%	a	a	a	a	a
	50%	a	a	a	a	a
Mortality	100%	NA	NA	NA	NA	a
	75%	NA	NA	NA	NA	a
	50%	NA	NA	NA	NA	b

Table S9.8 Chemicals used to prepare Green Algae Medium (GAM) and corresponding concentrations in the stock dispersions, as well as the volumes used for 1 L of GAM.

Stock solution	**Chemical**	**Concentration in stock solution [mg L^{-1}]**	**Volume of stock solution used for 1 L GAM [mL]**
A1	$MgSO_4 * 7\ H_2O$	37000	1
A2	$Na_2SiO_3 * 5\ H_2O$	56840	1
A3	$Ca\ Cl_2 * 2\ H_2O$	36000	1
A4	$NaNO_3$	339960	1
	H_3BO_3	4946	
A5	$K_2HPO_4 * 3\ H_2O$	42430	0,5
A6	$NH_4Fe(SO_4)_2 * 12\ H_2O$	321	3
	$Na_2EDTA * 2\ H_2O$	620	
	$MnSO_4 * H_2O$	20,16	
	$ZnSO_4 * 7\ H_2O$	7,68	
	$CuSO_4 * 5\ H_2O$	1,6	
	$(NH_4)_6Mo_7O_{24} * 4\ H_2O$	1,76	
	$Co(NO_3)_2 * 6\ H_2O$	0,48	
A7	Thiamin HCl	100	1
	Cyanocobalamine	0,5	
	Biotine	0,5	
A8	$NaHCO_3$	17900	3

Discussion

10 Discussion

The present work investigated how surface interactions and biological traits affect the exposure conditions of AgNP, how this relates to AgNP toxicity and how green algae and *Daphnia* respond to AgNP under limiting resource conditions.

Exposure conditions of test organisms in aquatic media can be defined by actual total silver concentration in the test dispersion and colloidal stability (agglomeration and dissolution of the AgNP) in the test medium. Both parameters, actual total silver concentration and colloidal stability, can be influenced by abiotic and biotic factors. Abiotic influences on AgNP exposure were the main focus in the first three parts of the results (5-7) of this work, while biotic influences were more prominent in the following parts. Still, biotic effects were already observed in Part 7 and abiotic effects also detected in Part 8, indicating the importance of both factors for describing AgNP exposure. The main influence on AgNP toxicity in Parts 8 and 9, however, was the response by the test organisms to resource limitation.

To link actual concentration and colloidal stability to toxicity, various analytical methods ranging from adsorption spectra measurements to separation of dissolved and particulate silver by ultracentrifugation, and additional short-term experiments were used. As shown in the detailed discussions of each results part, some more measurements would have been necessary to reveal all details of AgNP-surface interactions. The importance of analytics was asserted at the beginning of nanoparticle research (Handy et al., 2008; Kahru and Dubourguier, 2010), and characterisation of nanomaterials in the test medium has been established as a routine procedure. However, most studies still use nominal instead of actual concentrations and only a few include measurements of dissolved silver, especially in case of *Daphnia* studies. The results of the single parts, for example the comparison of analytical results obtained in Part 5 vs. 6 or the importance of the analytics for the interpretation of results in Parts 7 and 8, illustrate the importance of actual concentrations and assessment of colloidal stability in AgNP under test conditions throughout the exposure.

10.1 Abiotic influences on AgNP exposure

Medium composition has been shown to highly affect the colloidal stability of AgNP (Baalousha et al., 2013; Jin et al., 2010). However, the most used AgNP in this thesis, detAgNP, was sterically stabilized, so the influence of ions on colloidal stability was expected to be low (Tejamaya et al., 2012) and the possibility of reduced colloidal stability could not out compensate the additional work related to adapt the *Daphnia* cultures to new media conditions. Dissolution of AgNP has often been reported for tests with algae (e.g. Angel et al., 2013; Miao et al., 2009; Tuominen et al., 2013), and also in the present study, detAgNP partly dissolved in OECD algae medium (Part 9). A high influence of the stabilizer on dissolved silver fraction could be shown in a study using nine different coatings (Navarro et al., 2015), but the investigation time of only 2 h resulted in low dissolution for all AgNP. Dissolution of AgNP has been shown to increase with decreasing pH (Sigg et al., 2014). The pH values of Elendt M7 and all (modified) OECD algae media were comparable (7.4-8.2 vs. 7.8, in both Elendt M7 and all algae media, respectively); however, dissolution of detAgNP in Elendt M7 medium was only

about 2-3 % (Part 6), while the dissolution of detAgNP in OECD algae medium reached values of about 20 % in the standard composition and more than 50 % of total silver when nutrients were strongly reduced (Part 9). The increase in dissolution under limited nutrient conditions it most likely linked to the replacement of ions by sodium chloride, as chloride highly influenced the colloidal stability of AgNP (Tejamaya et al., 2012). In other studies, investigating effects of nutrient reduction on AgNP algae toxicity in natural freshwaters, dissolution was assumed to be low due to the large number of organic molecules present in these natural waters (Das et al., 2014; Norman et al., 2015). However, in a marine medium, dissolution as well as agglomeration were observed in the standard as well as in the nitrogen and phosphorous reduced media (Miao et al., 2009). However, the chloride content of algae medium remained below the chloride levels for Elendt M7 medium, also at the highest sodium chloride replacement (¼ nitrogen medium, 0.8 mol Cl L^{-1}; Elendt M7: 4.1 mol Cl L^{-1}), so the role of chloride for dissolution of detAgNP remains to be investigated. The results reported here, however, illustrate the importance of ionic silver measurements when AgNP toxicity in different media is compared.

As mentioned above, dissolution of detAgNP in the *Daphnia* medium, Elendt M7, was low and the same was observed for citAgNP (Part 6, 7, 9). This was also the case for carbonate coated AgNP and citrate coated AgNP in modified M7 media (Mackevica et al., 2015; Zhao and Wang, 2011). The more common phenomenon in *Daphnia* media is agglomeration of AgNP (e.g. Mackevica et al., 2015; Römer et al., 2011; Tejamaya et al., 2012). A 1:10 dilution of ISO as well as OECD medium has been recommended to reduce agglomeration (Roemer et al., 2011; Tejamaya et al., 2012). As mentioned above, this was not done in this work due to the long time required to adapt the test organisms to the new media conditions. Agglomeration may also be the reason for the lower degree of dissolution, as the total surface area (of all particles in dispersion) is smaller for larger agglomerates than for single particles which then reduces the area that can release silver ions into the medium. Agglomeration, as well as dissolution of detAgNP were observed during acute tests with *D. magna* depending on the ion composition of the medium (Cupi et al., 2016). Agglomeration of AgNP in Elendt M7 is most likely caused by the high number of divalent cations in this medium (about 10 times higher in Elendt M7 when compared to OECD algae medium), as divalent cations also induced agglomeration of citrate coated AgNP in another study (Baalousha et al., 2013). This hypothesis is supported by the results for colloidal stability of detAgNP in Elendt M7 medium: detAgNP was rather stable according to the adsorption peak wavelength (410 to 412 nm) in all experiments and the hydrodynamic peak diameter (Figure 10.1a). However, peak diameter measured in the experiments conducted at Copenhagen in Elendt M7 (DTU M7) were clearly higher (about 100 nm) than the peak diameter measured at Bremen in Elendt M7 (UB M7; about 60-70 nm).

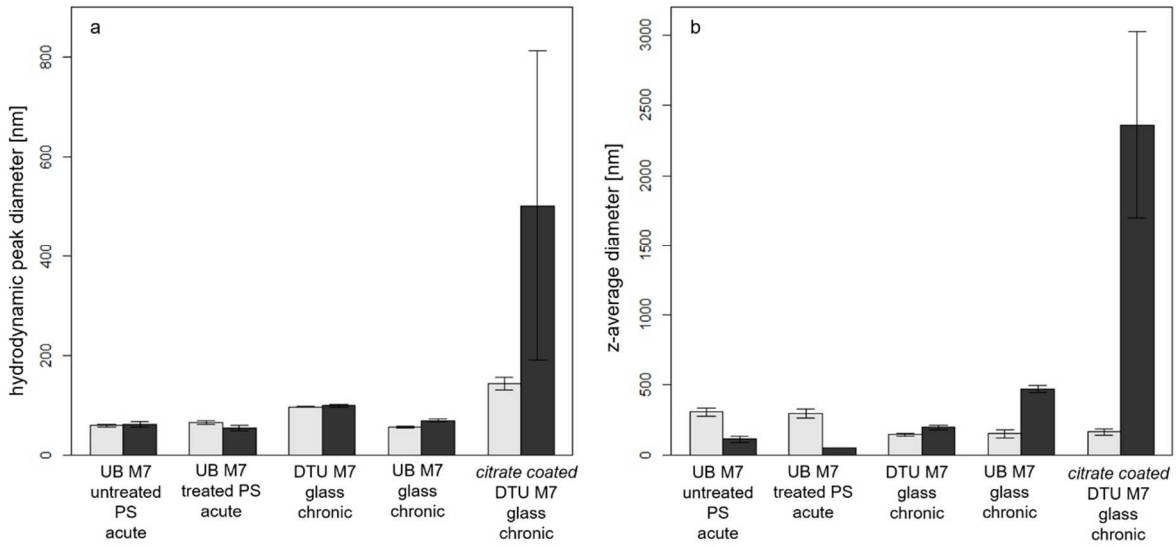

Figure 10.1 Overview of hydrodynamic diameters measured for both AgNP in the Daphnia medium, Elendt M,7 in all experiments of this work in relation to the used medium (UB M7 and DTU M7), the surface material of the test vessel, and the test design. Each size is given as mean value with standard error (n = 30 for acute tests, 9 for DTU M7, 20 for the chronic test in UB M7) in the beginning and the end of the characterization period (4 d for acute tests, 2 d for DTU M7, and 3 d for the chronic test with UB M7). PS: polystyrene, *citrate coated*: citAgNP. a) hydrodynamic peak diameter according to non-linear-least-square calculations, b) hydrodynamic diameter as z-average (cumulant result).

However, the comparison of z-average diameters (cumulant results, Figure 10.1b) show a small degree of agglomeration in DTU M7 (Part 7) and high degrees of agglomeration in the characterisation of detAgNP in the chronic test with UB M7 (Part 9). In contrast, strong decreases of z-average were found during acute exposure in UB M7 (Part 6). The most likely explanation for these deviations of z-average values is the sedimentation of large agglomerates that are then no longer included in the dynamic light scattering measurement. Sedimentation was observed during the short-term characterisation in DTU M7 (Part 7), but not for UB M7 (Parts 6 + 9).

However, sedimentation can also explain the increasing amount of silver lost from the measurement in the acute tests and the difference in lost silver fits well to explain the difference in size decrease between the two test designs (Part 6). The hypothesis on sedimentation is supported by the observation of the stock dispersions: in DTU M7, sedimentation was also detectable within days, while in stock dispersions prepared with UB M7, some black silver residues became visible at the bottom of the flasks only after weeks of storage. As this fact was also detectable for the same vial in comparable time frames, this cannot be linked to a difference in production charge or handling. Summarized, it can be said that the mean size (hydrodynamic peak diameter) of detAgNP in Elendt M7 medium is stable throughout typical exposure periods for daphnids and that large agglomerates will be lost from the medium by sedimentation. The difference in sedimentation during acute exposure was also influenced by the interaction between the stabilizing detergents and the test vessel (see also Part 6). The comparison of stock dispersions for chronic tests suggests higher degrees of sedimentation in

the DTU M7 compared to UB M7, which is supported by the higher peak diameter observed here. The main difference in Elendt M7 media between the two locations was the pH used for culturing and testing of the different *D. magna* clones: about 7.4 in Bremen and about 8.2 in Copenhagen. However, experiments on pH effects on detAgNP showed only minor pH effects, while the use of lower concentrations seemed to induce agglomeration in test dispersions compared to the stock (Cupi et al., 2016). As the concentration used for characterization in Copenhagen was ten times higher than in Bremen and higher sedimentation, a relation to concentration is not likely in this work. It is also possible that slight differences in ion composition due to the use of different stock solutions and bases (Milli-Q vs. demineralised water at DTU and Bremen, respectively) caused this effect, but due to the high ionic strength of Elendt M7 medium, such differences could also be expected to be low. Still, systematic investigations are needed to separate pH, ion composition and preparation process related effects and the clear difference to other works with detAgNP in DTU M7 (Cupi et al., 2016) support this finding. Overall detAgNP in Elendt M7 were relatively stable concerning the mean peak diameter, which confirms the relatively high stability of sterically stabilized AgNP. However, the results also show that especially for this kind of AgNP more systematic investigations are needed to evaluate the effects of test conditions on its behaviour. The observation that sedimentation most likely took place during all tests shows the high importance of this process for exposure conditions, especially for test organisms in contact with the bottom of the beakers.

As citrate coated AgNP were used for only one of the experiments, their colloidal stability is discussed in the corresponding section (Part 7). The comparison of colloidal stability of citAgNP and of detAgNP (Figure 10.1) illustrates the high influence of the stabilizer on colloidal stability. It is interesting to note that despite this clearly lower stability, actual silver concentrations in the medium were higher for citAgNP (Table 7.2), which seems contradictory at first. If initial concentrations were compared, test dispersion concentrations of detAgNP were already lower than those of citAgNP. This indicates some loss of detAgNP prior to or during test dispersion preparation and/or storage. As sonication and shaking were used prior to preparing subsequent dilutions for detAgNP, this loss cannot be explained by sedimentation and indicates some loss from the medium due to attachment to surfaces, like those of pipette tips or storage vials.
Even though the use of silver as core material suggests attachment to various surfaces (Welz and Sperling, 1997), the coating or surface covering also has a strong effect on attachment of AgNP to all surfaces present during the experiment (Part 7, Malysheva et al., 2016; Sekine et al., 2015; Song et al., 2011). Such differences have been shown for tannic acid and bPei (branched polyethyleneimine) coated AgNP using polypropylene, polycarbonate and glass test vessels (Sekine et al., 2015). Differences in AgNP attachment related to differences in surface material were also observed in some studies (Part 6, Malysheva et al., 2016; Sekine et al., 2015).

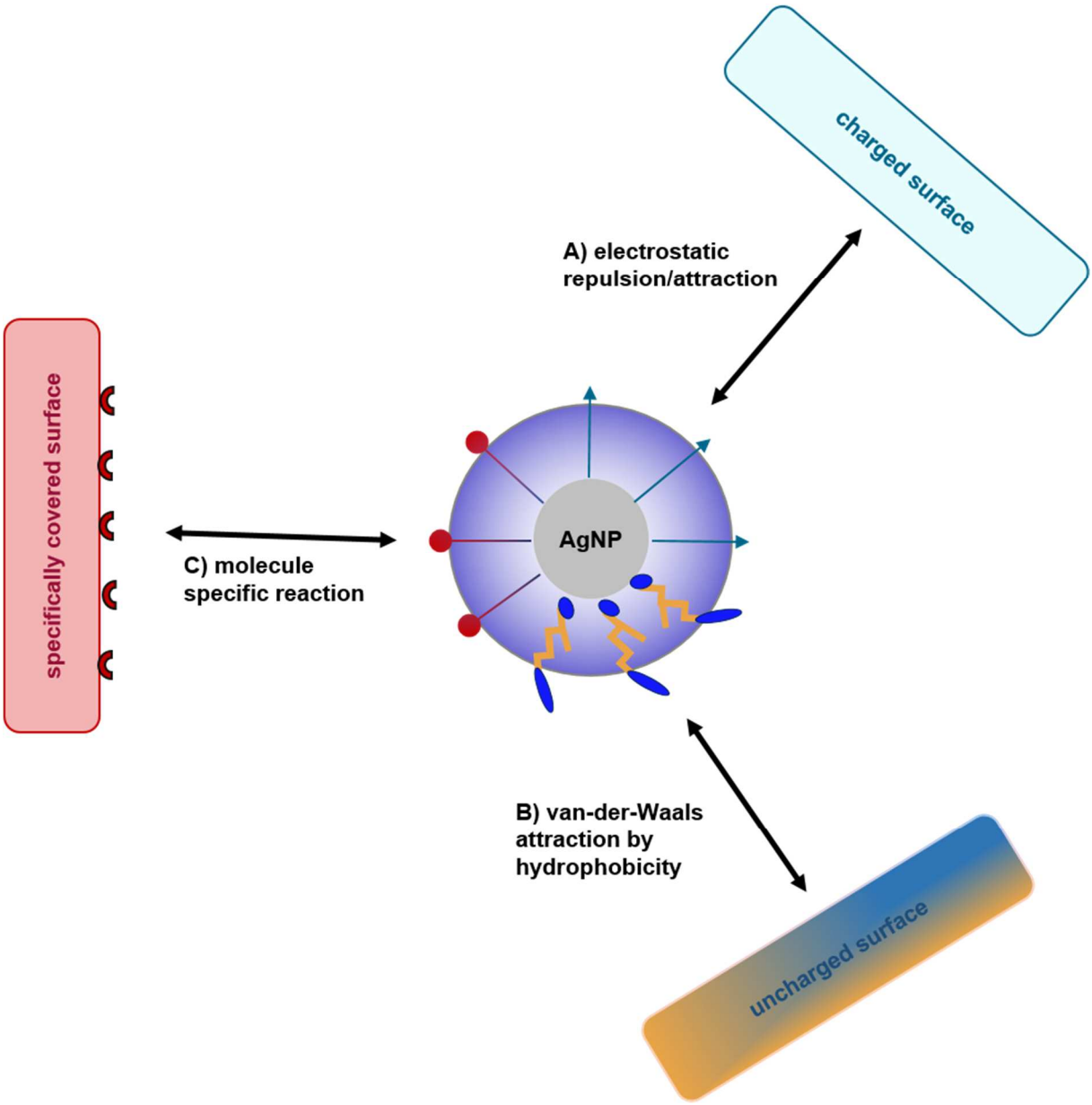

Figure 10.2 Schematic illustration of the main processes driving interactions between AgNP and surfaces present in a test system. As each process is highly influenced by the actual molecules present on the AgNP surface, different shapes and colours were used to illustrate each single interactions representing the main property of the covering molecules. A) interaction between the AgNP and the surface is dominated by surface charge, B) interaction between the AgNP and the surface is dominated by hydrophobicity, c) interaction between the AgNP and the surface is dominated by a specific interaction (e.g. a chemical reaction) between certain surface groups. Arrows indicate charges of the covering molecules (A), strongly orange and blue sections represent hydrophibic/lipophobic partitioning in the surface molecules (B), and red circles structures with a high reaction potential with specific surface structures (C).

Differences in the direction of surface charge may explain the different attachment between bPEI and tannic acid coated AgNP on glass vessel or polymer surfaces (Malysheva et al., 2016; Sekine et al., 2015, Figure 10.2, A). However, the direction of surface charge cannot

explain the difference in attachment between these two AgNP and polycarbonate, an uncharged surface, (Sekine et al, 2015), attachment of negatively charged AgNP to algae (Oukarroum et al., 2012), or the higher attachment of detAgNP to untreated polystyrene surfaces compared to hydrophilized polystyrene (Part 6).
In this context, hydrophobic interactions may play an important role (Figure 10.2, B). Song et al. (2011) showed that attachment of PVP, gum-arabicum and citrate coated AgNP increased with increasing hydrophobicity of the surface of silane and silica coated glass beads, and that this was correlated to the hydrophobicity of the coatings themselves. In case of attachment of bPei and citrate coated AgNP, attachment was not only influenced by electrochemical attraction, but also by hydrophobicity of the surfaces (Malysheva et al., 2016). Also for detAgNP, hydrophobic interactions most likely determined the attachment to untreated and hydrophilized polystyrene (Figure 6.5). Differences in hydrophobicity may have also affected differences in attachment to glass surfaces (test beakers) between citAgNP and detAgNP. Glass beads have been reported to be clearly negatively charged (-49 mV; Song et al., 2011). Thus, glass surfaces can be considered to be rather lipophobic and attachment of detAgNP is reduced to the repulsion of hydrophobic parts of the stabilizers. Still, the high attachment of negatively charged citAgNP to a most likely also negatively charged surface, contradicts the finding of relatively low adsorption to glass beads in 1mM $NaHCO_3$ (Song et al., 2011). However, addition of Ca^{2+} to the medium caused higher attachment of citrate coated AgNP on negatively charged silica surfaces (Thio et al., 2012). We hypothesized an interaction between the coating, calcium or magnesium ions in the Elendt M5 medium, and the glass surface, similar to their effect on citrate coated AgNP agglomeration (Baalousha et al., 2013), as explanation (in this case, the semi-circles in Figure 10.2, C would represent calcium ions). This calcium linked attachment could also explain the lower colloidal stability in Elendt M7 medium, compared to detAgNP that are not likely to be affected by divalent cations. A high influence of chemical surface groups was also observed for uncoated AgNP that attached to the thiol-group of a molecule, but not to its carboxyl-end (Bandyopadhyay et al., 1997). However, it was not stated whether the attachment occurred due electrochemical attraction by opposite surface charge or by a reaction between the thiol-groups and the silver core of the used AgNP.
Overall, main influences on AgNP attachment were molecule specific interactions related to electrostatic attraction for citAgNP and hydrophobicity for detAgNP. These results add to the investigations of other studies (Table 10.1) that support the importance of electrochemical and hydrophobic interactions for AgNP attachment and their relation to the properties of the stabilizers: in case of charge-stabilisation, the attachment is mainly affected by electrochemical differences, while for uncharged stabilizers, hydrophobic interactions are of higher importance. The results reported by Malysheva et al. (2016) for bPEI coated AgNP, however, illustrate that one stabilizer can be influenced by both forces, depending on the main property of the given surface. And the influence of calcium ions on electrochemical attraction (Part 7; Thio et al., 2012) shows that all aspects of a test system need to be considered for attachment ranging from test vessel surface properties, to medium composition and the chemical structure of the stabilizer. Thus, investigations on AgNP attachment to abiotic surfaces also represent basic information about their interaction with biological surfaces. For the present work, we can assume detAgNP to mainly attach to hydrophobic surfaces while citAgNP are likely to interact with positively charged surfaces as well as with negatively charged ones in presence of high divalent cations (e.g. calcium).

Table 10.1 Overview on results about attachment of AgNP to abiotic surfaces in relation to the attractive force, the corresponding surface property and the stabilizer used in this experiment.

Stabilizer/ Coating	Surface property influencing attachment	Attractive force	Reference
Citrate	Surface charge	electrochemical	Malysheva et al. ,2016
		Electrochemical, **mediated by Ca^{2+}**	Thio et al., 2012 Part 7
bPEI	Surface charge Hydrophobicity	electrochemical van-der-Waals	Malysheva et al. 2016
PVP	hydrophobicity	van-der-Waals	Thio et al. 2012 Song et al. 2011
Gum arabicum	hydrophobicity	van-der-Waals	Song et al. 2011
TWEEN 20/TAGAT® TO	hydrophobicity	van-der-Waals	Part 6

10.2 Biotic influences on AgNP exposure

Aside from test vessel surfaces, biological surfaces also interact with the AgNP in the test medium and may thus affect actual concentrations in the medium. However, any attachment to a biological surface is likely to be directly linked to toxicity – including "simple" consequences of strong attachment such as shading of algae (Ma and Lin, 2013) or moulting inhibition in *Daphnia* (Dabrunz et al., 2011). However, for AgNP only one other chronic study investigated the effect of AgNP on moulting (Gaiser et al., 2011) and none reported shading effects for algae.

Independent of shading effects, any attachment of AgNP to the algae surface is likely to be linked to toxicity. Glycodendrimer coated gold nanoparticles induced aggregation of algal cells which was most likely caused by an interaction between glycoproteins on the algal surface and the nanoparticle coating (Perreault et al., 2012), indicating that nanoparticles could also act as bridge between algae, comparable to calcium ions between citAgNP and glass surfaces (see 10.1, Discussion). In addition, also uncoated AgNP induced aggregation of algal cells (Oukarroum et al., 2012), and for some charges of citrate coated iron oxide nanoparticles agglomeration of feeding algae for *Daphnia* was observed as well (Baumann, 2014). These results indicate that agglomeration of algae may be an effect independent of the core material and the coating, but the mechanisms behind are not clear yet. However, the cell wall of algae may play an important role in this context, as no agglomeration of algae was observed for cell wall-lacking mutants (Perreault et al., 2012). A difference in cell wall morphology may have

also influenced the difference in sensitivity of the two algae species in the present study. *R. subcapitata* has been reported to possess a mucilage shell (Fawley et al., 2006), while the genus *Desmodesmus* was described by possessing submicroscopic structures in the cell wall (Guiry and Guiry, 2016) and information on mucilage was lacking. In addition, both algal species may also differ in their production of exopolymeric substances. As these can reduce the toxicity of AgNP and silver ions (Miao et al., 2009), any difference in produced amounts would cause a difference in observed toxicity. As the analysis of silver body burden in algae could not be established during this work, future research is needed to link algae cell wall morphology and excretion of exopolymers to AgNP toxicity (see also 7. Future research). Still, the algae species investigated in the present study clearly differed in their sensitivity to AgNP (Part 9). This difference was more pronounced than the ones reported in other studies (Dash et al., 2012; Oukarroum et al., 2012), but none of these added EC_{50} values for better comparability. Both studies assume that differences in cell wall structure caused varying levels of AgNP attachment and thus toxicity. However, we observed formation of colonies during AgNP exposure for *D. subspicatus* while *R. subcapitata* remained dispersed as single cells during exposure. An algal colony has a lower surface-to-volume ratio than a single cell, so for each single cell of the colony less surface area is exposed to the medium. *D. subspicatus* may thus be less sensitive to AgNP exposure compared to *R. subcapitata*, due to the comparatively simple explanation that it can reduce the surface area that can get into contact with the toxicant. The effect of colony formation has not yet been considered in AgNP toxicity to algae and further research is needed to separate the difference rising from colony formation from those rising from differences in cell wall structure and composition.

As stated in the beginning of this section, attachment of AgNP to the carapax of *Daphnia* has rarely been considered. Still, uncoated AgNP reduced the number of moults during acute tests and also partly during chronic exposure (Gaiser et al., 2011). Moulting inhibition may lead to overall growth inhibition, and the later was observed in other chronic experiments with AgNP (Mackevica et al., 2015; Zhao and Wang, 2011). However, the results of the chronic studies in this work did not reveal AgNP effects on growth or moulting (Parts 7 + 9). The carapax of *Daphnia* consists of chitin (Baumann, 2014), a polymer of N-acetylglucoasamine (Karp, 2010). This molecule is likely to be lipophobic due to the large number of oxygen atoms. In this case, low levels of attachment can be expected for detAgNP. However, the outer layer of the integument is covered with electron dense material (Halcrow, 1976) that is more likely to determine the attachment of AgNP. A more precise investigation of this material is required to estimate the attachment of detAgNP to the *Daphnia* surface. Still, based on the results obtained for growth and moulting in this work, attachment was assumed to be low (Figure 10.3, section 1) For citAgNP, no effect on moulting or growth were reported in one study (Part 7), while this was the case in the other chronic study using citAgNP (Mackevica et al., 2015). It is possible that the extent of attachment is highly affected by media composition, comparable to the attachment to abiotic surfaces (Figure 10.3, part 1). The results of this work indicate that attachment to the exoskeleton is not important for mediating AgNP toxicity, which is also supported by the results of other chronic tests (Blinova et al., 2013; Gaiser et al., 2011), but attachment may become important for some coatings/stabilizers in relation to the test conditions used (Mackevica et al., 2015; Zhao and Wang, 2011). More systematic research on AgNP attachment to the carapax and the influence of stabilizers and test conditions are needed to evaluate the importance of AgNP attachment for AgNP toxicity.

In case of ingestion of AgNP, morphological and behavioural properties influence the exposure scenario of the AgNP present in the medium. *D. magna* are able to actively filter particles in a size range of 150-640 nm from the water column (Brendelberger, 1991; Geller and Müller, 1981). As mentioned, some agglomerates in the present work passed the minimum size limit

and may be directly ingested. A relation between ingestion and agglomerate size was also reported for citrate and tannic acid coated AgNP (Zhao and Wang, 2012). Here, the larger agglomerates of about 400 nm were taken up in higher amounts than agglomerates of smaller size (slightly more than 100 nm). A similar result was obtained for 1,000 nm vs. 20 nm polystyrene beads (Rosenkranz et al., 2009). Other ingestion pathways would be passive ingestion for smaller AgNP via the water flow across the filtration apparatus or the ingestion of algae which are covered by AgNP. In case of passive uptake of AgNP, the main factor determining silver body burden would be media concentration of AgNP. In addition, passive uptake is likely to favour smaller particles, as these can be moved more easily by the water current. Despite the concentration dependence of silver body burdens (Table 7.5), higher body burdens were observed for the larger agglomerates of citAgNP compared to the more stable, smaller agglomerates of detAgNP. Active filtration is thus more likely than passive intake (Figure 10.3, part 1).

Alternatively, attachment of citAgNP to the algae present in the water column could have been higher and thus caused the described differences in body burdens. For carbonate coated AgNP, this process was the main pathway for uptake (Zhao and Wang, 2010). However, carbonate coated AgNP were additionally stabilized with cysteine which is likely to alter AgNP behaviour compared to untreated carbonate coated AgNP (Gondikas et al., 2012; Zhao and Wang, 2012) and may thus have reduced uptake via filtration. The body burdens measured in *Daphnia* at varying food concentrations (Part 9) were not significantly different, but showed a trend of increasing silver body burden with decreasing amount of food, so number of algae. Even though this result does not directly support dietary uptake of AgNP, it is also not an argument against dietary uptake: if numbers of AgNP were low enough that the increase in algae numbers resulted in a greater proportion of AgNP-free algae, the silver body burden would decrease with increasing amount of food. In addition, the interaction between the detAgNP and algae may strongly differ from the interaction of cit AgNP with algae. In summary, these results indicate the need of further investigations on the link between uptake and toxicity in relation to colloidal stability and coating/stabilizer.

Aside from the influences of feeding behaviour and algae surface on the uptake, so the internal exposure, the biology of the digestive system of *Daphnia* is also likely to affect the distribution of the AgNP in the organism. As the midgut section of the digestive tract is the only one not covered by chitin (Avtsyn and Petrova, 1986; Quaglia et al., 1976), the discussion of possible interactions between ingested AgNP and the digestive tract will be limited to this part. Up to now, no study addressing nanoparticular interactions with the digestive tract of *Daphnia* used AgNP. The results of the studies with other core materials than silver are ambiguous: interactions with midgut cells or translocations inside the organisms are reported in some studies (Heinlaan et al., 2011; Rosenkranz et al., 2009), but not in others (Khan et al., 2014; Lovern et al., 2008; Petersen et al., 2009). It is very likely that the surface properties of the used nanoparticle highly affect this interaction and thus the distribution of any nanoparticle inside the *Daphnia*. However, the close link between the higher silver body burden for citrate coated AgNP in *Daphnia* compared to detergent stabilized ones (41 % vs. 33 %, respectively; Part 7) and the corresponding difference in toxicity indicates a close link between ingestion and toxicity.

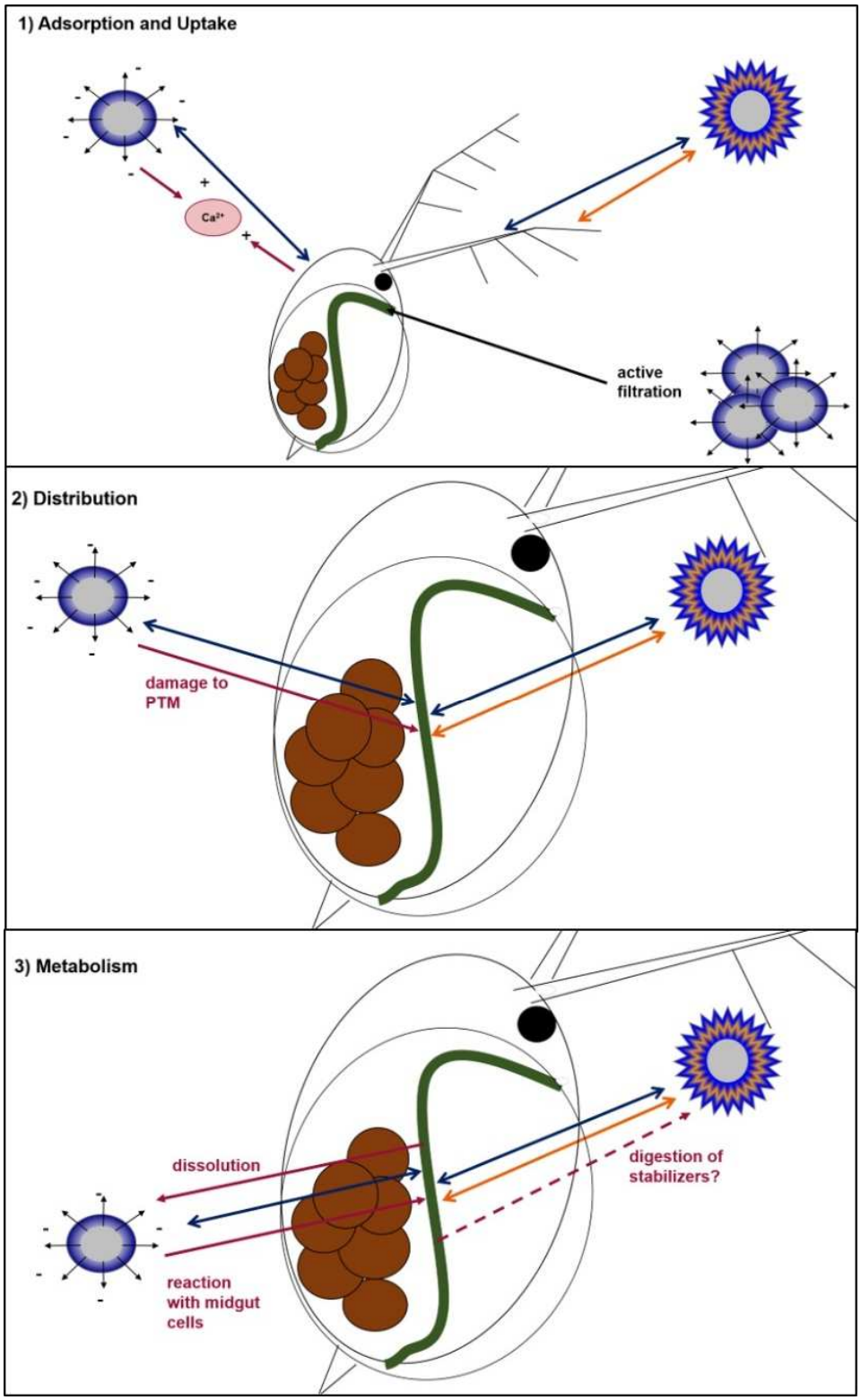

Figure 10.3 Schematic illustration of potential biotic influences of *D. magna* on detAgNP and citAgNP exposure observed in experiments conducted in this thesis in relation to the uptake pathway, starting with external attachment and ingestion (1), followed by processes in the midgut that affect the further distribution and toxic effects of both AgNP (2), to potential metabolic effects on both AgNP (3). Two-ended arrows indicate repulsion of AgNP from the surface or hindrance of interactions with biomolecules, one ended arrows indicate attraction with surfaces in section 1 and 2 and metabolic effects in section 3. Blue arrows represent steric hindrance, yellow arrows hydrophobic/lipophobic forces, red arrows molecule specific interactions.

In the midgut, the peritrophic membrane separates particles larger than 130 nm in the midgut lumen from the midgut surface (Hansen and Peters, 1997). Still, the much larger agglomerates of citAgNP acted more toxic than the smaller detergent stabilized counterpart (Part 7), also indicating a strong influence of the stabilizer on the interaction with the midgut surface. Two possible explanations would be linked to metabolic transformations: either citAgNP dissolved in the digestive tract or they exhibited a strong interaction with midgut cells, whenever smaller agglomerates passed the PTM. High degrees of dissolution of citrate coated AgNP have been observed after addition of high concentrations of cysteine to the test medium (Gondikas et al., 2012). As *Daphnia* use several enzymes to digest their food (Hasler, 1935; von Elert et al., 2004), cysteine or other thiol-rich molecules may be available for interactions with citrate. In addition, the coatings may interact in other ways with the enzymes, reducing their stabilizing properties for the AgNP. Consequently, dissolution of AgNP in the gut remains to be evaluated (Figure 10.3, part 3).
The difference in toxicity may be also linked to a mere physical effect instead of a true chemical interaction: reduced toxicity of TWEEN 80 coated AgNP was explained by the reduction of direct contact between cell wall and AgNP (Kvitek et al., 2008). As we assume a similar structure for the detergents around the silver core (Figure 4.2, Introduction), reduced toxicity may also be linked to sterical hindrance of contacts between enzymes or *Daphnia* midgut cells. However, higher hydrophobicity of the coating was related to higher toxicity to the bacterium *Bacillus subtilis* (Levard et al., 2012). As both stabilizers of detAgNP were more hydrophobic compared to citrate, the lower toxicity of detAgNP is somewhat contradictory to this finding. Still, the relation between hydrophobicity and toxicity could not be found for other bacteria species or fungi (Levard et al, 2012), indicating that toxicity of AgNP is influenced by additional factors that demand further investigation. An alternative explanation for the link between uptake and toxicity related to ingestion could be an interaction with the PTM rather than the midgut epithelium. If attachment of AgNP causes damage to the peritrophic membrane, the midgut epithelium is likely to suffer from physical damage (Figure 10.3, part 2). An effect on the peritrophic membrane and indirect effects of nanoparticle exposure were observed for exposure to iron oxide nanoparticles: bacterial numbers clearly increased when Daphnia were exposed to nanoparticles (Kwon et al., 2014). Increases in bacterial numbers could also cause deseases in *Daphnia* and thus reduce reproduction, growth or survival.

Even though the distribution and metabolism of both AgNP remain unknown in this work, the correspondence of body burden (=internal exposure) and toxicity observed in the results for Part 7 support the need for further studies in this field also for AgNP.

10.3 Effects of resource reduction on AgNP toxicity

The nutrient reduction for algae showed a clearer picture at first: fewer nutrients are correlated with higher toxicity, and this effect is more pronounced for the more effective growth nutrient phosphorous (Part 8). This result is in line with the results obtained for phosphorous enrichment on algal communities (Das et al., 2014; Norman et al., 2015). When dissolved fractions of AgNP are taken into account, however, this picture becomes more complex. In the two media with the lowest nutrient content (25 % nitrogen and 25 % phosphorous), dissolution of AgNP was higher than in all other algae media (Part 8; Discussion 10.1). Consequently, at least some part of the higher toxicity of AgNP at reduced nutrient conditions was caused by higher concentrations of ionic silver than by the reduction of nutrients. Also, reducing nitrogen

by 50% clearly increased AgNP toxicity showing similar levels of both total and dissolved silver when compared to the standard medium (OECD). The difference in AgNP toxicity between the 25 % and 50 % phosphorous media cannot not be explained by the dissolved silver fraction (Part 9), indicating an additional effect of the nutrients.

These results support previous findings about phosphorous effects on AgNP toxicity to algae using more standardised test conditions and and show the high importance of nitrogen supply for AgNP green algae toxicity. The difference between the two algae in their response to nutrient reduction indicates that respective nutrient demands and sensitivity alter the impact of AgNP on algae population composition.
The clear effect of nitrogen reduction on AgNP toxicity may be of concern for marine environments, where nitrogen levels are mostly low (Hecky and Kilham, 1988). However, studies testing the toxicity of AgNP to marine species already used seawater media and reported EC_{50} values were either higher than or similar to those of freshwater algae (Angel et al., 2013; He et al., 2016; Miao et al., 2009; Oukarroum et al., 2012).

Besides being affected by AgNP itself, algae serve as food source in ectoxicological tests with *Daphnia* and AgNP. Here, the reduction of algal numbers equals a reduction of resources and energy supply, while it is also likely to affect the uptake of AgNP attached to algae (dietary uptake, see Discussion 10.2). As the differences in body burdens were small while differences in toxicity were clearly detectable when a LOEC could be found (Table 9.2), we assumed that the reduction of energy supply is the more likely explanation for increased AgNP toxicity at lower food levels. However, the increase in toxicity did not follow the pattern for food reduction: while food reduction did mainly act on reproductive output, AgNP toxicity changed most drastically onset of reproduction and number of clutches (Part 9). The effects observed in food control were in line with patterns in the literature: decrease of offspring numbers and reduced growth (e.g. Boersma and Vijverberg, 1994; Guisande and Gliwicz, 1992), so according to the additional shortage of energy with increasing AgNP concentration, these endpoints should have been affected even more. However, this was not the case: two other endpoints that were not sensitive to food reduction were affected more strongly by AgNP at reduced food levels. Still, both these endpoints are closely related to reproduction, so that the model assumption: reproduction is limited first (Rinke and Vijverberg, 2005; Rinke and Petzoldt, 2003), is still valid. The only contradiction to the model is the increased mortality due to AgNP exposure at reduced food levels (Part 9), as self-maintenance should be reduced last.
When food levels were increased instead of reduced (Mackevica et al., 2015), the most sensitive endpoint was *Daphnia* growth, indicating a clear difference between either the two AgNP (citrate coated AgNP were used in Mackevica et al., 2015) or the *Daphnia* clones (Table 10.2). When citAgNP and detAgNP were compared in a chronic test (Part 7), no difference in endpoint sensitivity between the two AgNP was observed. In addition, the clone used for this comparison (Part 7) was the same as was used by Mackevica et al. (2015), but growth was not affected by citAgNP in this case (Tables 7.4, 10.2). It is thus unlikely that this difference in endpoint sensitivity is related to the used clone. This is supported by the high consistency of responses of different *Daphnia* species to food reduction (Boersma and Vijverberg, 1994; Giebelhausen and Lampert, 2001; Guisande and Gliwicz, 1992; McCauley et al., 1990). However, behaviour of citAgNP was different between the study presented here (Part 7) and the one on food quantity increase (Mackevica et al., 2015) that was most likely related to the modification of the Elendt M7 medium in Mackevica et al. (2015). It is interesting, however, that nominal LOECs for the different endpoints affected by citrate coated citAgNP are identical (Table 10.2), indicating a similar sensitivity despite the difference in response. Still, the

stabilizer strongly affected the toxicity at standard food levels (Part 7) and an influence on the most affected endpoints is also likely. Consequently, the effect resource variation on AgNP toxicity may also vary with the stabilizer.

However, the consequence of food reduction has been shown for the first time and the observed to increase the toxicity of AgNP for certain endpoints of the chronic test indicate that long-term effects in the environment are likely to vary over the year depending on food abundances.

Table 10.2 Overview on the test design and major results of the investigation of food increase (Mackevica et al., 2015) and decrease (Part 9) in relation to the AgNP comparison at standard food level (Part 7). AgNP toxicity is described by the most sensitive endpoint at the corresponding food regime and the largest change copared to the standatd food treatment in relation to the direction of food change (increase/decrease), the origin of the used *D. magna* clone, the tested AgNP, and the colloidal stability by main peak hydrodynamic diameter (HDD). In cases that LOEC values were observed, the corresponding nominal concentration is given in brackets as µg Ag L^{-1}. Offsping: mean cumulative number of offspring per surviving female, clutches: mean cumulative number of clutches per surviving female.

Food effect	Clone	AgNP	Main peak HDD	Most sensitive endpoint		Endpoint with the largest change of toxicity between food levels
				standard food level	highest deviating food level	
increase	DTU	citAgNP	55±5	Growth (30)	Growth (30) Offspring (30)	Offspring number (decreased toxicity)
decrease	UB	detAgNP	55±2	Offspring (11) Mortality (11)	Clutches (9)	Mortality (increased toxicity)
none	DTU	citAgNP	143±13	Offspring (30) Mortality (30)	NA	
none	DTU	detAgNP	96±1	Offspring (> 60) Mortality (> 60)	NA	

10.4 Positive effects of AgNP

In all chronic tests with *D. magna* in the present work, the lowest test concentration had some "positive" effect on the test organisms: size of test organisms was larger at 2.5 µg Ag L-1 compared to control values, so that further increase in AgNP concentrations showed a significant reduction in body size that was not detectable compared to control values (Table 9.2). The same effect was observed for cumulative number of neonates and of clutches in case of slight reduction of food quantity (Table 9.2). For citAgNP, significant increases were also

observed for cumulative number of neonates (Table 7.4). In result part 7, food quantities were accidentally reduced. As mortality in controls was comparably high and reproduction did not meet the validation criterion, one explanation would be a reduction of pathogenic bacteria by AgNP without further consequences for the daphnids. This was supported by the clearly higher toxicity of AgNP to various bacteria compared to daphnids (Table 4.2, Introduction). However, survival and reproduction were high in the other experiment (Part 9). In addition, only growth was affected under normal food conditions, while reproduction showed an increase in the 75 % food level only. As food levels were also below the recommended standard in Part 7, it is possible that the reduction of bacteria is important at low food concentrations only: when less food is available, the energy of the organism becomes limited and energy allocation inside the organism may experience trade-offs between single tasks, for example between self-maintenance and reproduction. In case of pathogenic bacteria present in the water column, more energy for self-maintenance is required that can then not be used for reproduction anymore. If low doses of AgNP inhibit bacterial growth while not causing cellular damage to the *Daphnia*, this energy can be allocated to reproduction again. When food is provided in sufficient amounts, this effect is masked by the additional energy that can be used for reproduction that increases to the maximum value and self-maintenance at the same time, and the indirect “positive” effect of AgNPs disappears. According to Rinke and Vijverberg (2005), energy is first allocated to self-maintenance and growth, before reproduction is taken into account. An increase in growth has also been reported in another study with citrate coated AgNP (Mackevica et al., 2015). However, positive effects were detectable for standard and increased food levels. More studies focussed on (indirect) positive effects are needed to evaluate the importance for the environment and the mechanism behind.

10.5 Evalution of AgNP toxicity

The EC_{50} values obtained for the two algae species in standard OECD medium are more close to the lower end of EC_{50} values reported in other studies, but still clearly higher than the lowest values (3.0 and 30 µg Ag L^{-1}; Angel et al., 2012; Navarro et al., 2015, respectively; Table 10.3). As this comparison is based on total silver contents, and dissolution of AgNP has been reported in several studies with algae including this work (Part 8; Angel et al., 2013; He et al., 2016; Miao et al., 2009; Navarro et al., 2015), comparisons between different studies need to be regarded with caution. Calculations of EC_{50} values based on ionic silver concentrations instead of total silver has been used in some cases to overcome this problem, but separate toxicity tests using only the AgNP or ionic silver fraction provide a better solution, as this considers that AgNP and ionic silver may act toxic simultaneously during the test without the removal of one fraction.

The toxicity observed for *D. magna* in this study was comparable to the results of other studies (Table 10.3) with acute and chronic exposure being more or less in the middle of the reported values, except for the chronic toxicity results obtained at DTU which were close to the upper limit under standard conditions. A comparison of AgNP toxicity to *D. magna* in this work with other studies reveal that lower EC_{50} or LOEC values was related to smaller hydrodynamic diameters than those measured in the present work in some cases (about 10-15 nm in Angel et al., 2013; Silva et al., 2014). However, the hydrodynamic diameter of PVP coated AgNP in the chronic study with the lowest LOEC in a chronic test was of similar size as in the present work (Part 9; Völker et al., 2013; about 60 nm of hydrodynamic diameter). A difference between

clones is unlikely in this special case, as the clone used for the chronic study with PVP coated AgNP (Völker et al., 2013) is the same that was used in Part 9. Consequently, this difference in toxicity can be related to the difference in surface coating without an effect of this stabilizer on colloidal stability. This comparison is supporting the conclusion on stabilizer mediated toxicity based on the results presented in this work (Part 7).

Beside confirming the toxic nature of AgNP, the results in this thesis support the initial conclusion that LOEC concentrations for AgNP are higher than predicted environmental concentrations (Batley et al., 2013; Gottschalk et al., 2013; Wigger et al., 2015). Even with additional stress by resource reduction (Parts 8 + 9), the critical values were still in the range of µg Ag L^{-1} (Tables 10.2 and 10.3). However, in case of increasing use of AgNP containing products, these levels could easily be reached and as product numbers claiming to contain AgNP (Figure 4.1), continuous supervision of environmental AgNP concentrations is recommended to ensure maintenance of environmentally innocuous conditions. The increasing toxicity at lower resource levels indicate that supervision is most important for oligotrophic environments.

Table 10.3 AgNP toxicity to *D. magna* and *R. subcapitata* in all experiments for this work compared to literature data using the lowest and highest toxicity data (if available). All values are given as µg Ag L^{-1} and as nominal concentration due to the missing comparability to the literature data in case of actual concentrations.

Test organism	AgNP toxicity	Test Medium	Toxicity criterion	Source
Raphidocelis subcapitata	72.65 ±1.48	OECD medium	EC_{50} (72 h)	Part 8
	48.88 ±1.79	¼ P medium	EC_{50} (72 h)	Part 8
	27.30 ±1.29	¼ N medium	EC_{50} (72 h)	Part 8
Minimal Toxicity	3.0±0.7	OECD medium	EC_{50} (72 h)	Angel et al. (2012)
Maximal Toxicity	1,630	OECD medium	EC_{50} (72 h)	Ksyazik et al. (2015)
Daphnia magna	33.7±9.9	UB M7 (ST)	EC_{50} (48 h)	Part 5
	22.4±3.8	UB M7 (MT)	EC_{50} (48 h)	Part 5
Minimal Acute Toxicity	1.0	Elendt M7	EC_{50} (48 h)	Allen et al. (2010)
Maximal Acute Toxicity	121	Elendt M7	EC_{50} (48 h)	Völker et al. (2013)
	30	citAgNP in DTU M7	LOEC (21 d)	Part 7
	>60	detAgNP in DTU M7	LOEC (21 d)	Part 7
	11	UB M7	LOEC (21 d)	Part 9
	9	UB M7 (50 % food)	LOEC (21 d)	Part 9
	11		LOEC (21 d)	
Minimal Chronic Toxicity	0.92	Elendt M7	EC_{10} (21 d)	Völker et al. (2013)
Maximal Chronic Toxicity	30	Elendt M7	LOEC (21 d)	Mackevica et al. (2015)
	148	natural river water	LOEC (21 d)	Blinova et al. (2015)

10.6 Environmental implications

In oligotrophic environments, food limitation for *Daphnia* stems from the low amounts of macronutrients, most commonly phosphorous (Hecky & Kilham, 1988), that limit algae growth and both, algae and *Daphnia* populations grow slowly (McCauley et al., 1988). In case of an exposure to low concentrations of AgNP (in the range of 1-10 µg Ag L^{-1}), algae are unlikely to respond with reduced growth, while *Daphnia* population growth is most likely reduced due to the effect of AgNP on mortality and maybe also reproduction and/or growth (Table 10.2, Discussion 10.3). Adult mortality was the most commonly affected endpoint in chronic studies (Parts 7 + 9; Blinova et al., 2013; Mackevica et al., 2015; Zhao and Wang, 2011). *Daphnia* have been shown to be a keystone predator for algae, exhibiting a strong influence on algal composition (Sarnelle, 2005) and on their total biomass (Lampert, et al., 1986). It is possible that the algae may profit from the reduced predation pressure, and may also profit from increasing phosphorous levels, as *Daphnia* have been shown to retain this nutrient more strongly than other grazers (Rothhaupt, 1997). This possible gain can also be expected under more nutrient rich conditions (Figure 10.4, algae abundances in spring and autumn: differences in peak hight and width between the upper and lower panel illustrate this indirect effect of AgNP). However, predation pressure is limited to a certain size class of algae (Brendelberger, 1991; Geller and Müller, 1981) and vulnerability of the algae to feeding (Sarnelle, 2005). Overall, it is likely that algae edible for daphnids and less sensitive to AgNP profit from low doses of AgNP, but at the same time this may be a disadvantage for not-edible algae in the phytoplankton community and also affect predator populations (indicated by the increased fluctuations and the higher abundances of algae during summer in the lower panel of Figure 10.4).

Another possible low food scenario for *Daphnia* are eutrophic lakes with high *Daphnia* densities that depleted their resources, which is a common phenomenon during summer (Figure 10.4, upper panel; Lampert, et al., 1986; Rinke and Vijverberg, 2005). Due to the dominant effect of AgNP on adult mortality at high food abundances (Part 9), population growth is probably already delayed before the occurrence of food limitation due to reduced reproduction of the surviving adults (Figure 10.4, effect A). In addition, recovery of populations may be delayed by AgNP (Figure 10.4, effect 1), as AgNP at low food levels caused a later start of reproduction and reduced the number of clutches (Table 10.2; Part 9). As a consequence, algae numbers may retain higher levels (compare upper and lower panel during summer in Figure 10.4). The green algae spring bloom already depleted some part of the nutrients, so that in early summer, other algae taxa dominate (Sommer, 1989). Due to differences in algae species sensitivity, also low AgNP concentrations may affect the phytoplankton community then (Figure 10.4, effect B). Under control conditions, algae populations are now mainly governed by competition for nutrients and predation pressure (upper panel, Figure 10.4). Nutrient limitation for single species may interact with AgNP exposure and change the composition of the phytoplankton community (Figure 10.4, effects 2 and 3). When phytoplankton densities reach a second maximum before the autumnal decline, *Daphnia* populations follow and increase as well. In this case, the effect of AgNP is comparable to the effect observed in spring (Figure 10.4, effect C compared to A): delaying and reducing population growth of *Daphnia*. Due to the decreasing light-energy input and the increasing predation pressure, algae populations decline again (Sommer, 1989). And again, a similar effect of AgNPs on *Daphnia* populations compared to the one in spring can be expected, so that AgNP toxicity is higher due to the low amount of food present in the lake (Figure 10.4, effect 4). Even though all effects are based on available literature, mesocosm experiments are needed to test these long-term effects of AgNP.

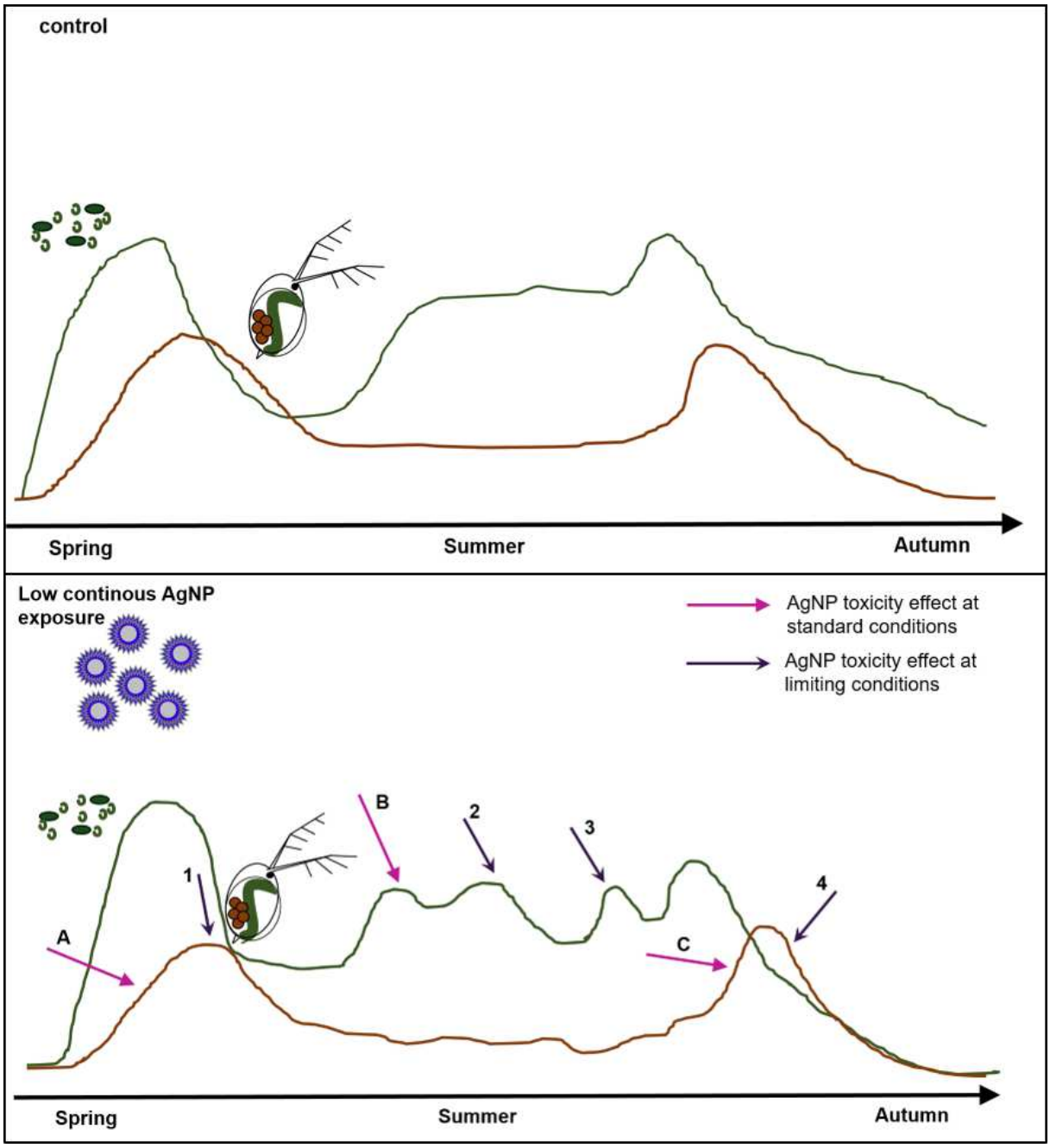

Figure 10.4: Schematic overview of phytoplankton and Daphnia population growth development during the growing season with (lower panel) and without exposure to low concentrations of AgNP (upper panel). The schematic growth curves in the control (without AgNP) is based on the PEG succession model for temperate lakes (Sommer et al., 1986, in Sommer, 1989). Green lines represent qualitative phytoplankton density development during the growing season, brown lines qualitative *Daphnia* population development. AgNP effects similar to standard conditions in ecotoxicological tests are marked by a pink arrow and potential increases of AgNP toxicity due to resource depletion.

11 Conclusions

The present work wanted to investigate how surface interactions affected AgNP fate and toxicity. Before addressing attachment to surfaces, the surfaces of the AgNP themselves needed to be considered: they determine if and how medium composition affects colloidal stability. The detAgNP were rather stable, but their colloidal stability in Elendt M7 for this work was quite different from the results reported in the literature. Additionally, the high degree of dissolution in algae medium could not be explained by effects caused by the test medium. However, sedimentation of large agglomerates was revealed as an important factor affecting the exposure scenario in Elendt M7 medium, at least when no algae were present. A similar process is also likely for citAgNP, but for this stabilizer larger agglomerates were observed in the medium. The formation of agglomerates could be related to the high concentration of divalent cations and they were most likely also responsible for the attachment to negatively charged surfaces. For detAgNP, hydrophobic interactions were shown to determine attachment to hydrophobic surfaces, which also could explain the maintenance of higher media concentrations in lipophobic test vessels over time. These differences in surface attachment mechanism were closely linked to the uptake and resulting toxicity of the AgNP, thus adding to the scarce information on this topic for AgNP.
The relation between uptake and toxicity was highly affected by the feeding behaviour of *D. magna* by determining possible uptake routes, as well as interaction partners inside the digestive tract. The importance of test species biology was also confirmed in the algae tests: aside from differences in sensitivity which may be expected for different test species, this difference in sensitivity may have also been affected by the species-specific ability to form colonies under unfavourable conditions and the response to AgNP exposure under nutrient limiting conditions also differed, indicating a possible effect of AgNP on algal community composition.

The second focus of this work was to determine the effect of resource reduction on AgNP behaviour and toxicity in an environmentally relevant context. In case of algae macronutrients, AgNP behaviour and toxicity were both affected, while for *Daphnia*, AgNP behaviour did not show a response to algae concentrations. However, both trophic levels showed an increase in AgNP toxicity when resources become limiting. Still, the sensitivity to AgNP of *D. magna* was clearly higher than the corresponding values of both green algae species also under the highest resource reduction levels used. For *D. magna*, the differentiation of endpoints allowed a comparison of resource and AgNP effects. It could be shown that resource limitation increased the toxicity for mortality and the sensitivity in case of start of reproduction and number of clutches, while having itself the clearest effect on cumulative number of neonates. This complex interaction between resource limitation and AgNP toxicity supports the high importance of long-term studies under environmentally relevant conditions – which could be shown to not only concern the range of test concentrations used, but moreover all test conditions from test vessel to resource availability.

12 Future perspectives

As can be seen from the large proportion that was used to discuss surface influences, this topic has been intensely studied in recent years. However, the numerous open questions remaining in this work related to specific surface interactions illustrate the importance of detailed knowledge on the surface chemistry of the nanoparticles and the abiotic and biotic surfaces present in the environment as well as during testing. In this context, modelling of surface interactions or T-SAR approaches may serve as support for experimental studies. These studies need to address how properties of (biological) surfaces such as structure, charge, or differences in surface composition at the molecular level between organisms and species affect the interaction with AgNP, to answer, for example, whether the attachment of negatively charged AgNP attach to algae surfaces can be related to divalent cation bridges.
In terms of biological and ecological influences, several open questions remain: to what extent are algae able to compensate AgNP exposure by exopolymer production or colony formation? How does this differ between species and algae communities in different habitats? How does an AgNP induced change in algal community size and their composition affect their grazers? In most studies, *Daphnia* were more sensitive than algae, but they may be additionally affected in cases where AgNP exposure decreases the abundance of edible algae as well. The complex interactions between resource limitation and AgNP toxicity also indicate the need for modelling studies and mesocosm experiments to relate the observed effects with *Daphnia* or zooplankton population growth.
However, it needs to be taken into account that actual environmental risk can be considered to be low and studies using AgNPs at the moment are better suited to improve our understanding of nanoparticle toxicity rather than improving risk assessment.

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13 Acknowledgements

This PhD wouldn't have been the same (and probably also not finished) without the support of so many people that made my PhD a great, interesting and (who could have guessed) instructive time.

First of all, I thank Juliane for trusting a marine biologist to successfully finish a freshwater PhD in a soil biology working group. It was a great opportunity and I am really thankful for all your support on my way to this thesis. Also to all other team members a huge thanks! Not only for your (endless) support in the lab (especially by Iris and Ute), but also for creating good working atmosphere and for all the nice social events from christmas parties to Rocky Horror Show cinema. And a special thanks to all current and former PhDs of the working group – that my PhD was a great time was mostly your part. Here, I definetely need to mention Thomas: his saying „Alles wird gut" became my mantra during writing – and it was a great help to not freak out at the end. I am also very grateful for the support of so many other people at the UFT during this work, including Jan, Jürgen, Stefan, and Sonja.

I also want to thank another important universary group of people: Anders Baun, Aiga, Lars, Signe, and Susanne from DTU – you made my stay at DTU a success in so many ways! Thanks for your nice welcome, and your support during my stay. I had a great time with all members of DTU environment and I am looking foward to meet some of you on conferences again.

As you all know: we need support at work, but our support at home is often crucial when work gets difficult. Whether a re-start of a failed AAS measurement, the need to write another application for financial support without having the time to do so, or whether everything was simply too much – I could always be sure to come home and meet my best friend and my greatest love – thank you for having been there and thank you even more for staying. Also to the rest of my family and my friends a huge thanks for their support and understanding. You made sure that I never forgot to balance myself and my life and helped me to find the right perspective on things. And in case that I needed some more "scientific" related friend support – thank you, Simone, Andrea, Anke, Birthe, and Jana for not only being friends, but also for providing scientific feedback to my work.

Appendix

14 Appendix

14.1 Methods and standard operating protocols used for this thesis

14.1.1 Green algae culturing at the University of Bremen

UFT Zentrum für Umweltforschung und Umwelttechnologie	Method description **GREEN ALGAE CULTURE** **FOR FEEDING ALGAE FOR *D. MAGNA*** Valid since: 01.07.2016 Version No.: **01**
Department 10	Author(s): Yvonne Sakka

· 1. General information / Introduction

· 1.1. Purpose and principle of the method

Description of the culture conditions and handling of the green algae cultures used for feeding the water flea (*D. magna*) cultures in the working group Filser.

· 1.2 Limits of the method

This kind of culture is suitable for feeding cultures for other organisms, not for ecotoxicological or ecological tests
This culturing protocol does not guarantee 100 % pure cultures
The culture conditions are optimised for *Raphidocelis subcapitata* (formerly *Pseudokirchneriella subcapitata*), other species may prefer different settings

· 1.3. Definitions / Abbreviations

- *R. subcapitata, Raphidocelis subcapitata*: green algae for feeding water fleas
- *D. magna, Daphnia magna*: Big water flea

· 2. Material and Methods

· 2.1. Devices

- pH-Meter (free of any contamination)
- climate room with 20 ± 1 °C
- Illumination (4400-8800 lux, as close to day light as possible, mixture of cold and warm white: L 36, W41 or W11, Osram)
- Laboratory cart for transports, once a week
- Sterile bench (1x per week)
- Air pumps (4 pieces, model: Marina 200: 4W, 180 l/min [the most important criterion], No. 1171354 – 62, Conrad)
- 10 L water canister with 10 L mark
- Eppendorf pipets (10, 200, 1,000 und 5,000 µL)

- Cell counting chamber (Neubauer, Paul Marienfeld GmbH)
- Microscope (20-fold Objective)
- Water filter with activated coal (Vario HP Untertischfilter, Carbonit Filtertechnik GmbH)
- Garden hose and adapter (Gardena, from building supplies stores)
- Repetitive pipet for partitioning of algae trace element and vitamin solution
- Small waste buckets for pipet tips
- Tally counter
- Inoculum needles and holder
- Laboratory scale
- Data sheets for cultures (see 5.3, part 3)

· 2.2. Consumables

- 5 L flasks (4 pieces, Schott, 45 mm opening width (GL 45), with pouring ring)
- Special lid for 5 L flasks (4 pieces, ordering details: Duran, "Schraubverschluss HPLC, GL 45, 4 Ports, komplett", No. 11 298 12)
- 1L flasks (30 pieces, Schott, 45mm opening width, with pouring ring and lid)
- 250 mL flasks (6 pieces, Schott, 45 mm opening width, with pouring ring and lid)
- Silica plugs (6 pieces, upper diameter 35 mm, lower diameter 29 mm, height 30 mm, with 2 openings [should be made by the UFT garage])
- Glass tubes (3 mm outer diameter, 12 pieces about 45 cm length, 6pieces about 30 cm length, 20 pieces about 15 cm length)
- Plastic tube pieces:
  a) 20 pieces, length about. 5 cm, 4mm outer diameter, autoclavable -> direct connection to glass tubes
  b) 30 pieces, length about 30 cm, 5mm outer diameter, autoclavable -> connection to aeration
  c) 3 pieces, length about 60 cm, 5 mm outer diameter -> conncection to aeration for the medium
- Tube connections (2 mm to 6 mm, autoclavable)
- Sterile filter for aeration of cultures (0.2 µm mesh size, No. 90491011, Minisart)
- Sterile filter for vitamin solution (0.2 µm mesh size, screw thread 45 mm width, easy-connecting tube adapter, e.g. No. 272-AC33.1, by Nalge Nunc of mercateo)
- Pipet tips (Eppendorf, all sizes)
- Pipet tips for the repetitive pipet (50 mL, sterile for vitamins, non-sterile for trace elements)
- Bell prover: 1L, 500mL, 250mL, and 100mL (company not important) to prepare stock solutions
- Reaction cups (2 mL, Eppendorf [others do not close well])
- 50 mL plastic bottle with lid: 4 pieces for deep freezing of vitamin stock solutions
- Aeration stones (4 pieces, non-dyed, 25x25x25 mm^3, No. 201, Hobby Dohse Aquaristik)
- Y-piece (tube connection, 2 times, No. 62750, Hobby Dohse Aquaristik)
- Tube clamps (10 pieces)
- Water proof pens (Edding, No. 751, red/blue)
- Edding
- Pen and pencils
- Tally counter
- Parafilm
- Lint free towels
- Laboratory gloves (protecting the cultures from contamination)
- Scaling boats
- Filter cartridge (model: Monoblock-Kartusche, Carbonit Filtertechnik GmbH)

· 2.3. Chemicals

- Ethanol, 70% and 100 %
- 3M HCl (to adjust the pH)

2.4. Solvents, substrates, and reagents

- Green algae medium GAM (see 3.3 and 5.3)
- Sterilium
- pH buffer solutions (4,7,9)

2.5. Used organisms

- *R. subcapitata*, strain No. 61.81, SAG, University of Göttingen

3. Preparations

3.1 Risk assessment

- Read Material Safety Datasheets of all media components and consider medium preparation for risk assessment
- Consider that glass can break (and can cause cuts)
- Consider use of autoclave (burns are possible)

3.2 Organization of materials and devices

- Reserve a place in the climate room permanently and inform colleagues about the regular use of the sterile bench
- Order required materials in time
- In case of medium preparation, reserve the autoclave for the following day, if possible 2 days ahead
- Coordinate demand of feeding algae with actual carers of the *Daphnia* culture: actual higher demand? Quality sufficient?

3.3 Further preparations

1. Prepare green algae medium (GAM)

Preparations: Make stock solutions
Prepare 250 mL trace element solution (AS, see 5.3, part2) one day before all other stocks, so that dissolution overnight is possible. Divide the AS stock in 4.5 mL aliquots (materials: repetitive pipet with 50 mL tip, 5 or 10 mL centrifugation tubes), use one (4 mL) for the preparation of stock solution A6 and deep freeze the others for future use. For preparing A6, the optimal order for preparation is: EDTA; iron and AS stock solution as last component. Prepare algae stock solutions A1 to A 5 according to the given recipe (see 5.3, part 1 and 2). The stock solution A8 is also prepared as described there, but it is divided into 4 to 5 portions of 200-250 mL in 250 mL glass flasks. The stock solution A7 (vitamins) it is prepared separately (see 3.3, part 2 below) and frozen afterwards. All stocks A1 to A6, and A8 are autoclaved and stored at room temperature in the dark.

Medium preparation:
Green algae medium is prepared either as 10 L or as 20 L using the plastic canisters reserved for GAM preparation. For this purpose, wash them repeatedly with demineralised, filtered water to remove all dust and remaining salt particles. Afterwards, fill the canister to about 5 L with demineralised, filtered water by using the medium-water tube. Now, the stock solutions are added according to the preparation list in the laboratory or the composition as listed in the appendix (5.3). However, vitamins and $NaHCO_3$ (sodium bicarbonate, A8) are not added now. This allows a better provision of the cultures with CO_2 during longer time periods and the possibility to adapt it to actual culture growth.
After addition of all required stock solutions, canisters are filled with demineralised, filtered water to the 10 L mark, and aerated using the medium aeration tubes and stones. The medium should bubble intensely overnight. To hinder dust entry, cover the openings with parafilm.
On the following day, adjust the pH to about 7.0. Consider the calibration of the pH-meter before use! If needed, prepare additional HCl (3M). Afterwards, fill the medium into 1 L glass flasks and autoclave (20 min, 121 °C, slow cool down). The medium is stored in the dark at room temperature until use.

2. Prepare vitamin stock solutions

Autoclave the required material (10 min, 135 °C, fast cool down): about 200 2mL reaction cups, 600 mL beaker or similar for transfer of solution, 1 L glass flask with lid. Book sterile bench and order vitamin powders, if necessary (check expiration date).

Due to the low concentrations of vitamins in the stock, higher concentrated pre-stock solutions need to be prepared: vitamin B12 and biotin are prepared as 0,01 g/L in 100 mL (20-fold higher). 50 mL each are used for the preparation of 1 L vitamin stock solution. The remaining 50 mL can be frozen for the next use.
The algae vitamin stock solution needs to be sterile filtered and divided into small aliquots.
Possibility 1: filtration by sterile filters and a 20 mL syringe in the sterile bench
Possibility 2: filtration by a sterile filter connected to a water flow-through pump
Possibility 2 is much faster and requires the filter and an autoclaved 1 L glass flask as consumables only, as the water flow-through pump is already installed in the laboratory (R0440). Thus, this method is described here.
Prepare the sterile bench and connect the sterile filter to the autoclaved glass bottle here. Bring them into the laboratory and connect to the pump. By opening the water flow, the pump creates a low-pressure and sucks liquid through the filter membrane. Pour the vitamin stock into the upper part of the filter until all is filtered. Disconnect the filter from the pump, not from the flask – then it is not sterile anymore and the filtration needs to be repeated. The division into aliquots will be done under the sterile bench. Prepare the required material (repetitive pipet with sterile 50 mL tip, autoclaved reaction cups and beakers, holder for reaction cups, freezing bags). Pipet 1.5 mL into each reaction cup, close and store in the bags for freezing afterwards. Label the bag with content, name and date. Remaining solution can be frozen as well in plastic flasks. Add an information about approximate number of aliquots on the flask.

3. Start a new algae culture

Book the sterile bench and check the required material (order anew, if needed).
List of material:
Autoclaved flask for the culture (5 L) including lid, glass tubes and connecting tubes
4 L GAM
A8: $NaHCO_3$ (3 ml per 1L)
A7: 4 Eppis vitamin solution (1 ml per 1L)
1 ml/5 mL Pipet
Sterile Pipet tips
Inoculum needles and holder
Spray bottle containing 70 % Ethanol
Sterilium
Laboratory gloves
Algae permanent culture (Do not use no.1, this for preservation of the cultures!)
Edding

Start the sterile bench, clean and prepare the required material. In case of problems with contamination of cultures, prepare all material except for algae and vitamins and start the UV-lamp for about 30 min.
To start the culture, add GAM, A8 and A7 into the culture flask. Retain 1 L of GAM at this step. Heat inoculum needle with the Bunsen burner until it is blazing. Place on the holder, take the algae culture in one hand and re-heat the needle. Open the algae culture, shortly heat the opening, and cool down the needle in algae-free agar at the bottom of the glass. Then transfer a small portion of algae onto the needle and solve them in the remaining GAM. Close the permanent culture of algae as soon as possible and clean the needle by heating. Close the GAM flask with the algae, shake thoroughly, and fill into the 5L culture flask. Label the new culture with name of the culture and date, connect sterile filters to the aeration connection tubes of the culture flasks, and clean up the sterile bench. The new algae culture can now be placed in the climate room and connected to aeration. For doing so, open the lid of the culture flask slightly, when connecting the first aeration tube or switch off the aeration, as increasing pressure may press algae solution out of the second aeration tube.
A new algae culture should be started every 3 weeks.

3.4 Data: Notes for data recording and analysis

- Every maintenance of the algae culture is noted in a culture-own datasheet
- It is cheaper to copy culture data sheets instead of printing new ones
- Old culture datasheets are not dumped, but kept in the archive

4. Maintenance of cultures

4.1. Culture conditions

- 20 ± 1 °C
- Permanent illumination
- Aeration with ambient air, so that cultures bubble intensely
- Maintenance of cultures is due once per week, preferable on the same day
- New cultures should be started sequentially, so that 2 are in the optimal growth phase (clearly green) (optimum time difference: 3 weeks, see 3.3, part 3).

4.2. Maintenance of cultures

1. Prepare required materials and store on the laboratory cart

List of materials:
1 L GAM per culture
3 mL $NaHCO_3$ per culture (more, if growth is insufficient)
1 mL vitamin solution per culture
1 empty 1L flask per culture with lid
Edding
Eppendorf pipets: 1 mL/5 mL
Sterile pipet tips
Spray flask containing 70 % ethanol (maybe in R 1350 near the sterile bench)
Laboratory gloves (maybe in R 1350)
Sterilium (maybe in R 1350)
Lint-free towels (for cleaning the sterile bench)

2. Start sterile bench and clean with ethanol
3. Prepare all material in the sterile bench
4. Add vitamins and $NaHCO_3$ into each litre of GAM, mix by shaking
5. Remove the cultures for maintenance from the aeration, loose the lid first to avoid splashing due to the high pressure!
6. Open the first culture in the sterile bench, pour about 700 mL into one of the empty glass flasks, and re-fill with the prepared GAM, heat lower part of glass tubes and close the culture flask.
7. Close the flask containing the 700 mL of algae dispersion and label (culture number, number of the removal of an algae subsample, date, name)
8. Repeat step 6 and 7 for all cultures
9. Clean up in the sterile bench, and switch off
10. Re-connect cultures to aeration, lose lids again for pressure equilibration and close again when aeration is connected and established
11. Potions of algae cultures (in the 1 L flasks) are used for feeding of water fleas, and connected to aeration in the Aquatox laboratory (R 0440) by silica bugs and shorter glass tubes (place is also illuminated) until use.

Notes:

- If a culture appears to be rather yellowish or turquoise-green, it should be disposed immediately and replaced with a fresh culture. Prior to feeding it to the water fleas (if no other culture is available), it needs(!) to be checked by light microscopy for cyanobacteria, fungi or diatoms. In doubt, baker's yeast (from the collembola culture) solution should be used as feeding replacement.
- A culture is considered as optimal (high quality), if it is strongly green coloured and algal density is about 35-40 $*10^6$ algae cells/mL. This corresponds to a count of 35-40 in one large counting square of the Neubauer counting chamber.

5. Additions / important notes and documents

- **5.1. Further applicable documents**
  - Method description "*Daphnia* culture"

- **5.2. Notes for disposal**

None

5.3. Appendix

1) Calculations and preparation og GAM

Coloured cells are added directly into the demineralised water for preparing the medium.

			Volume of medium in litres:		**10**
	Substance		**Conc. mg/L**	**Conc. ml/L**	**Volume ml**
Macro elements	A1	$MgSO_4 \cdot 7\ H_2O$	37000	1	10
	A2	$Na_2SiO_3 \cdot 5\ H_2O$	56840	1	10
	A3	$CaCl_2 \cdot 2\ H_2O$	36000	1	10
	A4	$NaNO_3 + H_3BO_3$	339960	1	10
			4946	1	10
	A5	$K_2HPO_4 \cdot 3H_2O$	42430	0,5	5
	A6	FeEDTA / trace elements			30
	A7	Vitamin solution			
	A8	$NaHCO_3$			

2) Stock solutions for GAM

Amount in 1L:

1) $MgSO_4 \cdot 7\ H_2O$: 37000 mg
2) $Na_2SiO_3 \cdot 5\ H_2O$: 56840 mg
3) $CaCl_2 \cdot 2\ H_2O$: 36000 mg
4) $NaNO_3$: 339960 mg

H_3BO_4: 4946 mg

5) $K_2HPO_4 \cdot 3\ H_2O$: 2430 mg
6) $Na_2EDTA \cdot 2\ H_2O$: 620 mg

$FeSO_4 \cdot 7\ H_2O$: 321 mg
AS: 4 mL

7) Thiamine hydrochloride: 100 mg

Cyanocobalamin (B_{12}): 0,5 mg
Biotin: 0,5 mg

8) $NaHCO_3$: 17900 mg

Amount per 1L in the trace element stock solution (AS):

MnSO4 • H2O: 5040 mg

ZnSO4 • 7 H2O 1920 mg
CuSO4 • 5 H2O: 400 mg
(NH4)6Mo7O24 • 4 H2O: 440 mg
Co(NO3)2 • 6 H2O: 1200 mg

3) Data sheet for the algae cultures

Algenkultur Erfassungsbogen GROßE FLASCHEN

	Kultur			**Kultur**		
Datum	**Medium**	**Gen.**	**Kommentar**	**Medium**	**Gen.**	**Kommentar**

14.1.2 Culture of *Daphnia magna* at the University of Bremen

UFT Zentrum für Umweltforschung und Umwelttechnologie	Method description **DAPHNIA CULTURE** Valid since: 01.07.2016 Version No.: **01**
Department 10	Author(s): Yvonne Sakka

1. General information / Introduction

1.1. Purpose and principle of the method

Description of the culture conditions and maintenance of the cultures of water fleas, *Daphnia magna*, in the owkring group Filser.

1.2 Limits of the method

- This culture is suited for the use in ecological and ecotoxicological tests with single clones; sexual reproduction and resting stage (ephippia) production are/should be avoided in this kind of culture.
- This culture does not provide genetic variability
- Culture conditions are optimised for the used clones of *D. magna*, other clones may prefer other settings

1.3. Definitions / Abbreviations

- *D. magna, Daphnia magna*: big water flea
- *R. subcapitata, Raphidocelis subcapitata*: green algae for feeding of *Daphnia* (see 5.3 for more information)

2. Material and Methods

2.1. Devices

- pH-Meter (free of any contamination!)
- climate room/chamber with 20 ± 1 °C and possibility to aerate the cultures (enter tube via the door)
- light table (min. 0.5 m x 0.4 m)
- Laboratory cart for transports, 2x per week
- 60 L Aquaria, 2 pieces, covered with aluminium foil, with lid, without illumination (lid needs to close well)
- Eppendorf pipets (10, 200, 1,000 und 5,000 µL)
- Cell counting chamber (Neubauer, Paul Marienfeld GmbH)
- Microscope (20-fold Objective)
- Water filter with activated coal (Vario HP Untertischfilter, Carbonit Filtertechnik GmbH)
- Garden hose and adapter (Gardena, from building supply stores)
- Water spray adapter for garden hose (Gardena, from building supply stores)
- Repetitive pipet for partitioning of vitamin solution
- Small waste buckets for pipet tips

- Tally counter
- Inoculum needles and holder
- Laboratory scale
- Data sheets for cultures (see 5.3, part 3)

2.2. Consumables

- Pasteur pipets with removed tip, alternatively 5 mL pipet tips with their tips cut off; all labelled with the actual names of the cultures (e.g A33) at the removed glass tip end or at the upper end of the plastic tip [use waterproof pens]
- Pipet "hats"
- Pipet tips (Eppendorf, all sizes)
- Pipet tips for the repetitive pipet (50 mL, non-sterile)
- 1L glass flasks (company not important, brown, with puring ring and lid): 21
- Bell prover: 1L, 500mL, 250mL, 100mL, and 50 mL to prepare the stock solutions
- 2 L measuring beaker with handle: 2 times
- 2 L glass beaker (slender/high form): about 20
- Watch-glass (15 cm diameter): about 20
- 600 mL beaker: about 5
- 50 mL beaker: about 5
- Large, flat water bowl: 1 per light table
- 15 mL centrifugation tubes: about 10 per month
- Reaction cups (1,5 mL and 2 mL, Eppendorf [others do not close well])
- 50 mL plastic bottle with lid: 4 pieces for deep freezing of vitamin stock solutions
- Water proof pens (Edding, No. 751, red/blue)
- Edding
- Pen and pencils
- Tally counter
- Parafilm
- Lint free towels
- Laboratory gloves (protecting the cultures from contamination)
- Filter cartridge (model: Monoblock-Kartusche, Carbonit Filtertechnik GmbH)
- Plastic tube pieces (length about 60 cm, 5 mm outer diameter, water resistant)
- Aeration stones (4 pieces, non-dyed, 25x15x15 mm^3, Hobby Dohse Aquaristik)
- Y-piece (tube connection, 2 times, No. 62750, Hobby Dohse Aquaristik)
- Tube clamps (15 pieces)
- Scaling boats

2.3. Chemicals

- Ethanol, 70% and 100 %
- See 5.3, part 1 and 2 for chemicals required for the culture medium

2.4. Solvents, substrates, and reagents

- Elendt M7 Medium (preparation in section 3.2)
- Green algae medium (see method description of green algae culture)
- Sterilium
- pH buffer solutions (4,7,9)

2.5. Used organisms

- *D. magna*: clone A-C: by UFZ Leipzig, in this working group prior to 2009; clone H-K: by IBACON GmbH, via Goethe University (Frankfurt), in this WG since 2009
- *R. subcapitata:* strain No. 61.81, SAG

3. Preparations

3.1 Risk assessment

- Read Material Safety Datasheets of all media components and include the information in the risk assessment
- Consider that glass can break and cause cutting wounds and that the autoclave can cause burns

3.2 Organization of materials and devices

- Keep a place in the climate chamber and the working place with the light table reserved for culture maintenance, also in case of high demand for ecotoxicological tests
- All beakers, containers etc. used for *Daphnia* MUST be washed with filtered, demineralised water after the dishwasher washing to remove residues from the detergents
- Order consumables in time
- Aquaria needs to be covered with aluminium foil to keep the medium in the dark
- Filling height of the aquaria (50 L) needs to be marked prior to their first use on the outside of the aquarium glass (not in contact with the medium, e.g. by filling a 5 L bell prover 10 times and emptying it into the aquarium)

3.3 Further preparations

1. Cleaning of medium Aquaria
   The *Daphnia* medium, Elendt M7, is prepared in 60 L aquaria. 50 L are prepared at the same time. If smaller volumes are needed, 5 L bell prover and the adapted recipe (use the existing excel file for calculations) can be used and glass flasks or small 10 L aquaria can be used for storage.
   Prior to medium preparation, the aquarium needs to be cleaned. First, salt residues and dust are removed by washing the inner surface with soap and sponges (marked by “M7”). To remove these residues, especially the edges need to be cleaned intensely, as it may also enter the edges “behind” the silicone. The lid is cleaned in the same fashion. Both pieces can be washed with demineralised water using the spray adapter for the hose. The aquarium can be placed upright at the edge of the sink on a reaction tube holder, so that water can flow directly into the sink. This avoids splashing and back problems. Aquarium and lid are washed until all foam is removed.
   Afterwards, the aquarium is washed with 100 % ethanol and paper towels, especially at the edges. Take care that the complete surface is well covered and washed with ethanol. Let soak for about 10 min. The lid will also be cleaned with the same procedure. Both pieces are again washed with demineralised water, now it needs to be filtered as well. In case of very low water pressure, washing can be started with non-filtered water and finished with filtered, demineralised water. It is important that the final step is done by the filtered form to remove all plasticisers remaining in the demineralised water. Aquarium and lid are then dried on laboratory paper.

2. Preparation of Elendt M7
   First, the required volume (50 L) is entered into the excel table (on Torp) to get the required volumes of all stock solutions. Alternatively, the print version in the laboratory (R0440) can be used. The demineralised, filtered water should run for about 30 min to ensure only fresh water without residues from the pipes is used. Afterwards, the water is filled into the aquarium by the medium-water tube. It can the fixed to add the stock solutions in parallel. The order of addition of stock solutions is not mandatory, but the stock solution 14, containing iron and EDTA should be added last. And vitamins are not added into the medium, but directly in the culture vessels during maintenance (see 4.1). If the marked filling height is reached, the water supply is ended and the tube removed. The aeration tube is cleaned with sterilium and let soak for about 1 min. Afterwards, the aeration tube is cleaned thoroughly with demineralised water and an aeration stone is added to the end. This will be placed on the bottom of the aquarium and aeration adapted, so that it is strongly aerated without flooding. From the next day on, the pH can be adjusted to values between 6.8 and 7.8. In general, pH values between 6.0 and 9.0 are suitable for daphnids. Before the pH can be considered to be stable, the pH of the medium needs to be adjusted about 3 times.

3. Preparation of vitamin solution
   The vitamin solution is prepared from a higher concentrated stock solution, as the concentrations are too low to be weighted directly. This stock solution is concentrated 5times

and remaining volumes can be frozen in plastic bottles until use. However, these bottles should not be thawed and re-frozen more than 2 times.
Correctly diluted vitamin solution will be divided into 650 and 950 µL aliquots in 1.5 mL reaction cups (by using the repetitive pipet). These are stored in freezing bags, labelled with content, volume, name and date and frozen until use.

3.4 Data: Notes for data recording and analysis

- Every maintenance of the algae culture is noted in a culture-own datasheet
- It is cheaper to copy culture data sheets instead of printing new ones
- Old culture datasheets are not dumped, but kept in the archive

4. Maintenance of cultures

4.1. Culture conditions

- 20 ± 1 °C
- 16:8 h light-dark cycle with 1110-1480 lux, cold-warm-white mixture
- 0.15 mg C per animal and day as food
- Max. 30 adults in 1,5 L medium
- Aeration of cultures is not mandatory, but helpful (single air bubbles need to be easily visible, strong aeration has negative effects)
- Maintenance takes place twice a week, e.g. Monday and Thursday
- 3 replicates per clone provide good results
- Form day 25 on, a follow-up culture with about 40 similar-sized neonates is started for each replicate of one culture. The number of neonates is reduced stepwise to 30 animals in total with every maintenance. Here, not more than 3 days of maintenance should be used to ensure a reduction to 30 is completed before the first reproduction takes place. If not, a density induced reproduction depression can occur.
- If the follow-up culture reproduced successfully for 2 times, the original culture can be discarded.

4.2. Maintenance of cultures

1. Control and adjust the pH in the medium (including stability of the pH, if possible only use media with stable pH values!)
2. Control, whether new follow-up cultures need to be started using the culture datasheets and adapt the number of beakers accordingly
3. Fill 2L beakers with 1.5 L medium
4. Add 150 µL vitamin solution per beaker
5. In case of breaks in between: cover beaker with watch-glasses to reduce dust entry (Daphnia may be trapped in long dust filaments)
6. Pour feeding algae from the 1 L flasks (in the laboratory, see method description “green algae culture”) in a 600 mL beaker, about 400 mL are usually needed
7. Fill 2times 990 µL tap water in a 2 Ml reaction cup and add two 100 µL samples of the feeding algae
8. Prepare the cell counting chamber
9. Shake the algae dilution thoroughly or vortex shortly (too much destroys the algae)
10. Fill 10 µL algae dilution into each side of the counting chamber
11. Count algae numbers (at least 1 large square per side, if less than 30 cells, more squares should be counted) and enter the two values in to the excel data sheet for feeding algae calculations (Torp) to calculate the required volume for feeding
12. Add feeding algae according to the results from point 11 into each beaker
13. Bring old cultures from the climate room/chamber to the laboratory, prepare folder with culture datasheets, tally counter, pasteur pipets for culturing, edding and pen for maintenance
14. Place a new beaker (filled completely: medium+vitamins+algae), an old culture beaker, the large glass bowl, the corresponding Pasteur pipet, and culture datasheet at the light table.
15. Label new beaker and place both beakers together on the light table, then transfer adults from the old into the new beaker and count each transferred animal using the tally counter (if adult organisms are “hidden” at the bottom of the beaker, a part of the old medium can be poured carefully into the glass bowl (step 16) to continue with the transfer. A pouring of adults should be avoided.)
    Note: cultures before their first reproduction can be poured directly into the glass bowl and reduced there. For removal, animals with deviating sizes (larger/smaller) should be chosen.

16. Pour the medium of the old culture including neonates or dead animals into the glass bowl, and guess the number of neonates using the pictures at the light table.
17. Fill the data into the corresponding data sheet
18. Cover the new culture with a watch-glass
19. Pour the old medium into a bucket (fish-food)
20. Repeat step 14 to 19 until all cultures are taken care for
21. Place the new beakers with the adult *Daphnia* into the climate chamber and connect to the aeration
22. Wash the old beakers and materials in the dishwasher with programme “A” (90 °C water temperature, and maximal washing with pure water) and wash again with filtered, demineralised water

5. Additions / important notes and documents

5.1. Further applicable documents

- OECD guideline No. 211
- Excel table for the calculation of Elendt M7
- Excel table for the feeding of *Daphnia*
- Method description “green algae culture”

5.2. Notes for disposal

- None, but remaining neonates can be fed to fish, e.g. at Yvonne’s or Annemarie’s place

5.3. Apendix

1. M7 Calculation
2. M7 stock solution preparation
3. Datasheet for the *Daphnia* culture (German)

1. **Calculations to prepare Elendt M7**

Coloured cells show volumes which are directly added into the demineralised water.

Volume of medium in litres: 50 <- enter here!

Substance		Conc. mg/L	Conc. ml/L	Volume ml	Amount mg	Amount g
Stocks part I			50	2500		
A	$CaCl_2 \cdot 2\ H_2O$	293800	1	**50**	14690	14,69
B	$MgSO_4 \cdot 7\ H_2O$	246600	0,5	**25**	6165	6,165
C	KCl	58000	0,1	**5**	290	0,29
D	$NaHCO_3$	64800	1	**50**	3240	3,24
E	$Na_2SiO_3 \cdot 5\ H_2O$	37325	0,2	**10**	373,25	0,37325
F	$NaNO_3$	2740	0,1	**5**	13,7	0,0137
G	KH_2PO_4	1430	0,1	**5**	7,15	0,00715
H	K_2HPO_4	1840	0,1	**5**	9,2	0,0092
Comb. Vitamine Stock		-	0,1	5	-	-
1	Thiamine hydrochloride	750	0,1	5	3,75	0,00375
2	Cyanocobalamine (B_{12})	10	0,1	5	0,05	0,00005
3	Biotine	7,5	0,1	5	0,0375	3,75E-05
Water [L]				47,325		

Stocks part II **Litre: 2,5**

Substance		Conc. mg/L	Conc. ml/L	Volume ml	Amount mg	Amount g
1	H_3BO_3	5719	2,5	**6,25**	35,74375	0,035744
2	$MnCl_2 \cdot 4\ H_2O$	7210	0,25	**0,625**	4,50625	0,004506
3	LiCl	6120	0,25	**0,625**	3,825	0,003825
4	RbCl	1420	0,25	**0,625**	0,8875	0,000888
5	$SrCl_2 \cdot 6\ H_2O$	3040	0,25	**0,625**	1,9	0,0019
6	NaBr	320	0,25	**0,625**	0,2	0,0002
7	$Na_2MoO_4 \cdot 2\ H_2O$	1260	0,25	**0,625**	0,7875	0,000788
8	$CuCl_2 \cdot 2\ H_2O$	335	0,25	**0,625**	0,209375	0,000209
9	$ZnCl_2$	260	1	**2,5**	0,65	0,00065
10	$CoCl_2 \cdot 6\ H_2O$	200	1	**2,5**	0,5	0,0005
11	KI	65	1	**2,5**	0,1625	0,000163
12	Na_2SeO_3	43,8	1	**2,5**	0,1095	0,00011
13	NH_4VO_3	11,5	1	**2,5**	0,02875	2,88E-05

	$Na_2EDTA \cdot 2 H_2O$	5000	2,5	**6,25**	31,25	0,03125
	$FeSO_4 \cdot 7 H_2O$	1991	2,5	6,25	12,44375	0,012444
14	Fe-EDTA solution	-	5	**12,5**	-	-
Water [L]				2,464375		

2. Stock solutions for Elendt M7 Medium

Amount per 1L:

A) $CaCl_2 \cdot 2 H_2O$: 293800 mg
B) $MgSO_4 \cdot 7 H_2O$: 246600 mg
C) KCl: 58000 mg
D) $NaHCO_3$: 64800 mg
E) $Na_2SiO_3 \cdot 5 H_2O$: 37325 mg
F) $NaNO_3$: 2740 mg
G) KH_2PO_4: 1430 mg
H) K_2HPO_4: 1840 mg

1) H_3BO_3: 5719 mg
2) $MnCl_2 \cdot 4 H_2O$: 7210 mg
3) LiCl: 6120 mg
4) RbCl: 1420 mg
5) $SrCl_2 \cdot 6 H_2O$: 3040 mg
6) NaBr: 320 mg
7) $Na_2MoO_4 \cdot 2 H_2O$: 1260 mg
8) $CuCl_2 \cdot 2 H_2O$: 335 mg
9) $ZnCl_2$: 260 mg
10) $CoCl_2 \cdot 6 H_2O$: 200 mg
11) KI: 65 mg
12) Na_2SeO_3: 43,8 mg
13) NH_4VO_3: 11,5 mg

Preparation of stock solution no. 14:
$Na_2EDTA \cdot 2 H_2O$: 5000 mg/L
$FeSO_4 \cdot 7 H_2O$: 1991 mg/L

Solve the EDTA first, add the iron salt and mix both thoroughly. Make up to 1 L and autoclave immediately. Divide the solution into 12.5 mL portions (for 50 L Elendt M7) in the sterile bench and freeze until use.

Preparation of vitamin solution (see also 3.3):
Thiaminhydrochlorid: 700 mg/L
Cyanocobalamin (B_{12}): 10 mg/L
Biotin: 7,5 mg/L

All vitamins need to be stored in the fridge. For biotin and B12, a higher concentrated stock needs to be made, while thiamine could also be directly weighted into the vitamin stock. If the recipe above is used, no further dilution is needed.

3. Erfassungsbogen Daphnienversuchskultur **Art: *Daphnia magna*** **Ursprung:** **Datum:**
Seite:

Ansatz (+Gen ges/urspr +Dat +Anz)						**Abiotik**			**Algensuspension**			**Anmerkungen**
Datum	Initial	Tag	Anzahl	Tot	Neonaten	Temp °C	pH	O_2 mg/L	Art	Zellzahl	Volumen	

14.1.3 Method used for the algae tests

UFT Zentrum für Umweltforschung und Umwelttechnologie	**Standard Operation Protocol** **ALGAE GROWTH INHIBITION TEST (ACCORDING TO OECD GUIDELINE NO. 201)** Valid since: 01.07.2016 Version No.: **01**
Department 10	Author(s): Yvonne Sakka, Sonja Faetsch

1 General information / Introduction

1.1. Purpose

Assessment of the toxicity of a (water soluble) substance or a substance mixture on the growth of an algae culture.

1.2. Principle of the method

Measurement of the concentration-dependent growth inhibition of algae by a substance/mixture within 72 h by cell counting.

1.3. Range of application of the method

Suitable for all water soluble substances or mixtures.

1.4. Limits of the method

The test needs to be conducted in OECD algae medium using shakers and illumination.

1.5. Definitions / Abbreviations

- EC_{50}: Effect concentration 50 = 50 % of control growth

2. Material and Methods

2.1. Devices

- 2 pH-Meter, one for the medium (free of any contamination), one for the test solutions
- Min/Max-Thermometer for water solutions

- Place in a climate chamber/room
- Illumination, near to sunlight: 4440-8880 lux, corresponding to 60-120 µE m^{-2} s^{-1} (attention: not more than 15 % variation over the whole area)
- Shaker: R 1370, AG Stolte at 80rpm **or** MTS 2/4 digital at 200 rpm (IKA Werke GmbH & Co. KG)
- Cell counter (Casy, R 1350, AG Stolte) or cell counting chamber (Neubauer, Paul Marienfeld GmbH & Ko. KG)
- Maybe: microscope (10 or 20 objectives for overview and counting, 40 for morphological details)
- Laboratory scale: measurement limits according to the test substance
- Eppendorf-Pipets: according to the pipetting scheme for the test substance
- Sterile bench (R1350)

Note: The Casy can only be used after personal introduction (Sonja Faetsch, AG Stolte)! It is not suited for tests with substances which can adsorp to its capillary system, e.g. silver or nanomaterials. If a cell counting chamber is used, fixation of samples is needed, as not all samples can be counted simultaneously. A good fixant here may be Lugol's solution (see 2.4).

2.2. Consumables

- Algae culture flasks (260 mL, ordering information: no.156800, Thermo Fischer Scientific): 1 per culture
- Algae test flasks (70 mL, ordering information: no.169900, Thermo Fischer Scientific): min.3 per concentration
- Glass flasks (1L, Schott, with pouring ring and lid): min. 6 for stock solutions
- Bell prover (1L), 6 for stock solutions
- Edding
- Casycups, if Casy is used
- Inoculation needles
- Scaling boats

- *Pipet tips (according to the pipetting scheme and 10 + 200 µL tips for sampling)*
- *Glass flasks (1L, Schott, with pouring ring and lid): for demineralised water + 1 for the medium*
- *Beaker (100ml, form not important): 1 per concentration to prepare test solutions*
- *Measuring cylinder for 70 mL*

Note: These consumable need to be autoclaved before use.

2.3. Chemicals

- Test substance (use only one charge!)
- pH-buffer: 7 and 9
- Ethanol and Sterilium for the sterile bench

2.4. Solvents, substrates, and reagents

- OECD algae medium (see Guideline Nr. 201)
- Casyton for the Casy (if used)
- Lugol's solution for algae fixation (if cell counting chamber is used)

2.5. Used organisms

In der AG Filser erfolgreich genutzt wurden:

- *Raphidocelis (formerly Pseudokirchneriella) subcapitata* (strain No. 61.81, SAG, University of Göttingen)
- *Desmodesmus subspicatus* (strain No. 86.81, SAG, University of Göttingen)
- *Chlorella vulgaris* (information not available)

3. Preparations

3.1 Risk assessment

- Read Material Safety Datasheets of all media components and consider them for the risk assessment
- Consider the test substance itself
- Avoid the contamination of controls by all means, so the working place dealing with control treatments needs to be free of any contamination (also from other sources), appointments with colleagues may be necessary
- Include the waste disposal

3.2 Organization of materials and devices

- Reserve place in the climate chamber/room (maybe test the required space beforehand)
- Organise introduction into the Casy, if needed
- Install min/max thermometer in pure water and control temperature: optimum values are 22 ± 1°C in the climate chamber, 20 ± 1°C in the climate room
- Install shakers, if needed

3.3 Further preparations

- Read the OECD guideline 201 and adapt this protocol according to it, if needed
- Check status of permanent algae cultures in laboratory R0510 (window sill), and order new strains in Göttingen, if necessary
- Create a pipetting scheme for all test solutions
- Autoclave all materials and demineralised water for the algae medium
- Prepare all media stock solutions and autoclave them (see 5.3)
- Prepare OECD algae medium (see 5.3)
- Start an algae stock culture in fresh algae medium (about 1 week before the start of the test; for longer time scales, an inoculum needs to be transferred into fresh algae medium under the sterile bench)

3.4 Data: Notes for data recording and analysis

- To control the validity of the test, controls need to be sampled daily to count their algae numbers
- Statistical analyisis can be made by R or ToxRat, the later is only useful when the test design was not modified

4. Test conduction

4.1. Test substrate/solution

- 70 mL per concentration (with 3 replicates à 20 mL) of the solved test substance in algae medium

4.2. Endpoints of the test

4 Mean algae numbers at the end of the test period (72 h)
5 Algae growth rate during the whole test period

The algae growth rate is more independent of the tested algae species and strain and thus the recommended endpoint.

The growth rate can be calculated as follows:

$$\mu_{i-j} = \frac{\ln X_j - \ln X_i}{t_j - t_i} \ (\text{day}^{-1})$$

For the algae growth rate for the whole test period the parameters are:

μ_{i-j}= algae growth rate

X_i = algae number at the beginning of the test

X_j = algae number at the end of the test

t_i =start of the test (= 0)

t_J = end of the test (=3)

Daily growth rates can be calculated using the corresponding values for the single days.

4.3 Standards, validity criteria and range of measurements

- standards (reference substances): benzalkonium chloride, potassium dichromate ($K_2Cr_2O_7$), or 3,5-dichlorophenol
- Validity criteria:
  a) minimal daily growth rate in controls of 0.92 d^{-1}, corresponding to a 16-fold increase in algae numbers compared to the initial cell concentration during the complete test period (attention: for *R. subcapitata* and *D. susbspicatus* higher growth rates are given, see Appendix of OECD guideline no. 201)

  b) the differences between daily growth rates (day 0-1, 1-2, 2-3) of controls should not exceed 35 %. [calculation: standard deviation of daily growth rate of all replicates divided by the mean of all daily growth rates in all replicates]

  c) the differences of the complete growth rate (day 0-3) of all controls does not exceed 10 %, below 7 % for *R. subcapitata* und *D. subspicatus* [calculation: mean of all results of b]

4.4. Experimental design

- 1 control without test substance
- If needed: additional controls, e.g. pH controls or solvent controls
- if helpful: positive control with reference substance
- use of min. 6 concentration levels, a maximum of 8 is suggested, if one person is working alone at the Casy. The limiting factor in this case is the time required for measurements at the device, as all concentrations need to be finished within the afternoon. If a fixation with Lugol's solution is done, samples can also be counted days

after finishing the test. However, in this case, the Casy cannot be used, as the capillary system may be damaged by the iodine. For counting by microscope, a pilot test to determine the time required to count one sample can help to plan the test.

- 3 replicates per concentration, 6 replicates in the controls due to the higher variance in algae growth

4.5. Test protocol

1. Start of the test

Preparation of the sterile bench. Counting of the algae number in s subsample of the algae stock culture (by Casy or counting chamber). Take care that the stock culture is not contaminated by taking the subsample. Calculate the required volume to start the algae test. The initial algae number depends on the test species. A list of initial algae numbers can be found on page 4 (No. 21) in the OECD guideline Nr. 201. For *R. subcapitata* it is $5*10^3$-10^4 mL^{-1}.

Afterwards, all test solutions are prepared according to the pipetting scheme. Beforehand, the pH of the test medium needs to be measured in a subsample and if necessary, adjusted to 8.1 using HCl oder NaOH (it should deviate more than 0.5 units also during the test in the controls). Prepare all sterile material in the sterile bench and prepare the test solutions. Control the pH in subsamples of the test solutions and adjust as well, if needed (not more than 0.5 units difference to the control). Add the corresponding volume of algae and place test flasks on the shaker. The starting time of the test is the mean value of the addition of algae into all treatments. At this time, subsamples for cell counts will be taken the following day. Subsampling and counting is not done on day 0, here algae numbers equal the nominal initial algae number. If in doubt, it can still be measured.

2. daily controls/working steps

Every 24 hours at the starting time of the test, a subsample will be taken out of the test flasks and the algae number will be determined. For test solutions, this step can be excluded until day 3, but for controls it is mandatory to evaluate the validity of the test. If information on the toxicity mode is wanted, all treatments should be measured, e.g. to determine whether algae may recover from exposure. The volume of the subsample is related to the used counting method (Casy vs. counting chamber), so support by a more experienced person or a pilot test is required to find the most suitable volumes here. Take care of potential contamination of the treatments and also for personal risks or contamination of the working place during subsampling, counting (!), and disposal of subsamples.

In case that test vessels are not placed randomly on the shaker, daily rotation is recommended to avoid spatial effects.

3. Data analysis

To get a first overview, algae numbers at the end of the test (yield) vs. the concentration can be used. For further analysis, calculation of algae growth rates is required. In case of using R, these need to be further transformed into percentages of the control growth. For doing so, the mean of all controls serves as 100 % basis and is thus not directly but indirectly included in the concentration-effect curve.

5. Additions / important notes and documents

5.1. Further applicable documents

12. OECD guideline No. 201

5.2. Notes for disposal

13. According to the test substance

5.3. Appendix

Composition of OECD algae medium:

10 mL stock solution 1

1 mL stock solution 2

1 mL stock solution 3

1 mL stock solution 4

per 1 L demineralised water

Overview on the algae stock solutions:

Name	Chemical	Concentration (g/L)
stock solution 1: macro nutrients	NH_4Cl	1.5
	$MgCl_2 * 6H_2O$	1.2
	CaCl2*2H2O	1.8
	$MgSO_4 * 7H_2O$	1.5
	KH_2PO_4	0.16
stock solution 2: iron	$FeCl_3 * 6H_2O$	0.064
	$Na_2EDTA*2H_2O$	0.100
		Concentration (mg/L)
stock solution 3: trace elements	H_3BO_3	185
	$MnCl_2 * 4H_20$	415
	$ZnCl_2$	3**
	$CoCl_2 * 6H_2O$	1.5**
	$CuCl_2 * 2H_2O$	0.01**
	$Na_2MoO_4 * 2H_2O$	7**
stock solution 4: sodium bicarbonate	$NaHCO_3$	50,000 (50 g/L)

** prepare higher concentrated stock solutions and dilute for preparing this stock (minimum 1:10)

Preparation of the medium:

Weight and solve all salts according to the stock solution overview. Autoclave all stock solutions at 121 °C for 20 min, and store in the fridge (4 °C).

Autoclave demineralised water (according to required medium volume), flask and pipette tips. Prepare OECD algae medium according to the composition given above under the sterile bench and store in the fridge until use.

14.1.4 Method used for the acute test with D. magna at the University of Bremen

UFT Zentrum für Umweltforschung und Umwelttechnologie	Standard Operation Protocol **DAPHNIA IMMOBILIZATION TEST (ACCORDING TO OECD GUIDELINE NO. 202)** Valid since: 01.07.2016 Version Nr.: **01**
Department 10	Author(s): Yvonne Sakka,

1. General information / Introduction

1.1. Purpose

Evaluation of the acute toxicity of a (water soluble) substance or a mixture of substances to the Big Water flea, *Daphnia magna*.

1.2 Principle of the method

Measurement of the concentration-dependent immobilization of *D. magna* neonates by a substance or substance-mixture. For this purpose, a standard test design (according to guideline no.202) and a miniaturised design (Baumann et al., 2014) are available. The latter is of special interest, if a large number of tests need to be made in parallel or the amount of test substance is limited, as less animals and material are needed here.

1.3. Range of application of the method

Suitable for all water soluble substances or mixtures.

1.4. Limits of the method

The test needs to be conducted in Elendt M7 or M4 medium with a pH between 6.8 and 8.4.

1.5. Definitions / Abbreviations

- *D. magna: Daphnia magna*; Big water flea
- EC_{50}: Effect concentration 50 = 50% of the animals show an effect (are immobilised)

2. Material and Methods

2.1. Devices

4. 2 pH-Meter, one for the medium (free from any contamination), one for the test solutions
5. Min/Max-Thermometer for watery solutions
6. Repetitive pipet (max. pipetting volume: 50ml)
7. Place in climate chamber/room
8. If needed: LED-illumination (1110-1480 lux, cold white, not more than 15-20 µE m^{-2} s^{-1})
9. In case of illumination: timer
10. Light table

- Laboratory scale, measurement range according to test substance

- Maybe: bell prover for preparing a test stock solution
- Eppendorf-Pipets (according to the pipetting scheme for the test substance)

2.2. Consumables

a) Miniaturised test:

- 24-well multiwell plates: 1 per 2 concentrations (No. 83.1836; Sarstedt)
- 600ml Beaker: 1 per concentration to prepare the test solution
- Edding

11. Pipet tips (according to the pipetting scheme for the test substance)
12. Pipet tips for the repetitive pipet (50 mL)
13. Scaling boats

b) Standard test

- Glass vials with screw-off lids: 4 per concentration
- 600ml beaker: 1 per concentration to prepare the test solution
- Edding

14. Pipet tips (according to the pipetting scheme for the test substance)
15. Pipet tips for the repetitive pipet (50 mL)
16. Scaling boats

2.3. Chemicals

- Test substance (use one charge only!)
- pH buffer solutions: 4, 7 and 9

2.4 Solvents, substrates, and reagents

- Elendt M7 Medium

2.5. Used organisms

- *D. magna*: 10 animals per concentration (miniaturised design)/ 20 animals per concentration (Standard design), < 24 h old at test start

3. Preparations

3.1 Risk assessment

- Read Material Safety Datasheets of all media components and consider them for the risk assessment
- Consider the test substance itself
- Avoid the contamination of controls by all means, so the working place dealing with control treatments needs to be free of any contamination (also from other sources), appointments with colleagues may be necessary
- Glass may break and cause cuts
- Include the waste disposal

3.2 Organization of materials and devices

- Book place in a climate chamber/room (maybe test required space beforehand)
- Book the repetitive pipet
- Control material and order missing things
- Install the thermometer and check the water temperature: 20 ± 1°C is optimum
- If needed: programme the light cycling (16:8 h light-dark)

3.3 Further preparations

- Read the OECD guideline 202 and adapt this protocol, if needed
- Create a pipetting scheme for the test substance
- Define the immobilisation criteria for yourself, usually 15 s without movement (= leaving the place) after touching gently with a needle

- Control the health status of the *Daphnia* cultures: Are there problems with survival or reproduction? Do not start a test using such cultures, it will most likely not meet the validity criteria!
- Chose a culture for the test: optimal health and at least 3 reproductions took place, but the culture is not older than 30 days (in emergencies, cultures can be used after one reproduction)
- A day prior to the test, the daphnia cultures need to be synchronised = remove all offspring (neonates) carefully from the culture and note the time – until this time the following day, the test can be started
- On the test day, the neonates should be collected several hours prior to the test start to decide whether they are enough for conducting the test and to allow the neonates to recover from handling for an optimal results of the test

  Example: The start os the test is planned for 2pm >> Synchronisation on the previous day needs to be finished until 3pm (1h buffer for the start of the test) >> collection of neonates on the starting day of the test at about 10am >> about 4 h for recovery of neonates and to prepare the test solutions

3.4 Data: Notes for data recording and analysis

- Statistical analysis can be done by R or ToxRat – this can only be used for the standard design without modifications

4. Test conduction

4.1. Test substrate/solution

- Per concentration:
  2 mL per well in 10 wells = 20 mL (Miniaturised design)/ 10 mL per glass vial in 4 vials = 40 mL (Standard design)

4.2. Endpoints of the test

6 Immobilisation of neonates after 48 h

4.3 Standards, validity criteria and range of measurements

- Standard (reference substance): potassium dichromate ($K_2Cr_2O_7$); EC_{50} after 24 h: 0.6 – 2.1 mg/L
- Validity criteria:
  1) Mortality in the control, and all solvent controls: not succeeding 10%
  2) Oxygen content in all treatments is > 3 mg/L

4.4. Experimental design

a) Miniaturised design
10 replicates per concentration with 1 *Daphnia* per replicate
4-6 repeats of the complete test

b) Standard design
4 replicates per concentration with 5 *Daphnia* per replicate
3-4 repeats of the complete test

For both designs:

- 1 control without test substance
- Maybe further controls, e.g. in case of large pH differences when test substance is added, or if solvents are used
- If needed: positive control with potassium dichromate
- Min. 6 different concentrations, max. 15 for a single person. Limiting influence of the time to fill all test vessels and the addition of neonates into the vessels.

4.5. Test protocol

1. Test start

Control the pH in the test medium and adjust, if necessary
Collect all neonates out of the synchronised cultures into a beaker with fresh medium. Count during collection using the tally counter and decide whether a test can be started (10 or 20 per concentration, but add about 20 % to regard for background juvenile mortality and handling related death). Maybe design and pipetting scheme can be adapted to the neonate number. Feed the neonates with some feeding algae and place them back into the climate chamber.
Label all test vessels and prepare the materials needed for test solution preparation. Prepare the test solutions according to the pipetting scheme and control the pH in all test solutions. If the measurement starts in the lowest concentrated solution, drying with paper towels or at the edge of the beaker in between of two concentrations is sufficient for transfer of the pH sensor. This reduces the time needed for measurements.
Fill all test vessels with their corresponding concentration and volume, adapt the speed of the repetitive pipet to "medium" to avoid splashing. If necessary, test solutions need to be acclimatised in the climate chamber/room (e.g. in summer). Take the neonates out of the climate chamber and add them into the test vessels: use the pipetting adapter and start with the controls (work with increasing concentration), so that the same pasteur pipet can be used for all treatments. Suck in *Daphnia* carefully, take care that no animal is directly sucked to the opening and thus closing it, and let them sink back into the pipet tip (hold the tip vertically). Place the required number of neonates into the test vessel. Note the time required to finish this step: the mean can be used as test start and control time the following day. Store all test vessels in the climate chamber.

2. Daily work

Control the immobilisation of neonates each 24 h at the starting time of the test: place on the light table and touch gently with a needle, if no movement is immediately visible. Consider that this needle needs to be cleaned when the concentration is changed. Control the temperature and note it in the laboratory book for the test. At the end of the test, also the pH should be measured in one test vessel per concentration.
In case that test vessels are not placed randomly on the experimental area, rotate them every 24 h to avoid spatial effects.

3. Data analysis

a) Miniaturised design
As true replicates are missing, one test can be evaluated by the use of a binomial general model. If more than one test is conducted (recommended), the number of immobilised neonates per concentrations serves as result and the test repeats as replicates.

b) Standard design
The easiest analysis is the use of ToxRat and the number of immobilised neonates per replicate as raw data.

4. Modifications of the test:

Cetylalcohol can be added into all test solutions to reduce the surface tension that may entrap neonates.

5. Additions / important notes and documents

5.1. Further applicable documents

14. OECD guideline No. 202
15. Baumann et al., 2014
16. Method description "Daphnia culture"
17. Method description "green algae culture"

5.2. Notes for disposal

18. Pasteur pipets can be disposed in the (contaminated) glass waste
19. Do not forget: adapt the cleaning of all re-usable material to the test substance

5.3. Appendix

None

14.1.5 Method used for the chronic test with D. magna at the University of Bremen

UFT Zentrum für Umweltforschung und Umwelttechnologie	Standard Operation Protocol **DAPHNIA REPRODUCTION TEST (ACCORDING TO OECD GUIDELINE NO. 211)** Valid since: 01.07.2016 Version Nr.: **01**
Department 10	Author(s): Yvonne Sakka

Note: The conduction of the *Daphnia* reproduction test demands an active participation in maintenance of the cultures and requires experience in handling of the animals. The decision of the suitability to conduct the test is made by the actual supervisor of the *Daphnia* cultures (currently: Tonya Gräf).

1. General information / Introduction

1.1. Purpose

Evaluation of the reproductive (chronic) toxicity of a (water soluble) substance or a mixture of substances to the Big Water flea, *Daphnia magna*.

1.2. Principle of the method

Measurement of the number of offspring per individual organism in relation to the concentration of a test substance/mixture within 21 days, also onset of reproduction and status of the offspring (dead/unhatched or alive) is noted.

1.3. Range of application of the method

Suitable for all water soluble substances or mixtures.

1.4. Limits of the method

The test needs to be conducted in Elendt M7 or M4 medium with a pH between 6.8 and 8.4. If the pH for the test deviates from the pH of the culture, an adaptation of the animals to the deviating conditions may be needed. In this case, the adaptation needs to be coordinated with the supervisor(s) of the *Daphnia* cultures.

1.5. Definitions / Abbreviations

- *D. magna: Daphnia magna*; Big water flea
- EC_{50}: Effect concentration 50 = 50% of the animals show an effect

2. Material and Methods

2.1. Devices

17. 2 pH-Meter, one for the medium (free from any contamination), one for the test solutions
18. Min/Max-Thermometer for watery solutions
19. Repetitive pipet (max. pipetting volume: 50ml)

20. Place in climate room (attention: a chamber cannot be used for this test!)
21. If needed: LED-illumination (1110-1480 lux, cold white, not more than 15-20 µE m^{-2} s^{-1})
22. In case of illumination: timer
23. Light table

- Laboratory scale, measurement range according to test substance
- Eppendorf-Pipets (according to the pipetting scheme for the test substance)
- Centrifuge (Sigma, 3-18K; Rotor: H-12159) and re-usable centrifugation tubes (60mL volume)
- Cell counting chamber (Neubauer, Paul Marienfeld GmbH & Co. KG)

2.2. Consumables

- 100 mL beaker (slender/high shape): 10 per concentration and clean, new ones for each medium exchange
- 600 mL beaker: 1 per concentration to prepare the test solution
- Edding
- Pasteur pipets with tips removed with “hats”: 1 per concentration
- Pasteur pipets (short tips): 1 per concentration
- Petri- dishes, glass: 1 half per conducting person
- Pipet tips (according to the pipetting scheme for the test substance)
- Pipet tips for the repetitive pipet (50 mL for test media, 5 mL for feeding algae)
- Scaling boats
- Datasheets: 1 per concentration (see 5.1 and 5.3)

All materials listed can be ordered in the chemical inventory of the University.

2.3. Chemicals

- Test substance (use one charge only!)
- pH buffer: 4, 7 and 9

2.4. Solvents, substrates, and reagents

- Elendt M7 Medium
- Green algae medium for feeding algae (see 5.1)

2.5. Used organisms

- Feeding algae (*Raphidocelis subcapitata*): strain no. 61.81, SAG, University of Göttingen
- *D. magna*: 10 animals per concentration, < 24 h old at test start

3. Preparations

3.1 Risk assessment

- Read Material Safety Datasheets of all media components and consider them for the risk assessment
- Consider the test substance itself
- Avoid the contamination of controls by all means, so the working place dealing with control treatments needs to be free of any contamination (also from other sources), appointments with colleagues may be necessary
- Glass may break and cause cuts
- Include the waste disposal

3.2 Organization of materials and devices

- Book place in a climate room (about 3 concentrations fit onto one tablet)
- Book the repetitive pipet
- Control material and order missing things
- Print datasheets (1 per concentration)
- Programme light-dark cycle (16:8 h light:dark)
- Install the thermometer and check the water temperature: 20 ± 1°C is optimum (room temperature varies between 18-20 °C in winter and 20-22 °C in summer)
- Prepare the Pasteur pipet tips and label with treatment name/concentration, store in a beaker until use

- Prepare working place in the climate room with light table, datasheets in a folder, Petri dishes, Pasteur pipets, paper towels, and min/max thermometer

3.3 Further preparations

- Read the OECD guideline 211 and adapt this protocol, if needed
- Create a pipetting scheme for the test substance
- Define the immobilisation criteria for yourself, usually 15 s without movement (= leaving the place) after touching gently with a needle
- Control the health status of the *Daphnia* cultures: Are there problems with survival or reproduction? Do not start a test using such cultures, it will most likely not meet the validity criteria!
- Chose a culture for the test: optimal health and at least 3 reproductions took place, but the culture is not older than 30 days (in emergencies, cultures can be used after one reproduction)
- A day prior to the test, the daphnia cultures need to be synchronised = remove all offspring (neonates) carefully from the culture and note the time – until this time the following day, the test can be started
- On the test day, the neonates should be collected several hours prior to the test start to decide whether they are enough for conducting the test and to allow the neonates to recover from handling for an optimal results of the test

  Example: The start os the test is planned for 2pm >> Synchronisation on the previous day needs to be finished until 3pm (1h buffer for the start of the test) >> collection of neonates on the starting day of the test at about 10am >> about 4 h for recovery of neonates and to prepare the test solutions

3. Data: Notes for data recording and analysis

- Place datasheets into a separate folder and place it close to the light table
- Prepare an excel file to data analysis, an example can be found on Torp
- Use Image J to measure the length of the animals at the end of the test
- Statistical analysis can be done by R (one-factorial ANOVA with post-hoc analysis and Bonferroni correction) or ToxRat – this can only be used without modifications

4. Test conduction

4.1. Test substrate/solution

- Use 50 mL test solution per beaker for calculations of required volumes, add at least one more to regard the pipetting behaviour of the repetitive pipet, so at least 550 Ml per concentration
- Vitamins can be added into the test solution either before or after filing it into the 100 mL beakers, according to availability
- Feeding algae can be added either daily or with every media exchange, in tests with nanomaterials the exact time of addition needs to be defined to ensure homogenous exposure conditions. This may also be required for other materials interacting with the algae.

· 4.2. Endpoints of the test

24. Number of living offspring (neonates) per surviving *D. magna*
25. Number of dead and unhatched neonates per surviving *D. magna*
26. Start of reproduction
27. Number of reproductions (= broods)
28. Length at the end of the test

Additional endpoints:

29. Mortality of adult *D. magna*
30. Number of moults

· 4.3 Standards, validity criteria and range of measurements

- Standard (reference substance): not defined
- Validity criteria:

1) Mortality in controls not more than 20% (2 animals)
2) Number of living neonates in control: min. 60 (mean)

· 4.4 Experimental design

20. 10 replicates per concentration
21. 1 control without test substance
22. Maybe further controls, e.g. in case of large pH differences when test substance is added, or if solvents are used
23. Usually concentration clearly below the acute EC_{50} value are used to avoid mortality of adults during exposurel
24. More than 6 levels (including control) cannot be managed by a beginner, for an experienced person 9-10 are the maximum range

· 4.5. Test protocol

1. Test start

Control the pH in the test medium and adjust, if necessary
Collect all neonates out of the synchronised cultures into a beaker with fresh medium. Count during collection using the tally counter and decide whether a test can be started (10 or 20 per concentration, but add about 20 % to regard for background juvenile mortality and handling related death). Maybe design and pipetting scheme can be adapted to the neonate number. Feed the neonates with some feeding algae and place them back into the climate chamber.
Fill 50 mL algae culture into each of the re-usable centrifugation tubes. Centrifuge for 10 min at 4480 rpm (Programme 2), pour out the supernatant, and resuspend algae in 10 mL Elendt M7. Fill resuspended algae in a beaker and wash centrifugation tubes again with 5 mL Elendt M7. Pout this washing medium also into the beaker. Create a 100-fold dilution of a subsample (2 times: 990 µL tap water, 10 µL algae solution) and count this using the cell counting chamber and a microscope. Use the mean values per large counting square of each side to calculate the required volume of feeding algae with the prepared excel file (available on Torp). The amount of carbon is either fixed (0.1-0.2 mg C $animal^{-1}$ d^{-1}) or increasing (see excel file), in this case, the day used to calculate the required carbon content is the mean day of the medium exchange period (e.g. day 0 to 2-> day 1).
Label all test vessels and prepare the materials needed for test solution preparation. A dilution series is not recommended here, due to the large volumes required in this case. In parallel to test solution preparation, the pH of test solutions can be measured. If the measurement starts in the lowest concentrated solution, drying with paper towels or at the edge of the beaker in between of two concentrations is sufficient for transfer of the pH sensor. This reduces the time needed for measurements.
Prior to filling the beakers (test vessels), decide whether one tip is used per media exchange (with increasing concentration) or one tip is re-used for one concentration at every media exchange. In this case, a safe storage of tips needs to be ensured.
Finally, feeding algae are added. If necessary, test solutions need to be acclimatised in the climate chamber/room (e.g. in summer). Take the neonates out of the climate chamber and add them into the test vessels: use the pipetting adapter and start with the controls (work with increasing concentration), so that the same Pasteur pipet can be used for all treatments. Suck in *Daphnia* carefully, take care that no animal is directly sucked to the opening and thus closing it, and let them sink back into the pipet tip (hold the tip vertically). Place the required number of neonates into the test vessel. Note the time required to finish this step: the mean can be used as test start and control time the following day. Store all test vessels in the climate chamber.

2. Daily work

- Control temperature and note in the experiment folder, take care that the sensor is placed into demineralised water and re-fill, if needed.
- Control the mortality and note, if observed
- Counting of neonates:
  Neonates are caught with the Pasteur pipets for the corresponding concentration and counted. For counting, caught neonates are placed on the Petri dish with as less medium as possible. This will form a drop of test solution on the surface of the dish. Place the pipet vertically on the glass surface (press slightly) and suck in the medium carefully without sucking in the neonates again. The test medium is transferred back into the test vessel. The remaining neonates are immobilised and can be counted. Per dish about 3 test vessels can

be analysed. Here, the toxicity of the test substance needs to be considered for re-filling of different concentration levels and personal handling.

Note: If test vessels are not placed randomly in the climate room, daily rotation is required to reduce spatial effects.

3. Media exchange

3.1 Preparations

Follow the descriptions for the start of the test (part 1 of this section). The only difference to the initial procedure will be the adaptation of the required algae volume, if increasing amounts of food are used.

3.2 Media exchange

Transfer the test organisms into the new beakers using the broken pasteur pipets for the corresponding concentration (broken end into the "hat"). Take care that no neonate is transferred or the adult is injured by the transfer, and avoid that beakers are messed up (e.g. transfer from beaker 1 to 5 instead to 1 again). After the transfer of adults, moults and neonates can be counted. At least in one beaker per concentration, the pH is also measured. Further measurements of oxygen content, salinity, or water hardness are possible according to the behaviour of the test substance in the medium. However, consider that all devices are contaminated with the test substance after use. Used beakers are emptied (> chemical waste) and cleaned according to the test substance.

The media exchange and daily measurements should be done at approximately the same time of the day to reduce variability of results. The media exchange needs to take place every 2 to 3 days.

4. Data analysis

For all endpoints aside from mortality and start of the reproduction, all numbers for each surviving (!) female are added (cumulant result). These results are used as raw data for the statistical analysis, usually results are plotted as mean with standard errors vs. the concentration of the test substance. Mortality is not a typical endpoint of this test, but can be analysed by using a binomial generalised linear model using numbers of alive and dead animals per concentration as matrix.

5. Allowed modifications

Modifcations of feeding rhythm:

It can also be fed daily and/or a constant amount of algae (corresponding to 0.15 – 0.2 mg C/d). For daily feeding, store resuspended algae cool (4 °C) and dark for about 3 days. However, allow algae to warm up to room temperature prior to feeding.

Modifcations of media exchange:

Media exchange can also take place daily and the transfer of test organisms with 5 mL pipet tips (end cut off) is also possible. For this modification, the volume of test medium transferred together with the animal should be compared – the lower volume is preferable. Consider also the possibility to injure the test organism for each method.

5. Additions / important notes and documents

5.1. Further applicable documents

7 OECD guideline No. 211
8 Excel datasheet for calculate the required volume of feeding algae
9 Excel datasheet for data analysis of the chronic *Daphnia* test, as well as the word or pdf version for printing
10 Method description "*Daphnia* culture"
11 Method description "green algae culture"

5.2. Notes for disposal

- Pasteur pipets can be disposed in the (contaminated) glass waste
- Do not forget: adapt the cleaning of all re-usable material to the test substance

14.1.6 Measurement protocol of the AAS measurement

UFT Zentrum für Umweltforschung und Umwelttechnologie	**Method description** **QUANTIFICATION OF SILVER IN WATERY SOLUTIONS BY ATOMIC ABSORPTION SPECTROMETRY** Valid since: 01.07.2016 Version Nr.: **01**
Abteilung 10	Author(s): Yvonne Sakka (based on documents written by Jan Köser and Alica Rother, AG Stolte)

1. General information / Introduction

1.1. Purpose

- Quantitative measurement of concentration of silver in watery solutions, most dominantly test media of ecotoxicological tests. Adaptations for the measurement of silver in test organisms or soil eluates/extracts exist, but are not included in this description to avoid confusion. Measurement protocols are available from Alica Rother (AG Stolte).

1.2 Principle of the method

- Measurement of the absorption of gaseous silver at an atom-specific wavelength after heating of the liquid sample.

1.3. Range of application of the method

- Suitable for all watery solutions with a silver concentration > 5 µg Ag L^{-1} (standard) or > 1 µg Ag L^{-1} (modified sampling procedure, see 4.3)

1.4. Limits of the method

- Very high concentrations of chloride in the solution can cause sedimentation of AgCl (silverchloride), which disturbes the quatification. Additional validation of the measurement for this kind of test medium are required.
- Acids and water used to prepare all calibration solutions and make the sample preparation need to be free of any residues of silver, so that salpetric acid and water need to be produced by distillation (see 2.3).
- All samples should be 100 µL aliquots in reaction cups (1.5 mL) size) which are stored in the dark at 4 °C. For storage, addition of 10 µL 1 % HNO_3 is required (see 2.4)

1.5. Definitions / Abbreviations

- AAS: Atom-absorption-spectrometry
- HCl_{con}: concentrated chloric acid
- $HNO_{3,conc}$: concentrated salpetric acid
- H_2O_{dd}: double distilled water
- Aqua $regia_{dil.}$: mixture from chloric and salpetric acid (composition of a diluted version see 2.4)

2. Material and Methods

Note: All devices and materials belong to the working group Stolte. While planning the measurements ask for costs for use of devices and materials.

2.1. Devices

- Graphite furnace atomic absorption spectrometer (Solaar, Unicam; AG Stolte)
- Autosampler (AG Stolte)
- Equipment for a graphite cuvette exchange (plastic forceps and alignment tool)
- Eppendorf-Pipets: 200 and 1.000 µL (use only the ones labelled for AAS)
- Place under the hood
- Holder for reactions cups
- Holder for 50 mL centrifugation tubes
- 2 Thermoblocks (56°C and 95 °C can be chosen; 24 places for 1.5 mL reaction cups)
- Table centrifuge (for 1,5 mL Eppendorf rection cups)
- Vortex

2.2. Consumables

- 15 and 50 mL centrifugation tubes (single use)
- 5 and 10 mL plastic forceps for single use
- 1,5 mL reaction cups (preferable: by Eppendorf)
- Pipet tips (use only those marked for AAS)
- Brown Polyethylen flasks, 100 mL
- Cups for the AAS-Autosampler (large ones are used repeatedly, small ones are for single use only)
- Bell prover (1 L and 100 mL)
- Edding
- Laborarory gloeves (Nitrile or similar)

2.3. Chemicals

- H_2O_{dd}
- Salpetersäure 65 % (HNO_3, 1,4 g/mL, distilled)
- Salzsäure konzentriert 37 % p.a. (HCl, 1,18 g/mL)
- Argon (puritiy degree 4.6 is sufficient, it corresponds to 99,996 %)

2.4. Solvents, substrates, and reagents

- Aqua $regia_{verd.}$ (for 48samples: 22,5 mL H_2O_{dd}, 22 mL HCl_{conc} und 5,5 mL $HNO_{3,conc}$)
- 1 % HNO_3 (for 1 L: 989 mL H_2O_{dd} und 11 mL $HNO_{3,conc}$; for 50 mL: 49,45 mL H_2O_{dd} und 0,55 mL $HNO_{3,conc.}$)

- Silver calibration solution for measurements: 20 µg Ag/L in 1 % HNO_3 (prepare fresh, do not store!)
- Silver stock and calibration solutions: 10 g Ag/L, 1g Ag/L ,100 mg Ag/L, 10 mg Ag/L in 1 % HNO_3 (stored in the brown polyethylene flasks, see 5.3)
- Matrixmodifier: Palladium 1g/L

2.5. Analysed Organisms

Already measured for the working group Filser:

- Mites
- Collembola
- Daphnia

3. Preparations

3.1 Risk assessment

- Material Safety Datasheets for both acids and silver need to be included
- Consider the development of heat at the oven of the device during measurements
- Consider each step of sample preparation and measurement, so that the working with the acids does not cause risks to the worker or other peoplet
- Include disposal of solid and liquid wastes

3.2 Organization of materials and devices

- Organize the introduction to the AAS device with Alica Rother
- Coordinate the sample size and measuring plan with Alica (Is the AAS free?)
- Check the material for completeness and functioning, including the dates on the calibration solutions
- Organize the required number of Thermoblocks (1x WG Stolte, 1x WG Filser)

3.3 Further preparations

- Make new calibration solutions (if existing ones are older than 6 months), see 5.3
- Change the graphite cuvette directly before the measurement (Introduction by Alica)

3.4 Data: Recommendatios for data collection and analysis

- Export results as .csv oder .txt
- Calculate the corresponding silver concentrations yourself (more exact than the results from the device):
  1. Calculate the mean of all blanks (1 % HNO_3) and substract this mean from all adsorption-area results of the calibration
  2. Create a calibration curve using the corrected adsorption-area values as “x” and nominal concentrations as y-axis. Determine the start of the curve as zero for both coordinates.
  3. Background correction of the samples: 1. Substract the blank mean from all samples, 2. Substract the mean of the Aqua regia from all samples
  4. Transform all background corrected adsorption area results into silver concentrations using the equation from the calibration curve.
  5. Correct for dilution of the samples: using the standard method without dilution of your sample this would be a multiplication by 10.

4. Procedure

4.1. Time plan

Day 1: sample preparation (=ashing)

Day 2: resuspension and measurement of samples

4.2. Sample preparation (Day 1)

12 Place samples under the hood
13 Fill HCl_{conc} and $HNO_{3,conc}$ into 50 mL centrifugation tubes
14 Add 80 µL HCl_{conc} and 20 µL $HNO_{3,conc}$ into each 110 µL sample volume (sample + 1 % HNO_3)
15 Close all reaction cups, vortex for about 5 seconds
16 Centrifuge for 30 seconds at 10.000 rpm (corresponds to 6710 g)
17 Open all reaction cups and place them into the thermoblock
18 Ash at 56 °C overnight (let the liquid evaporate)

Note: If liquid remains the next morning, increase the temperature to 95 °C. Not used acids from day 1 can also be used the following day, but only, if they are definitely not contaminated. If you are in doubt, dispose them.

4.3 Resuspension of samples (Tag 2)

- take reactions cups out of the thermoblock and close after cooling them down (now, they could be stored, if needed) or directly continue with the sample preparation
- prepare 50 mL Aqua $regia_{dil.}$ in a centrifugation tube: 22,5 mL H_2O_{dd}, 22 mL HCl_{conc}, 5,5 mL $HNO_{3,conc}$ (adapt volume, if needed, 50 mL are sufficient for 48samples without further dilution using the standard preparation method)
- add 1 mL Aqua $regia_{dil.}$ to all samples
- close all reaction cups and vortex for about 30 seconds
- centrifuge for 5 min at 10.000 rpm (6710 g)
- if the AAS device is already ready for measurements: label the autosampler cups according to the sampling list; if the device is not ready yet, label a second set of reaction cups
- transfer 700 µL supernatant from each centrifuged sample into the corresponding autosampler cup/reaction cup
- prepare the 20 µg Ag L^{-1} calibration solution (add 50 µl of silver calibration solution 2 with 10 mg Ag L^{-1}, see 5.3, into 550 µl $HNO_{3,conc}$ in a 50 mL centrifuge tube and make up to 25 mL with H_2O_{dd} (using the mark of the tube.); alternatively: add 50 µl of silver calibration solution 2 directly into 25 mL 1 % HNO_3)

Modifications:

a) concentrations above the range of the measurement (>20 µg Ag L^{-1}) at least in some samples:

31. follow the sample preparation until the end
32. label additional autosampler cups
33. fill all with 900 µL Aqua $regia_{dil.}$
34. add 100 µL from the sample needing the dilution
35. mix the diluted sample by repeated pipetting (very important!)

36. make further dilutions accordingly until you expect the sample to be in the measuring range (0.5 – 20 µg Ag L^{-1}), note: a dilution factor of 10 has been proven to be useful
37. all dilutions as well as the original sample need to be placed into the autosampler for measurement, note: start with the highest dilution to avoid contamination of samples

b) concentrations below the range of the measurement (<0.5 µg Ag L_{-1}):

- follow the sample preparation protocol for the first day (Ashing) only
- reduce the volume of Aqua $regia_{dil.}$ for resuspension (down to 300 µL is possible without further modifications, minimum is 200 µL Aqua $regia_{dil.}$, but in this case, smaller autosampler cups need to be used).
- The supernatant is then:
  500 µL Aqua $regia_{dil.}$: 400 µL
  300 µL Aqua $regia_{dil.}$: 250 µL
  200µL Aqua $regia_{dil.}$: 150 µL

4.4. Preparation of the AAS device

- Open the Argon supply (tap on the right behind the computer): check first, whether the tap is closed (to the left), if yes, open the lever slowly and increase gas supply using the tap to 1,4 bar
- Open the cooling water supply (tap on the left behind the computer, „WKV“), about 1/4 turn is optimal
- Start computer (Password: aas), start software „solaar32“ (Password: solaar)
- Switch the oven and the measuring device on

<u>Adjust the parameter of measurements:</u>

- Check the method details (folder-symbol):

Name: Silber Jan/Victor 12µl
3 repeats per measurement
Acceptance level: 90%
Wavelength 328,19 nm
Zeeman-correction
Calibration with 5 steps

- Create a new data frame („Data“), use a name similar to: „User_Name for Data“

<u>Check parameters of the device</u>

- Open lid
- Check the position of the silver lamp (hollow cathode lamp), enter the correct position and chose the silver lamp in the software (“lamp-symbol“ for opening the software package), change status to “on”
- Control that lamp starts to shine
- Adjust optical parameters (Rainbow-symbol):
  Control the voltage on the photomultiplier at a wavlenegth of 328,1 nm: it should be 450 – 550 V, but more important is the relative output, it should be about 80 %. If needed, adjust optical parameters by re-positioning the lamp using the screws behind the bulb holder.
- Blank control [“00“ symbol]

- Close the lid
- Close the optical parameter window

Adjust the sample capillary:

- Switch on the capillary supervision camera software („GFTV“ Symbol)
- Switch on the camera in: method development
- Control that graphite cuvette appears as circle without unevenness or attaching condensation water
- Check whether condensation water is visible on the outside of the oven
- Move the sample capillary into the graphite cuvette (symbol of a circle with a hook inside) and control its position:
  The capillary should be directly in the middle of the opening of the cuvette. Control this positioning directly above the opening and by the camera picture (also in the midlle and not on the floor of the cuvette, about 1/3 distance should be remaining below the capillary).
- Adjust the capillary using the srews (on top and at the right side of the autosampler)
- Control the capillary position again: it should meet the correct position at least 3 times
- Switch off the camera (do not forget!) and close the window of the software

Direct preparations for measurements:

- Adjust the life time („Orders“ -> Adjust life time),but check that last value was noted in the user book for the device and add, if missing
- Clean the sample capillary 3times, and transfer the used solution into the drainage after each cleaning using the plactic pipettes
- Clean the graphite cuvette 2times (red-yellow symbol)

4.5. Measurement

- Enter programme sequence in the method details (folder symbol):
  Start with blank and calibration, repeat the blank and Aqua regia$_{dil.}$ as sample 1; each 10 samples add a re-standardisation (chose standard 4!) and a blank before and after this re-standardisation
- Positioning of large autosampler cups:
  R1: silver calibration solution 20 µg/L in 1 % HNO_3

  R2: about 1 mL Matrixmodifier (100 µL Palladium solution into 900 µL 1% HNO_3/ incase of >30samples: 120 µL Palladium solution + 1080 µL 1% HNO_3)

  R3 and R4: 1 % HNO_3, filled rather completely
- Place autosampler cups (filled) into the sample plate in accordance with the programme sequence in the method details; remember to add Aqua regia$_{dil.}$ as sample 1 (all blanks and standards will be used from the large autosampler cups/ if device was not used for some time, just place sample 1 and proceed to the next step (control measurement results), if this was done successfully, all other samples cen be added)
- Control measurement results:
  Adjust method details: 1 repeat (instead of 3) and remove elimination of outliers
  Chose single measurement (green cuvette symbol)
  And measure after each other:

➢ blank (signal without peak, about zero)

- Re-standardisation (reproducible between 0,35 A·s and 0,45 A·s)
- Sample 1 (signal without peak, between -0.010 A·s and 0.010 A·s)

- Clean the graphite cuvette
- Adjust method details for the complete measurement: repeats=3 ; elimination of outliers=on
- Blank („00“ Symbol)
- Start the analysis (“>>” symbol; duration about 4 hours for 2samples, about 7 h for 48samples)
- Control the measurement quality (taking the sample without splattering, peak without unevenness) for about 3 measurements
- Repeat at least once per hour, including gas and cooling water supply

4.6 Post-processing and Clean up

- Clean the graphite cuvette
- Clean the autosampler capillary 3 times
- Close Argon and cooling water supply
- Switch off the AAS devices (not the PC!)
- Export data frame as .txt/.cvs to your folder on the computer and to your USB
- Note use and life time in the book for the device, add which graphite cuvette was used
- Turn off the computer
- Dispose all solid and liquid wastes
- Clean up the hood and other working places used

5. Additions / important notes and documents

5.1. Further applicable documents

- Most recent version of this document from the WG Stolte (Alica Rother)

5.2. Notes for disposal

- All acids and silver solutions can be disposed together. Waste canister needs to be kept under the hood, due to the production of gaseous chloride.

5.3. Appendix

Preparation of silver calibration solutions (1 L each, adapted according DIN 38 406 part 18):

Silver Stock Solution (1000 mg Ag L^{-1}):

Weight 1,5748 g $AgNO_3$ and fill them into a 1 L bell prover. Add 11 mL of $HNO_{3,conc.}$ (65%, d = 1.4 g/L; distilled!) and make up to 1 L using H_2O_{dd}.

Silver Calibration Solution 1 (0,1 mg/L=100 µg/L):

Add 10 mL of the silver calibration solution 2 into a 1 L bell prover, add 11 mL of $HNO_{3,conc.}$ (65%, d = 1.4 g/L; distilled!), and make up to 1 L using H_2O_{dd}.

Silver Calibration Solution 2 (10 mg/L):

Add 10 mL of the silver stock solution into a 1 L bell prover, add 11 mL of $HNO_{3,conc.}$ (65%, d = 1.4 g/L; distilled!), and make up to 1 L using H_2O_{dd}.

Silver Calibration Solution 3 (100 mg/L):

Add 100 mL of the silver stock solution into a 1 L bell prover, add 11 mL of $HNO_{3,conc.}$ (65%, d = 1.4 g/L; distilled!), and make up to 1 L using H_2O_{dd}.

14.2 Currciculum vitae

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	General and theoretical ecology, Professor Filser, department of Biology, University of Bremen
08/2014 - 09/2014:	Research stay
	Technical University of Denmark (DTU), Department of Environmental Engineering, Professor Baun, Copenhagen
10/2009 - 08/2011:	Master of Science
	Marine Biology
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14.3 List of publications

14.3.1 Publications:

Chowdhury, A. M., Sakka, Y. Filser, Nutrient deficiency increases sensitivity of green algae towards silver nanoparticles, submitted manuscript

Sakka, Y., Völkel, A., Filser, J., Food reduction adds stress to chronic silver nanoparticle toxicity for *Daphnia magna,* submitted manuscript

Sakka, Y., Köser, J., Filser, J, 2016, How test vessel properties affect the fate of silver nitrate and sterically stabilized silver nanoparticles in two different test designs used for acute tests with *Daphnia magna*, under review

Sakka, Y., Mackevica, A., Skjolding, L. M., Baun, A., Filser, J.;2016, Influence of the stabilizers on silver nanoparticle toxicity in a chronic test with *Daphnia magna*; Aquatic Toxicology, Vol. 117, pages 526-535

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 Vol. 21; pages 2201-2213

14.3.2 Contributions to scientific meetings

Platform presentations

Filser, J., Hackmann, S., Köser, J., Lesnikov, E., Röhder, L., **Sakka, Y.**; Species, speciation and environment – risk of silver nanoparticles; 41.Tagung der Gesellschaft der Ökologie (GfÖ); 5. – 09.September 2011; Oldenburg

Poster

Sakka, Y., Mackevica, A., Skjolding, L. M., Baun, A., Filser, J.; Influence of the stabilizers on silver nanoparticle toxicity in a chronic test with Daphnia magna; SETAC Europe 25th Annual Meeting, 03.-07.05.2015, Barcelona, Spain

Sakka, Y., Baumann, J., Bertrand, C., Köser, J., Filser, J.; Effekte der Miniaturisierung des akuten Daphnientests auf die Toxizität von Silbernitrat und Silbernanopartikeln (NM-300K); 18.Jahrestagung der SETAC GLB; 23. – 26. September 2013; Essen

Sakka, Y., Baumann, J., Siol, A., Filser, J.; Schadstoffe im Leitungswasser – Aktivkohlefilter als einfache Abhilfe; Jahrestagung der SETAC GLB und der Fachgruppe Ökotoxikologie und Umweltchemie der GDCh; 10. – 13. September 2012; Leipzig

14.4 Supervised student theses

14.4.1 Master theses

05/2014-03/2015: Asif Moinur Chowdhury;

Interaction between nutrient supply and AgNP toxicity tested with two different green algae

14.4.2 Bachelor theses

04/14-09/14: Katharina Pilgram;

Toxicity of eluates of marine sediment cores

05/2013-01/2014: Alexander Völkel;

Interaction between food limitation and AgNP toxicity in the test organism *D. magna*

03/2013-09/2013: Sina Schüler;

Mixture toxicity of two antibiotics to *Daphnia magna*