Effects of Silver Nanoparticles on Bacterial Biofilms

Effekte von Silber-Nanopartikeln auf bakterielle Biofilme

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Geboren am 16.06.1983 in Bendorf

Angenommene Dissertation

Zur Erlangung des akademischen Grades eines Doktors der Naturwissenschaften

Fachbereich 3: Mathematik/Naturwissenschaften

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Datum der Disputation: 09.03.2020

Abstract

Because silver nanoparticles (Ag NPs) are broadly applied in consumer products, their leaching will result in the continuous release of Ag NPs into the natural aquatic environment. Therefore, bacterial biofilms, as the prominent life form of microorganisms in the aquatic environment, are most likely confronted with Ag NPs as a pollutant stressor.

Notwithstanding the significant ecological relevance of bacterial biofilms in aquatic systems, and though Ag NPs are expected to accumulate within these biofilms in the environment, the knowledge on the environmental and ecological impact of Ag NPs, is still lagging behind the industrial growth of nanotechnology.

Consequently, aim of this thesis was to perform effect assessment of Ag NP exposure on bacterial biofilms with ambient Ag NPs concentrations and under environmentally relevant conditions.

Therefore, a comprehensive set of methods was applied in this work to study if and how Ag NPs of two different sizes (30 and 70 nm) affect bacterial biofilms i.e. both *monospecies* biofilms and freshwater biofilms in environmentally relevant concentrations (600 - 2400 µg l^{-1}).

Within the first part of this work, a newly developed assay to test the mechanical stability of *monospecies* biofilms of the freshwater model bacterium *Aquabacterium citratiphilum* was validated. In the first study, to investigate the impact of Ag NPs on the mechanical stability of bacterial biofilms, sublethal effects on the mechanical stability of the biofilms were observed with negative implications for biostabilization.

Furthermore, as it is still challenging to monitor the ecotoxicity of Ag NPs in natural freshwater environments, a mesocosm study was performed in this work to provide the possibility for the detailed investigation of effects of Ag NPs on freshwater biofilms under realistic environmental conditions. By applying several approaches to analyze biofilms as a whole in response to Ag NP treatment, insights into the resilience of bacterial freshwater biofilms were obtained. However, as revealed by t-RFLP fingerprinting combined with phylogenetic studies based on the 16S gene, a shift in the bacterial community composition, where Ag NP-sensitive bacteria were replaced by more Ag NP-tolerant

species with enhanced adaptability towards Ag NP stress was determined. This shift within the bacterial community may be associated with potential detrimental effects on the functioning of these biofilms with respect to nutrient loads, transformation and/or degradation of pollutants, and biostabilization.

Overall, bringing together the key findings of this thesis, 4 general effect mechanisms of Ag NP treatment have been identified, which can be extrapolated to natural freshwater biofilms i.e. (i) the identification of Comamonadaceae as Ag NP-tolerant, (ii) a particular resilient behaviour of the biofilms, (iii) the two applied size fractions of Ag NPs exhibited similar effects independent of their sizes and their synthesis method, and (iv) bacterial biofilms show a high uptake capacity for Ag NPs, which indicates cumulative enrichment.

Zusammenfassung

Dadurch, dass Silber-Nanopartikel (Ag NPs) vielfältig in Konsumartikeln eingesetzt werden, führt deren Auswaschung zu einer kontinuierlichen Freisetzung von Ag NPs in natürliche Gewässer. Dadurch werden bakterielle Biofilme, welche die vorherrschende Lebensform von Mikroorganismen in der aquatischen Umwelt darstellen, sehr wahrscheinlich mit diesen in Form eines Umweltschadstoffes konfrontiert.

Ungeachtet der bedeutsamen ökologischen Relevanz von bakteriellen Biofilmen in aquatischen Systemen und obwohl erwartet wird, dass Ag NPs in diesen Biofilmen in der Umwelt akkumulieren, liegt der Wissensstand hinsichtlich der umweltbedingten und ökologischen Auswirkung von Ag NPs hinter dem industriellen Wachstum der Nanotechnologie zurück.

Demzufolge ist das Ziel dieser Dissertation, die Wirkungsbeziehung der Ag NP-Exposition gegenüber bakteriellen Biofilmen mit Ag NP-Immissionskonzentrationen und unter umwelt-relevanten Bedingungen zu erbringen.

Infolgedessen wurden eine umfassende Reihe an Methoden angewendet, um zu untersuchen ob und inwiefern Ag NPs in zwei verschiedenen Größen (30 und 70 nm) und in umweltrelevanten Konzentrationen (600 - 2400 µg l^{-1}) bakterielle Biofilme, d.h. *monospecies*- und Süßwasser-Biofilme, beeinträchtigen.

Innerhalb des ersten Teils dieser Arbeit wurde ein neu entwickelter Assay validiert, um die mechanische Stabilität von *monospecies*-Biofilmen des Bakteriums *Aquabacterium citratiphilum* zu untersuchen. In der ersten Studie, welche den Einfluss von Ag NPs auf die mechanische Stabilität von bakteriellen Biofilmen untersucht hat, wurden subletale Auswirkungen auf die mechanische Stabilität dieser Biofilme mit negativen Implikationen für die Biostabilisation festgestellt.

Weiterhin wurde eine Mesokosmus-Studie konzipiert und durchgeführt, innerhalb der die Auswirkungen von Ag NPs auf Süßwasser-Biofilme eingehend unter realistischen Umweltbedingungen untersucht werden konnte, da es derzeit technisch noch sehr anspruchsvoll ist, die Ökotoxizität von

Ag NPs in der Umwelt von Binnengewässern zu untersuchen. Innerhalb dieser Studie wurden verschiedene Methoden zur Untersuchung der Biofilmeigenschaften eingesetzt und damit Erkenntnisse über die Resilienz von bakteriellen Süßwasser-Biofilmen gewonnen. Demgegenüber konnte mittels t-RFLP *fingerprinting* und phylogenetischen Untersuchungen basierend auf der Sequenzanalyse des *16S*-rRNA-Gens nachgewiesen werden, dass die Exposition der Biofilme mit Ag NPs zu einer Verschiebung innerhalb der Zusammensetzung der bakteriellen Biofilmgemeinschaft führt, in der Ag NP-sensitive Arten von Ag NP-toleranten Arten, die besser an Ag NP Stress adaptiert sind, verdrängt wurden.

Diese Verschiebung innerhalb der bakteriellen Biofilmgemeinschaft könnte die Biofilm-Leistungen beeinträchtigen, die intakte Biofilme auszeichnen, wie etwa den Abbau erhöhten Nährstoffeintrags, die Umwandlung und/oder den Abbau von Schadstoffen sowie Biostabilisation.

Durch das Zusammenführen der wichtigsten Erkenntnisse dieser Dissertation konnten 4 generelle Wirkmechanismen durch die Ag NP-Behandlung identifiziert werden, die auf natürliche Süßwassersysteme übertragbar sein könnten: (i) Comamonadaceae wurden als Ag NP-tolerant identifiziert, (ii) Biofilme zeigen ein partiell resilientes Verhalten, (iii) die beiden eingesetzten verschiedenen Ag NP-Größen führten zu vergleichbaren Ergebnissen unabhängig von deren Größe oder Synthesemethode, (iv) Bakterielle Biofilme verfügen über eine hohe Aufnahmekapazität für Ag NPs, die auf eine kumulative Anreicherung hinweist.

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1.1. General introduction

Silver nanoparticles (Ag NPs) are ubiquitous in many consumer products (Vance et al., 2015) due to their versatile application possibilities compared to their homologous bulk material.

Especially their antimicrobial properties have led to broad applications in consumer products (e.g., textiles, food packaging, surface treatment of medical devices, and household cleaning appliances) (Fabrega et al, 2011). The antimicrobial properties of Ag NPs are supposed to be attributed to their small size and large surface area, which enables interaction with the microorganisms and, secondly, to their high surface area to volume ratio with the resulting higher release of silver ions compared to larger particles (Rai & Gade, 2009).

Overall, the main part of Ag NPs, which have been leached out from various sources, is firstly introduced into sewage treatment plants (STPs) (if STPs are available) before entering the aquatic environment (Blaser et al., 2008). Because sulfidization processes in sewage systems can be incomplete in wastewater treatment plants, Ag NPs are expected to be discharged into rivers at least partly in the metallic form (Kaegi et al. 2013). Ag NPs are expected to accumulate in surface waters (Benn & Westerhoff, 2008) and in the uppermost sediment layer in aquatic systems (Bradford et al., 2009). Hence, the predicted environmental concentrations of released silver from Ag NPs range from 0.1 pg l^{-1} to 1 µg l^{-1} in the water column and from 2 µg kg^{-1} to 14 mg kg^{-1} in sediments (Blaser et al. 2008; Gottschalk et al. 2013, Giese et al., 2018). Nevertheless, these predicted environmental concentrations are based on general assumptions without considering local conditions. Thus, actual accumulations will likely be highly variable. Furthermore, flood events may lead to a short-term release of Ag NPs into the water column resulting from sediment resuspension, as has been shown for a wide range of chemical compounds (Wölz et al. 2009).

Moreover, Ag NPs can accumulate in microbial biofilms (Ikuma et al. 2015), which are commonly found on all natural and man-made interfaces in aquatic systems. Biofilms are multicellular and mostly multispecies communities embedded in their self-excreted extracellular polymeric substances (EPS) forming a slimy, sticky matrix (Battin et al., 2016; Flemming & Wingender, 2010; Gerbersdorf &

Wieprecht, 2014). Bacterial biofilms are regarded as the dominant lifestyle of microorganisms in the environment (Flemming & Wingender, 2010; Kolter & Greenberg, 2006).

Furthermore, biofilms in streams and rivers are key sites of enzymatic activity (Romaní et al., 2008), including organic matter cycling, ecosystem respiration and primary production and, as such, being the base of the food chain, transferring carbon to higher trophic levels (Battin et al., 2016).

Apart from water, which is by far the largest component of the EPS matrix with 97 – 99% (Christensen & Characklis, 1990), the EPS matrix contains a mixture of macromolecules including proteins, polysaccharides, and DNA, which represent the major classes of constituents. Owing to polar functional groups (anionic and cationic), apolar compounds, and groups with hydrogen-bonding potential, the EPS matrix is very sticky (Flemming & Wingender, 2010). Moreover, the multitude of binding sites correlates with intermolecular chemicophysical interactions between the biopolymers, which accounts for mechanical stability in biofilms. The mechanical stability is crucial for biofilms to withstand hydrodynamic conditions in flowing waters in natural riverine systems where flow velocities are in the in the range of 0.1 - 6 m s^{-1}. Furthermore, the mechanical stability of biofilms has implications for biostabilization (Gerbersdorf & Wieprecht, 2014). Biostabilization is crucial for the cohesion of sediments in aquatic environments (Gerbersdorf & Wieprecht, 2014).

Metal cations including Ag^+ have shown to impair the mechanical stability, which has been explained with their affinity to bind to functional groups of exopolysaccharides and proteins in the EPS matrix (Chen & Stewart, 2002; Chaw et al., 2005). Thus, the network of physicochemical interactions, which is necessary for mechanical stability, might be disturbed and/or interrupted. Ag NPs are known to release Ag^+ ions and may even serve as a continuous source for ions (Navarro et al., 2008; Kroll et al., 2016; Gil-Allué et al., 2015; Li and Lenhart, 2012; Metreveli et al., 2016). Moreover, the accumulation of Ag NPs can result in enhanced local concentrations of released Ag^+ ions.

Consequently, continually increasing concentrations of Ag NPs in the aquatic environment might impair the ability of biofilms to withstand potentially harsh hydrodynamic conditions in flowing waters and might have adverse effects on biostabilization. Although there are a few studies reporting adverse

effects of Ag NPs on the biomass and structure of biofilms (Schaumann et al. 2015), investigating the mechanical stability of bacterial biofilms in response to Ag NP has been neglected.

Furthermore, applied concentrations of Ag NPs in ecotoxicological studies range from 0.1 µg l^{-1} – 200 mg l^{-1} with most frequently used concentrations in the upper range of mg l^{-1} (e.g. Fabrega et al., 2011). Hence, in many cases, applied concentrations of Ag NPs in ecotoxicological studies among the literature are much higher than predicted environmental concentrations of Ag NPs (Giese et al., 2018) and do not represent environmental conditions (Fabrega et al., 2011). Taken together, toxicity assessment of Ag NPs on mechanical stability of bacterial freshwater biofilms with environmentally relevant concentrations of Ag NPs has been urgently required.

Notwithstanding the ecological relevance of bacterial biofilms and though Ag NPs are expected to accumulate within these biofilms in the environment, effects of Ag NPs to aquatic bacterial biofilms are poorly understood. This is reflected by inconsistent findings as a consequence of Ag NP exposure among the literature. Reductions in biomass (Kroll et al., 2016; Fabrega et al., 2011a), alterations in bacterial community composition (Fabrega et al., 2011a, Yang et al., 2014, Das et al., 2012) and reduced metabolic activities (Das et al., 2012, Gil-Allué et al., 2015) have been reported. Contradictory effects have been shown for wastewater biofilms which were highly tolerant to Ag NP treatment (Sheng & Liu, 2011) and for marine biofilms which were only negligibly affected (Bradford et al., 2009). Pertaining to the effects of Ag NPs on bacteria in aquatic environments, a range of habitats, such as marine biofilms (Fabrega et al, 2011a; Bradford et al, 2009), activated sludge (Sheng & Liu, 2011; Yang et al., 2014), surface stream water (Das et al., 2012), and freshwater biofilms (Kroll et al., 2016; Gil-Allué et al., 2015), have been investigated. Nevertheless, only a few studies have addressed freshwater biofilms although the biofilms of streams and rivers are of high interest. These habitats receive waste effluents of STPs and are most likely to be confronted with Ag NPs (Kroll et al., 2016; Gil-Allué et al., 2015). In this area there is a critical knowledge gap, which needs to be filled up because of the relevance of freshwater biofilms in important ecosystem functions. The composition of the bacterial community in these freshwater biofilms as well as the structure of the biofilms substantially contribute

to the ecosystem functions such as biodegradation with respect to nutrient loads, transformation and/or degradation of pollutants, and biostabilization. For example, Actinobacteria and Alphaproteobacteria which are commonly found in stream biofilms (Manz et al., 1999; Brümmer et al., 2000; Zwart et al., 1998, Zwart et al., 2002) are known for their variable metabolic properties, such as the production of secondary metabolites (Lechevalier & Lechevalier, 1967) and the degrading of complex organic compounds (Newton et al., 2011). Consequently, an absence of these classes in freshwater biofilms due to Ag NP exposure would impoverish some functional properties of the biofilms such as biodegradation with respect to nutrient loads as well as transformation and/or degradation of pollutants.

In summary, studies to broaden the knowledge of the impact of Ag NPs on freshwater biofilms of rivers with respect to biofilm structure and bacterial community composition are urgently required, not least in order to assess the risk of released Ag NPs into aquatic systems.

Nevertheless, as noted above, applied concentrations of Ag NPs in ecotoxicological studies are in the range of mg l^{-1} and do obviously not represent environmental conditions (e.g. Fabrega et al., 2011). Moreover, monitored time periods with average dose regimes of ~24 h (e.g. Fabrega et al., 2011) might be too short to mirror an environmental relevant scenario (Fabrega et al., 2011).

Furthermore, as it is still challenging to monitor the ecotoxicity of Ag NPs in natural freshwater environments, there is a need for suitable experiments to facilitate this task. Studies performed in mesocosms provide the possibility for the detailed investigation of effects of AgNPs on freshwater biofilms under realistic environmental conditions. Thus, studies which address artificially lotic biofilms treated with environmentally relevant concentrations of Ag NPs as well as covering a long evaluation period that mirrors an environmental relevant scenario are much needed.

Overall, bioavailability of Ag NPs by bacteria and the resulting bactericidal effects seem to be dependent on the size, shape, surface coating, surface charge, surface structure and area, solubility, Ag^{+} release, solution chemistry, Ag NP mediated reactive oxygen species (ROS) production and aggregation state of the Ag NPs (Fabrega et al., 2011, Xiu et al., 2012). These distinct physicochemical

properties can be determined by the type of NP syntheses (Fabrega et al., 2011). Consequently, to strive for a broader picture about toxicity mechanisms, it is necessary to investigate the effects of Ag NPs synthesized by various types of syntheses (e.g. by top-down technique and bottom-up approach). In addition, because smaller particles have been claimed to be more toxic than bigger ones (Lok et al., 2007; Pal et al., 2007; Radniecki et al, 2011), more work is needed to compare Ag NP triggered effects pertaining to different sizes of Ag NPs.

In the following paragraphs the properties of Ag NPs, their transformations, fate and bioavailability in nutrient media and in the environment are described in more detail. Furthermore, deduced from this, the currently known mechanisms of toxicity of Ag NPs on bacteria and bacterial biofilms together with the ecological relevance of bacterial freshwater biofilms with some further implications and rationales for this work are discussed in detail.

1.2. Nano, -materials, -particles, -technology

The term nano is derived from "*nanos*", the Greek word for dwarf, and means extremely small. Hence, nanomaterials are typically specified as materials containing particles for 50% or more in the size of 1-100 nm (Commission Recommendation on the definition of nanomaterial, 2011). Nanomaterials can be formed deliberately or accidentally and they can be of anthropogenic or of natural origin. In nature, they are formed, for example, as soot particles in fires or can be synthesized by magnetotactic bacteria, a polyphyletic group of bacteria, as magnetite nanoparticles called magnetosomes (Bennet et al., 2015). Nanoparticles (NPs) are part of diesel exhaust or arise during welding operations.

However, in nanotechnology NPs are manufactured specifically, mainly to produce nanomaterials, which are of assistance to the industry or medicine. The concept of nanotechnology was first introduced 1959 by the physicist Richard P. Feynman in his lecture *There's plenty of room at the Bottom* (Feynman, 1959), but the term was characterized by Norio Taniguchi in the year 1974, who described nanotechnology as *the processing of separation, consolidation, and deformation of materials by one atom or one molecule* (Taniguchi, 1974).

The advantages and benefits of nanotechnology as a multidisciplinary science and for industrial application are reflected in the facts that well established functionalities can be introduced into smaller dimensions and, moreover that the properties of NPs substantially differ from the corresponding bulk material. For example, in conjunction with the well-known effect of hyperthermia, magnetic iron nanoparticles are applied to target selectively cancer cells, which are then killed by increasing the temperature (Singh & Sahoo, 2014). Altered physical properties of NPs with respect to melting point, surface charge, mechanical stability, and surface properties can be accompanied by different chemical properties. More precisely, the drastic increase in the specific surface area can be proportionally correlated with chemical reactivity. Thus, the novel physicochemical properties of nanomaterials are coming along with improved electronical, optical, and mechanical properties for application in a wide range of areas including medicine, cosmetics, renewable energies, environmental remediation, and electronic devices (López-Serrano et al., 2014; Fabrega et al., 2011). Their versatile applications are also due to the fact that NPs can comprise of various organic and inorganic materials such as carbon (nanotubes, fullerenes, etc.), metal oxides (zinc oxide, titanium dioxide, iron oxide, etc.), metals (silver, gold, iron, etc.), polymers (e.g. silicon dioxide), and semiconductor particles i.e. quantum dots (cadmium sulphide and cadmium selenite). The total global market size of nanotechnology products in 2014 exceeded 1 trillion US dollars and is expected to grow up to 3 trillion US dollars in 2018 (Emashova et al., 2016). The main area, where nanotechnology is being applied, is in consumer products (López-Serrano et al., 2014). In the recent past, there were up to several thousand products containing nanomaterials and the largest share is attributable to the health and fitness category (Project on Emerging Nanotechnologies (2013). Consumer Products Inventory. Retrieved [Accessed 11/05/2016], from http://www.nanotechproject.org/cpi; Vance et al., 2015). According to the Nanotechnology Consumer Product Inventory (CPI) metals and metal oxides are listed in 37% of the products and represent the largest nanomaterial composition group (Vance et al., 2015).

Titanium dioxide (TiO_2), silicon dioxide (SO_2), and zinc oxide (ZnO) are the nanomaterials, which are manufactured mostly worldwide (on a mass basis) and the global annual production of silver

nanoparticles (Ag NPs) represents only 2% of that of TiO_2 NP (Vance et al., 2015). However, in terms of quantity Ag NPs are most widely inserted in 24% of the products (Vance et al., 2015).

Though nanomaterials show great potential to combat environmental pollution by applications of nanotechnology in the sustainable development of renewable energy, air-, water-, and soil-remediation such as adsorption of Pb^{2+}, Cu^{2+}, and Cb^{2+} ions, and removal of barium ions, Pb, Cr, and nitrate from water, to name a few (Das et al., 2015), nanoproducts themselves can be considered as pollutants.The widespread use of nanomaterials and the expected continual increase in nanomaterial production might result in NP's rapid incorporation and accumulation within the environment via various process routes, such as manufacturing, product use and waste disposal (Holden et al., 2014). Even though for the past several years, the number of studies pertaining to the physical, chemical and biological properties of nanoparticles as well as the understanding of nanotoxicity has increased significantly (Fatisson et al., 2013), the knowledge on the environmental and ecological impact of nanomaterials, in particular with respect to the prevalently applied Ag NPs, is still lagging behind the industrial growth of nanotechnology (Holden et al., 2014).

1.3. Silver nanoparticles

1.3.1. Applications

Silver as a precious metal is relatively inert in its metallic state, but silver ions, which e.g. are supposed to be released by silver coming into contact with wound fluid, are highly reactive and are claimed to exhibit antibacterial properties (Rai et al., 2009). These antibacterial properties of silver compounds and ions have been extensively used both for hygienic and medical benefits for centuries (Tolaymat et al., 2010; Rai et al., 2009; Chen & Schluesener, 2008). With the discovery of penicillin in the 1940s and the subsequent production of many other antibiotics and disinfectants, silver lost its importance as an anti-infective agent (Tolaymat et al., 2010; Rai et al., 2009).

Because Ag NPs exhibit altered physicochemical and biological properties compared to the bulk material and due to the emergence of antibiotic-resistant bacteria, in the last decade, renewed interest has arisen in silver in the form of Ag NPs as an antibacterial agent for applications in medicine (e.g.

wound dressings, scaffold, skin donation, recipient sites, sterilized materials in hospitals, medical catheters, contraceptive devices, surgical instruments, bone prostheses, artificial teeth, bone coating), the health industry (e.g. cosmetics, lotions, creams, toothpastes, laundry detergents, soaps, surface cleaners, room sprays, antimicrobial paints), home applications (e.g., washing machines, air and water filters), food storage containers, and textiles (Tolaymat et al., 2010; Fabrega et al, 2011; El-Nour et al., 2010). The antibacterial properties of Ag NPs are supposed to be attributed to their small size and large surface area, which enables interaction with the microorganisms and, secondly, to their high surface area to volume ratio with the resulting higher release of silver ions compared to larger particles (Rai & Gade, 2009).

Other unique properties of Ag NPs open up a wider spectrum of technologies and fields of application. The optical properties can be deduced from the distinct localized surface plasmon resonance (LSPR), a UV-vis (ultraviolet-visible) absorption band that is not present in the spectrum of the bulk material (Tolaymat et al., 2010; Haes & Van Duyne, 2002). Thus, the fluorescence and the surface plasmon resonance render Ag NPs operational in sensing applications such as detection of DNA sequences, laser desorption/ionization mass spectrometry of peptides, real-time probing of membrane transport in living microbial cells, colorimetric sensors for measuring ammonia concentration, biolabeling, optical imaging of cancer, biosensors for detection of herbicides, and glucose sensor for medical diagnostics (Tolaymat et al., 2010).

The large surface area of Ag NPs, which is accompanied by high surface energy and reactivity features catalytic properties being useful in many reactions such as CO oxidation and benzene oxidation for phenol (Tolaymat et al., 2010).

In electrotechnology Ag NPs are applied in inks, optoelectronics, nanoelectronics, sub-wavelength optics, and data storage devices, to name a few (Tolaymat et al., 2010).

In general, size and shape (i.e. rod-shapes, prisms, tubes, and spheres) essentially determine the properties and resulting uses of Ag NPs. For instance, the width and position of SPR peak depend mainly on the particle size and shape and the thermodynamic properties of silver nanoparticles (e.g.,

melting point and molar heat of fusion) are directly proportional to particle diameter (Jiang et al., 2007; Luo et al., 2008; Basavaraja et al., 2008; Tolaymat et al., 2010). Furthermore, smaller Ag NPs have claimed to exhibit a higher antibacterial toxicity (El-Nour et al., 2010) and triangular Ag NPs have shown a higher antibacterial activity against the bacterium *Escherichia coli* than spherical and rod-shaped nanoparticles (Pal et al., 2007). Thus, two Ag NPs made of the same bulk parent material, but differing in size and/or shape may exhibit different physical, chemical and biological properties and uses (Fabrega, 2009). In order to precisely produce sizes and shapes for particular applications, Ag NPs are synthesized using various techniques.

1.3.2. Syntheses

Synthesis techniques are generally categorized into top-down and bottom-up approaches (Prabhu & Poulose, 2012). The top-down technique is a physical approach, in which the size of silver in its bulk form is mechanically reduced to nanoscale utilizing methods including evaporation/condensation, lithography, and laser ablation (Amendola et al., 2007, Tolaymat et al., 2010, Abou El-Nour et al., 2010). However, it has been evaluated that the bottom-up approach was applied in the prevailing majority of Ag NPs syntheses compared to the top-down technique (Tolaymat et al., 2010). This has been attributed to a higher manufacturing precision via the bottom-up approach with respect to size, shape and surface of the synthesized Ag NPs leading to a higher applicability compared to Ag NPs generated by the top-down approach (Fabrega et al., 2011; Tolaymat et al., 2010).

Ag NPs can be synthesized in various shapes such as 1-D objects (e.g., thin films), 2-D objects (e.g., nanowires, nanorods) and 3-D objects (e.g., spheres) (Muraviev, 2005; Tolaymat et al., 2010), of which spheres are the mainly existing shape accounting for approximately 90% of Ag NPs produced by bottom-up technique (Tolaymat et al., 2010). The bottom-up (also known as self-assembly) technique is a chemical approach in which silver ions in solution are reduced by the subsequent addition of a reducing agent resulting in the formation of silver atoms (Ag^0), small silver clusters or aggregates (Abou El-Nour et al., 2010, Tolaymat et al., 2010, Fabrega et al., 2011). The predominantly used silver salt precursor providing the silver ions is silver nitrate (Ag NO_3) due to its low cost and chemical stability

(Lee et al., 2007; Tolaymat et al., 2010). Besides the use of organic solvents, water is the solvent which is prevalently applied to solubilize the reacting agents in the synthesis process (Tolaymat et al., 2010). The colloidal chemical reduction of Ag NO_3 is performed with borohydride, citrate, ascorbate, polyethyleneimine (BPEI) or other reductants.

More recently, another bottom-up procedure, in which Ag NPs are synthesized by bacteria, fungi, and plant extracts has been applied (Prabhu & Poulose, 2012). Ag NPs are produced by this biosynthetic method in reduction/oxidation processes which are catalysed by intracellular and extracellular microbial enzymes or plant phytochemicals (Prabhu & Poulose, 2012). Nucleation and growth of the Ag NPs are mediated by proteins and macromolecules, which also serve as nontoxic stabilizing agents (Fabrega et al., 2011; Prabhu & Poulouse, 2012). This method promises a more environmentally friendly and sustainable approach by avoiding toxic chemicals (Prabhu & Poulouse, 2012).

1.3.3. Stabilization of Ag NPs

Because 'bare' Ag NPs are often not stable in solution, the supplemental use of stabilizing agents (also known as capping agents or coatings) including citrate, other organic molecules (e.g. sodium dodecyl sulfate (SDS)), or polymers (e.g. polyethylene glycol (PEG)) are required to prevent the formation of large aggregates and agglomeration (Fabrega et al., 2011, Tolaymat et al., 2010). Stabilizing agents modify the physicochemical and morphological characteristics of manufactured Ag NPs and thus may interfere with the properties, behavior, and toxicity of Ag NPs (Tolaymat et al., 2010). For example, the application of sodium citrate as a reducing agent produces a negatively charged Ag NP which may show different behaviour and interaction than a positively charged Ag NP produced with polyethyleneimine (BPEI) (Tan et al., 2007; Tolaymat et al., 2010). Furthermore, it has been observed that citrate-stabilized Ag NPs impacted dissolution pertaining to a lesser release of Ag^+ ions compared to Ag NPs stabilized with polyvinyl pyrrolidone (PVP) or tannic acid (Yang et al., 2011; Dobias & Bernier-Latmani, 2013). This has been attributed to several mechanisms resulting in a lowering in solubility. Ag^+ ions may bind to the carboxylic groups of the organic acid leading to the retention of the ions and/or citrate may

serve as a reductant, which reduces the oxide layer at the surface of the Ag NPs back to Ag^0 (Dobias & Bernier-Latmani, 2013, Liu & Hurt, 2010; Li et al., 2011).

Citrate is the most commonly described stabilizing agent accounting for 27% and 50% of the stabilizing agents used in general and specific applications, respectively (Tolaymat et al., 2010). Other widely used stabilizers are polyvinyl pyrrolidone (PVP) (~ 18%) and amines (~ 8%) (Tolaymat et al., 2010).

Stabilization of Ag NPs is based on electrostatic, steric, or electrosteric repulsion. Electrostatic stabilization is often mediated by charged, anionic species (e.g. halides, carboxylates, or polyoxoanions). The charge of these species forms an electrical double layer leading to coulombic repulsion between the Ag NPs, which overcomes the attractive short range of van der Waals forces responsible for aggregation (Abou El-Nour et al., 2010; Fabrega et al., 2011). For example, citrate-stabilized Ag NPs are stabilized by electrostatic repulsion (Sharma et al., 2014). However, the chemical composition of the solvent medium with respect to ionic strength and medium components strongly affects the dispersion behaviour of the electrostatically stabilized Ag NPs (Fabrega et al., 2011).

Steric stabilization, though far more poorly understood but less susceptible pertaining to external solution conditions, is mediated by a cover of polymers (typically organic materials) preventing the particles to get close in the range of attractive forces (Abou El-Nour et al., 2010, Fabrega et al., 2011). PEG- and PVP-stabilized Ag NPs are typical examples for steric stabilization (Sharma et al., 2014).

In summary, because ionic strength and solvent components can have a great impact on size and size distribution of the Ag NPs, characterization with respect to the physicochemical properties of the Ag NPs is important to understand and control Ag NP synthesis and applications (Reidy et al., 2014; Abou El-Nour et al., 2010). Moreover, the knowledge about well characterized Ag NPs used in products, in toxicological exposure media and found in the environment may provide an assessment for the fate, transport and toxicity of Ag NPs in the environment (Fabrega et al., 2011; Tolaymat et al., 2010).

1.3.4. Characterization of Ag NPs

1.3.4.1. Methods and tools for characterization

There is a number of methods suited for separation, extraction, and enrichment of NPs as well as for assessing different parameters of NPs including size, shape, and surface area, which are based on different physical principles (Reidy et al., 2013). These different methods are marked by distinct advantages and limitations and thus, the application of a particular method needs to be targeted to the composition of the sample and aimed at the analytical output. It also has to be mentioned that often several methods are used in parallel with a certain discrepancy in obtained data, which can be explained by different underlying measurement principles and assumptions, different sample preparation protocols, and different base of size distribution (mass, number) (Reidy et al., 2013).

1.3.4.2. Separation and fractioning techniques

Mostly, in order to characterize nanoparticles (NPs), a first step is to separate and enrich NPs from their matrices with the aim to preserve the actual state of NPs with respect to aggregation, coating and indication for chemical transformation (Schaumann et al., 2015). Separation of NPs from liquid samples does not require drying or extraction (Schaumann et al., 2015). Common used techniques for liquid and/or environmental samples are ultracentrifugation, cross-flow ultrafiltration, size exclusion chromatography (SEC), hydrodynamic chromatography (HDC), sedimentation field flow fractionation (Sed-FFF), and flow-field flow fractionation (AF4) (Fabrega et al., 2011; Schaumann et al., 2015). A pre-concentration step is required as well as NP-membrane interactions can occur both for Sed-FFF and AF4, which can be disadvantageous (Fabrega et al., 2011). But these methods are non-destructive and exhibit a high size resolution, which generates benefits (Fabrega et al., 2011). AF4 provides a high size resolution from particles in the range from 1 nm to several µm basing on their diffusion coefficients compared to Sed-FFF, which is used to assess particles bigger than 50 nm in size based on particle mass distribution (Schaumann et al., 2015). For SEC, the scope of application is below 100 nm pertaining to the size of NPs. Although HDC exhibits a lower resolution compared to FFF techniques, this method provides a robust separation range, high reproducibility, high recoveries, and simultaneous detection

and quantification of NPs (Schaumann et al., 2015). Using HDC, NPs are separated in a capillary or column filled with uniform microspheres, in which the retention time of the NPs is attributed to the size (for particles > 5 nm, effective diameter), but also to their shape (Schaumann et al., 2015).

1.3.4.3. Microscopy-based techniques

Scanning electron microscopy (SEM) and transmission electron microscopy (TEM) are powerful tools to assess size, shape and aggregation/agglomeration of NPs, by which TEM is currently the most commonly applied electron microscopy technique (Reidy et al., 2013; Fabrega et al., 2011). Microscopic analysis require sample preparation such as supercritical drying (SCD), N_2-shock freezing, or freeze drying, what leads to drying artefacts with lower informative value pertaining to the pristine particle aggregation behavior and the morphology of organic NP coatings (Schaumann et al., 2015). Thus, if NPs are characterized by electron microscopy solely, drawing conclusions about the behaviour of NPs in suspension is limited (Reidy et al., 2013).

Applying atomic force microscopy (AFM) delivers a high resolution in determining size, shape and aggregation/agglomeration of NPs, but is also limited with respect to drying artefacts as well as a poor lateral accuracy (Fabrega et al., 2011, Schaumann et al., 2015). In contrast to the rather traditional microscopy techniques SEM and TEM, AFM measures three-dimensional images that provide data about particle height and volume (Abou El-Nour et al., 2010).

1.3.4.4. Light scattering based methods

Dynamic light scattering (DLS) and nanoparticle tracking analysis (NTA) provide information about the particle size based on the scattering of light by particles (Reidy et al., 2013). DLS is commonly applied in order to obtain data about the characteristics of NP in solution pertaining to hydrodynamic diameter, nanoparticles behavior regarding agglomeration and dissolution, as well as size distribution and polydispersity index (Reidy et al., 2013). Because DLS can lead to an overestimation of the hydrodynamic diameter, it is best suited for spherical particles (Fabrega et al., 2011; Reidy et al., 2013) and with the latest technology, from particles in the range from 0.1 nm to several µm. DLS is less effective for more complex samples, which are heterogeneous, polydispers or contain other nanosized

entities such as protein clusters, because no distinction can be made between particles of different origin (Reidy et al., 2013). NTA is an efficient method for this task, because individual nanoparticles in a suspension can be microscopically determined and distinguished on a particle-by-particle basis (Reidy et al., 2013).

1.3.4.5. Spectroscopic methods

Ultraviolet-visible spectroscopy (UV-vis) is applied in order to gain insights into size, shape, and aggregation and dissolution behavior of metallic NP by showing the plasmon resonance (Abou El-Nour et al., 2010). The shift in the adsorption maximum gives an indication for particle size and shape (Reidy et al., 2013). However, it is rather a qualitative approach, because interactions with ions and biomolecules can affect the peak (Reidy et al., 2013).

Inductively coupled plasma mass spectrometry (ICP-MS), inductively coupled plasma optical emission spectrometry (ICP-OES), and atomic absorbance spectrometry (AAS) allow total elemental composition in bulk to be quantified with limitations about NP information (Fabrega et al., 2015). ICP-MS is isotope specific and heavy elements can be detected and quantified in complex samples with a detection limit in the range of ng l^{-1} (Schaumann et al., 2015).

More recently, single-particle ICP-MS (SP-ICP-MS) has been developed, by which single particles can be detected with a size limit of 18 nm for Ag NPs and at very low concentrations (Schaumann et al., 2015). On the basis of strongly diluted samples, which have to be injected droplet by droplet, spike signals of vaporised single particles can be detected (Schaumann et al., 2015; Reidy et al., 2013). Indeed, in conventional ICP-MS a millions of droplets are measured simultaneously allowing only the quantification of total elemental concentration (Reidy et al., 2013). Using the promising method SP-ICP-MS, the height of the spike signal is proportional to the mass of the particles and the frequency of the peaks is correlated with the number concentration of NP (Schaumann et al., 2015; Reidy et al., 2013).

Surface enhanced Raman spectroscopy (SERS) is a surface-sensitive technique relying on inelastic scattering of monochromatic light, by which the resulting signal is enhanced by molecules adsorbed

on rough metal surfaces. Though showing some limitations, SERS is increasingly finding application for the investigation of environmental contaminants such as the interaction of Ag NPs with organic coatings of natural origin (natural organic matter, NOM) (Schaumann et al., 2015).

SEM and TEM equipped with energy-dispersive X-ray spectroscopy (EDX) and by X-ray absorption spectroscopy (XAS) are methods for the study of inorganic coatings, because the determination of the coating delivers important information about the environmental fate of NPs (Schaumann et al., 2015). In consideration of sample adjustments pertaining to the ionic strength and coatings being at least a few nm thick, size differences between uncoated und coated NP and the chemistry of inorganic coatings can be determined (Schaumann et al., 2015). For example, using SEM-EDX, extended X-ray absorption fine structure (EXAFS), and near-edge X-ray absorption fine structure (XANES), the degree of sulfidation in correlation to the size of Ag NPs could be identified based on variable S/Ag ratios on Ag NPs aged in wastewater (Kaegi et al., 2013; Schaumann et al., 2015).

1.3.4.6. Distinction between Ag NPs and dissolved Ag^+ ions

It is still challenging to characterize NP as they exist in their material composite or in the natural environment (Reidy et al., 2013). In this context, it is also a crucial issue for understanding the biological impacts of Ag NPs to distinguish between the effects of dissolved Ag^+ ions and Ag NPs (Schaumann et al., 2015). For all mentioned techniques above there is a certain signal overlapping between dissolved Ag^+ ions and Ag NPs (Schaumann et al., 2015), though there are several approaches coming close to a separation. For instance, SP-ICP-MS is a promising technique allowing the distinction between Ag NPs and Ag^+ ions, because Ag NPs deliver a spike signal compared to a continuum signal produced by Ag^+ ions (Mitrano et al., 2012; Schaumann et al., 2015). However, this method is limited to Ag NPs bigger than 18 nm in size (Schaumann et al., 2015). In ecotoxicology media, the combination of ultracentrifugation followed by subsequent ICP-OES of the supernatant allows the separation of Ag NPs bigger than 2 nm and Ag^+ ions (Metreveli et al., 2016).

Overall, because environmental samples usually exhibit a certain complexity and heterogeneity, a combination of several techniques is required (Reidy et al., 2013; Schaumann et al., 2015). Thus,

coupling ICP-MS to SEC, HDC, and capillary electrophoresis is a suitable approach for analysis of environmental samples (Schaumann et al., 2015). For example, the combination of AF4, SEC and HDC with DLS and multiangle light scattering (MALS) detectors allows the shape of the NP to be characterized (Schaumann et al., 2015). To characterize NPs in complex media and at low concentrations, online coupling of HDC and SP-ICP-MS was efficient to derive data about elemental mass and effective hydrodynamic diameter for each particle (Rakcheev et al., 2013, Schaumann et al., 2015).

Because Ag NPs can undergo a series of transformations in aqueous solutions and in particular in the environment with significant changes including in stability (e.g. aggregation/agglomeration, dissolution) and surface chemistry, it remains challenging to distinguish between coated/uncoated, dissolved, nanoparticulate, and bulk particles (Fabrega et al., 2011). Furthermore, characterization is complicated due to the expected very low concentrations in natural aqueous samples (Fabrega et al., 2011).

1.3.5. Fate and transformations of Ag NPs

1.3.5.1. Transport and fate of Ag NPs in the aquatic environment

Due to the widespread use of Ag NPs in consumer products and the expected growth of nanotechnology, Ag NPs may increasingly be released into the environment through several routes of entry. This kind of pollution may be caused during synthesis, during manufacturing and incorporation of the Ag NPs into products, during the period of use of these products (e.g. washing of clothing containing Ag NPs), and while recycling or disposal of products and Ag NPs (Fabrega et al., 2011). According to a study by Blaser et al., (2008), 15% of total silver released into water systems in the EU comes from Ag NPs incorporated into textiles and plastics. Other materials, which can release silver are municipal solid waste (circuit boards, old black-and-white films, photographic prints, dental fillings, non-recycled coins and old silverware and silver-oxide batteries), waste from electrical and electronic equipment, industrial waste (photo laboratories, film and print production, dentists and hospitals), hazardous waste (silver-oxide batteries and waste from dentists), imported silver waste form the major

part, cosmetics and outdoor paint (Blaser et al., 2008). Besides a rather indirect introduction of Ag NPs into the aquatic environment through emissions of thermal waste treatment plants (TWTPs), the main part of silver released from Ag NPs is firstly introduced into sewage treatment plants (STPs) before entering the aquatic environment (Blaser et al., 2008). As mentioned above, the release of Ag NPs from outdoor paint during runoff events can be another significant source for Ag NPs entering the waste water stream and the aquatic environment (Kaegi et al., 2010). It has been detected that more than 30% of the initial mass of silver was released into the environment with Ag NPs of <15 nm in size after one year, of which 80% was lost during the first eight runoff events (Kaegi et al., 2010). Overall, the leaching of Ag NPs from various sources may lead to accumulation of Ag NPs in surface waters (Benn & Westerhoff, 2008) and in the uppermost sediment layer in aquatic systems (Bradford et al., 2009). It can be expected that the majority of Ag NPs present in aquatic environments precipitate and sediment (Metreveli et al., 2015), because in a laboratory study it has been shown that only 4.9% of the originally added mass of Ag NPs retained in the water column (Griffitt et al., 2009). Hence, the predicted environmental concentrations of released silver from Ag NPs range from 10 pg l^{-1} to 1 µg l^{-1} in the water column and from 2 µg kg^{-1} to 14 mg kg^{-1} in sediments (Blaser et al. 2008; Gottschalk et al. 2013). Recently, a revised concept predicted the environmental concentrations of Ag NPs to be ranging from 0.1 pg l^{-1} to a few ng l^{-1} in the surfaces of fresh water and up to ~750 µg kg^{-1} in the sediments of fresh water (Giese et al., 2018).

Ag NPs undergo complex and highly dynamic physical, chemical, and biological transformations in aqueous solutions, which additionally may vary between the different compartments beginning from biological/ecotoxicological media in laboratory studies over the waste stream, in particular with regards to STPs, to the natural aquatic environment. The water chemistry can be responsible for aggregation, dissolution, or stabilization of Ag NPs (Chambers et al., 2013). For instance, dissolution of Ag NPs is influenced by the presence of dissolved oxygen (Liu and Hurt 2010; Lowry et al. 2012), pH value and natural organic matter (Liu et al. 2007). Aggregation processes are caused by pH, ionic strength, divalent counter-ions (Ca^{2+} and Mg^{2+}), monovalent anions (Cl^{-}), polysaccharides, and proteins

(Metreveli et al. 2015; Bradford et al., 2009; Fabrega et al., 2009a; Metreveli et al., 2016; Fabrega et al; 2011). Furthermore, the intrinsic properties of Ag NPs, such as the particle morphology, zeta potential, surface coating, and Ag NP concentration further influence these transformation processes. Against this background, the need for the characterization of Ag NPs in all biological/ecotoxicological media and environmental compartments in parallel with the toxicity assay is again underlined (Fabrega et al., 2011; Reidy et al., 2013). In addition, the behavior of Ag NPs in biological media and water may be varied by the impact of biomolecules (Reidy et al., 2013).

1.3.5.2. Behavior of Ag NPs in biological/ecotoxicological media

Biological media used to cultivate microorganisms and other aquatic organisms and to monitor ecotoxicological tests commonly contain various constituents and additives such as organic substances (e.g. proteins, carbohydrates, lipids), halides (e.g. Cl^-), multivalent cations (e.g. Ca^{2+} and Mg^{2+}), and other substances at variable concentrations, which has been measured to affect the stability of Ag NPs (Metreveli et al., 2016) .

Generally, the Dejaguin–Landau–Verwey–Overbeek (DLVO) theory is used to calculate and explain aggregation of NPs in solution. The stability of NPs can be quantitatively determined on the basis of effects of van der Waals attraction and electrostatic repulsion. Thus, it has been demonstrated that the aggregation correlates positively with the particle number concentration (e.g. Metreveli et al., 2016). Stebounova et al. (2011) also reported a similar linear relationship between the initial concentration of Ag NPs and their stability. In accordance with the DLVO theory is the observation that polymer-coated Ag NPs aggregated to a lesser degree than unspecific-coated Ag NPs, which is explained by a higher surface charge of polymer-coated Ag NPs (Stebounova et al., 2011).

Furthermore, besides high ionic strength, which increases aggregation rates, the type of cations and anions affect the aggregation of Ag NPs (e.g., Cl^-, $SO4^{2-}$, NO^{3-}, Ca^{2+}) (Metreveli et al., 2016). Chloride is known to promote aggregation of Ag NPs (Chambers et al., 2013; Tejamaya et al., 2012; Li et al., 2010) and forms $AgCl^0_{(s)}$ shells resulting in bridging Ag NPs (Chambers et al., 2013; Li et al., 2010).

Moreover, monovalent cations have found to increase aggregation to a lesser degree than divalent cations, of which Ca^{2+} has a bigger influence on the aggregation rate of citrate-stabilized Ag NPs than Mg^{2+} (e.g. Huynh & Chen, 2011, Metreveli et al., 2016). This effect might be explainable with a higher affinity of Ca^{2+} to interact with citrate resulting in the formation of complexes (Huynh & Chen, 2011). Due to the presence of other components i.e. organic substances in biological media, processes responsible for the stability of Ag NPs are even more complex. Thus, the presence of natural organic matter (NOM) is known to exert a contradictory influence on aggregation behaviour in dependency on the type and concentration of NOM as well as the presence of multivalent cations (Metreveli et al., 2016). A positive impact on stability has been reported for the surfactant and medium additive Tween 80 (Metreveli et al., 2016; Li et al., 2011), which might be explained by a partial steric stabilization (Li & Lenhart, 2012).

Moreover, because aggregation probably leads to sedimentation of the Ag NPs, the concentration of NPs available for organisms in toxicity tests (particularly in the aqueous phase) might be diminished, which can hamper the interpretation of ecotoxicological test results (Metreveli et al., 2016).

Not only aggregation is determined by the chemistry of the medium but also dissolution of the Ag NPs significantly depends on the ambient conditions. Dissolution i.e. the release of Ag^{+} ions from Ag NPs occurs under aerobic conditions. Consequently, in the presence of oxygen, Ag_2O is formed on the Ag NP surface and Ag^{+} ions subsequently dissolve into the aqueous solution (Levard et al., 2012). Generally, the potential for releasing Ag^{+} ions is increased with decreasing size of Ag NPs (Reidy et al., 2013). Ion release is enhanced with decreased pH (Levard et al., 2012) and increased temperature (Liu et al., 2010). Furthermore, dissolution is increased with increasing chloride conditions, but chloride ions can bind Ag^{+} ions, which results in poorly soluble, precipitating AgCl complexes (Chambers et al., 2013; Reidy et al., 2013). Contrary, dissolution decreases due to the addition of NOM (humic or fulvic acids) (Liu et al.,2010; Reidy et al., 2013) or as a consequence of proteins and protein fragments in the medium adsorbed on the surface of Ag NPs (Metreveli et al., 2016; Durán et al., 2015). Furthermore,

the carboxylic groups of citrate in citrate-stabilized Ag NPs may bind released Ag^+ ions and thus antagonize dissolution (Dobias & Bernier-Latmani, 2013, Liu & Hurt, 2010; Li et al., 2011).

Overall, it has to be mentioned that the chemistry of the media employed in experiments mostly differs from natural conditions in the aquatic environment and may not be representative for the behaviour of Ag NPs in the environment (Reidy et al., 2013).

1.3.5.3. Fate of Ag NPs in sewage treatment plants

Transformation processes of Ag NPs discharged in sewer systems and sewage treatment plants (STPs) seem to be dominated by sulfidation due to the discharge of sulfur from human activities (Kaegi et al., 2011; Kaegi et al., 2013). Because in sewer pipes concentrations of sulfide are relatively high, Ag NPs probably may be sulfidized to form Ag_2S before entering the STP (Levard et al., 2012; Nielsen et al., 2008). The probability for the transformation of Ag NPs into Ag_2S is even higher in STP due to the higher levels of sulfides. Thus, Ag_2S NPs have been identified in final stage sewage sludge material (Kim et al., 2010), suggesting that a passivating Ag_2S surface layer was formed at the Ag NP surface (Kaegi et al., 2011; Reidy et al., 2013). Furthermore, it has been proven that STP are very efficient in retaining Ag NPs in the activated sludge (Kaegi et al., 2013). Nevertheless, silver might be labile between sulphur (2II) species (e.g., sulphides and thiols), which enables an exchange between inorganic sulphide species in the aqueous phase (Reidy et al., 2013; Adams & Kramer, 1999) providing onward movement of Ag NPs to the aquatic environment. Moreover, even though high concentrations of sulfide were sufficiently present in STPs, complete sulfidation of the Ag NPs did not occur (Kaegi et al., 2011).

Overall, though the main part of Ag NPs seems to be diverted in STP (Kaegi et al., 2013), a small fraction will nonetheless continually be released into the aquatic environment.

1.3.5.4. Fate of Ag NPs in the aquatic environment

Similar to the behaviour of Ag NPs in biological media, transformation of Ag NPs in natural freshwater systems is determined by both aggregation and dissolution. Generally, dissolution of Ag NPs occurs depending on the availability of dissolved oxygen, is enhanced with decreasing particle size and depends on the type of coating (Liu & Hurt 2010; Lowry et al. 2012; Levard et al., 2012; Dobias &

Bernier-Latmani, 2013). For instance, the dissolution rate was higher for PVP-stabilized and tannic acid-stabilized Ag NPs than for citrate-stabilized Ag NPs (Dobias & Bernier-Latmani, 2013). Because dissolution rates of Ag NPs are diminished by increasing pH values and increasing concentration of natural organic matter (NOM), dissolution in freshwater might be mitigated due to the presence of NOM and environmentally relevant pH values, which are neutral or slightly alkaline (Liu & Hurt 2010; Lowry et al. 2012; Metreveli et al., 2015). Furthermore, it has been observed that aggregation of citrate-stabilized Ag NPs in surface water leads to decreasing dissolution rates (Li & Lenhart, 2012). Nevertheless, because the toxicity of Ag NPs is often claimed to be mainly attributed to dissolved Ag^+ ions (Liu et al., 2010; Reidy et al., 2013), continuously discharged Ag NPs to the aquatic environment might affect aquatic life including microorganisms (Fabrega et al., 2009a), invertebrates (Das et al., 2013), and fishes (Lee et al., 2012). Moreover, because dissolution of Ag NPs was never complete, Ag NPs may have the potential to persist in the environment for extended periods of time (Dobias & Bernier-Latmani, 2013). Though the released Ag^+ ions can precipitate as insoluble silver salts (e.g., AgCl or Ag_3PO_4) (Kittler et al., 2010), new formation of Ag NPs from dissolved Ag^+ ions of parent Ag NPs can also occur (Glover et al., 2011; Akaighe et al., 2011). The nucleation of new Ag NPs relies on chemical and photo reduction processes under conditions of high humidity, presence of light (Glover et al., 2011), presence of humic acids and adequate ambient temperatures (Akaighe et al., 2011).

As discussed earlier in chapter 3.5.2., aggregation is controlled by the presence and composition of divalent cations, such as Ca^{2+} and Mg^{2+}, as well as monovalent anions, such as Cl^- (Metreveli et al. 2015), which are common and essential components of freshwater environments. Furthermore dissolved organic matter (DOM), NOM, as well as intrinsic properties of Ag NPs, such as the particle morphology, zeta potential, surface coating, and Ag NP concentration, further influence aggregation and aggregation reversibility i.e. disaggregation. For instance, citrate-stabilized Ag NPs have been reported to aggregate rapidly in river water (Li & Lenhart, 2012), whereas humic substances as representatives of NOM seemed to provoke disaggregation of citrate-stabilized Ag NPs (Schaumann et al., 2015; Fabrega et al., 2009a). Furthermore, because it has been shown that shear forces in the form of

mechanical shaking provoked disaggregation of citrate-stabilized Ag NPs in Rhine river water (Metreveli et al., 2015), this might be relevant for natural waters due to strong shear forces evoked by the flow velocity in riverine systems. Hence, it has been concluded that seasonal variations in the chemical composition of natural waters may trigger a cyclical release of previously aggregated Ag NPs from sediments (Metreveli et al., 2015). Nevertheless, because aggregation results in sedimentation (Metreveli et al., 2015), it is very likely that Ag NPs accumulate in microbial biofilms, which are commonly found on all natural and man-made interfaces in aquatic systems.

1.4. Microbial biofilms

1.4.1. Formation of microbial biofilms

Microorganisms can exist in two life forms - as planktonic cells or accumulated at interfaces as sedentary biofilms, which are regarded as the dominant lifestyle of microorganisms in the environment (Flemming & Wingender, 2010; Kolter & Greenberg, 2006). Most, if not all, bacteria can switch reversibly between a nomadic and a sedentary lifestyle, in which they can produce biofilms (Kolter & Greenberg, 2006). Biofilms are multicellular and often multispecies communities embedded in their self-excreted extracellular polymeric substances (EPS) forming a slimy, sticky matrix (Battin et al., 2016; Flemming & Wingender, 2010; Gerbersdorf & Wieprecht, 2014). With the formation of a biofilm as a lifestyle that is fundamentally different from the planktonic form (Flemming & Wingender, 2010), growth, metabolic activity, and survival of biofilm cells is benefitted compared to planktonic cells (Gerbersdorf & Wieprecht, 2014). For example, cells associated to biofilms surrounded by EPS exhibit a higher protective value against antibiotics, toxicants, and predators. Furthermore, gene transfer is facilitated within the same species or to other species (vertical and horizontal gene transfer) due to a high cellular density. Hence, biofilms appearing e.g. as films, mats, flocs, and sludge (Wimpenny et al., 2000) are very successful in colonizing nearly all kinds of interfaces such as sediments, riverbeds, macrophytes, pipes, teeth, catheters, inflamed mucous membranes, and open sores (Battin et al., 2016; Gerbersdorf & Wieprecht, 2014).

Planktonic bacterial cells and biofilms show proteomic, physiological (Kolter & Greenberg, 2006), and ecological (Davey & O'Toole, 2000) distinctiveness. For instance, bacteria being motile by means of a single or multiple polar or subpolar flagella differentiate into EPS-producing cells and lose their flagella at the beginning of biofilm formation, which is genetically controlled (Kolter & Greenberg, 2006). The formation of a biofilm occurs in several steps, comprising the initial adhesion (step 1), microcolony formation (step 2), maturation (step 3) and cellular detachment (step 4) and is subjected to quorum sensing.

1.4.1.1. Adhesion

At the initiation of biofilm formation bacteria adhere to an abiotic or biotic surface, which they reached randomly or supported by chemotaxis by means of molecular diffusion, gravity, or self-propulsion, respectively (Dunne, 2002; Gerbersdorf & Wieprecht, 2014). The attachment can be divided into a primary or reversible adhesion and a secondary or irreversible adhesion phase. Primary or reversible adhesion is characterized by weak intermolecular forces such as van der Waals forces and electrostatic repulsion due to the double layer of counterions (DLVO theory). The repulsive forces are present due to both negatively charged bacterial cell surfaces and substratum. To bridge this repulsive barrier, the bacteria make use of their pili or fimbriae, which are predominantly hydrophobic proteinaceous appendages (Gerbersdorf & Wieprecht, 2014). Furthermore, due to hydrophobic interactions between the appendages and the substratum adhesion is additionally strengthened (Dunne, 2002; Gerbersdorf & Wieprecht, 2014). By almost closing the gap between the bacteria and the surface, short-range polar forces including hydrogen bonds, ionic bonds, and hydrophobic interaction become relevant and the bacteria start to produce EPS, which represents the secondary or irreversible adhesion phase (Gerbersdorf & Wieprecht, 2014). Once a bacterium has successfully adhered, it can recruit other bacteria by means of quorum sensing (QS) (Gerbersdorf & Wieprecht, 2014), which is bacterial intra- and interspecies communication using chemical signal molecules, resulting in the second phase of biofilm formation –microcolony formation (step 2).

1.4.1.2. Microcolony formation and maturation

The microcolony can host single- or multispecies communities of bacteria, which is influenced by environmental parameters (Kolter & Greenberg, 2006). The three dimensional development of the biofilm is promoted by growth, recruitment and EPS production resulting in aggregates of microorganisms surrounded by EPS. Water-filled channels, which cross the round-shaped architecture provide exchange of substances such as nutrients, enzymes, metabolites, waste products and other solutes (Sutherland, 2001) as well as facilitating quorum sensing and mutualistic symbioses. Furthermore, the structural complexity of the microcolony is influenced by co-aggregation and co-adhesion of the biofilm cells in dependence on ionic strength, temperature and pH (Busscher & van der Mei, 2000). Continuing EPS secretion, which ensures cohesion in the biofilm (Flemming & Wingender, 2010) accompanied by colonization of other organisms including prokaryotic cyanobacteria, eukaryotic green algae, diatoms, and protozoa (Gerbersdorf & Wieprecht, 2014) leads to maturation of the biofilm. Because oxygen concentration and pH can be decreased in the bottom layers of a matured biofilm (Watnick & Kolter, 2000), particular microenvironments can be inhabited reflecting the metabolic diversity of bacteria (Kolter & Greenberg, 2006).

1.4.1.3. Cellular detachment

In mature biofilms some cells detach from the extracellular matrix, disperse in the environment and regain the planktonic lifestyle to colonize new niches. Microbial interferences in response to internal stress in the biofilm such as changes in nutrient availability, pH, temperature, and oxygen can lead to the activation of specific enzymes, which break down the extracellular matrix and enhance biofilm dispersion (Picioreanu et al., 2001; Rendueles & Ghigo, 2012). Other factors influencing cellular detachment and dispersion are erosion, sloughing, human intervention, grazing, and abrasion (Percival et al., 2000).

1.4.2. Biofilm structure

1.4.2.1. Architecture of biofilms

As a result of various environmental conditions and the diversity of the biofilm community, a correspondingly wide variety of biofilm structures can be found (Kolter & Greenberg, 2006). For instance, biofilms can be smooth and flat, rough, fluffy or filamentous (Flemming & Wingender, 2010). Furthermore, mushroom-like structures can bloom on submerged surfaces under sufficient nutrient availability and hydrodynamic conditions such as laminar and turbulent flows can affect biofilm structure (Wimpenny et al., 2000; Kolter & Greenberg, 2006; Davey & O'toole, 2000). Moreover, the composition of exopolysaccharides and proteins in EPS can shape the architecture of a biofilm, because in most biofilms EPS can account for over 90% of the dry mass compared to microorganisms, which account for less than 10% (Flemming & Wingender, 2010).

1.4.2.2. EPS matrix

Apart from microbial cells, the EPS matrix contains a mixture of macromolecules including proteins, polysaccharides, and DNA, which represent the major classes of constituents. These are interspersed with minor amounts of peptidoglycan, lipids, phospholipids and other cell components, whereas water is by far the largest component of the EPS matrix with 97 – 99% (Christensen & Characklis, 1990). Thus, in this highly hydrated environment, in which the water seems to be retained entropically, embedded cells might be protected from drying out during drying events (Flemming & Wingender, 2010). Moreover, the water traversing the fine structures of the matrix provides the basis for diffusion processes such as quorum sensing or exchange of metabolites (Sutherland, 2001).

The EPS matrix can contain a high proportion of **proteins** that can exceed the amount of polysaccharides which has been demonstrated for environmental and sewer biofilms as well as for activated sludge (Flemming & Wingender, 2010; Jahn & Nielsen, 1998). Generally, these proteins can broadly be divided into three classes as biopolymer-degrading enzymes, EPS-modifying enzymes, and structural proteins (Flemming & Wingender, 2010). Thus, the matrix may be considered as an "immobilized enzyme system" in which the enzymes adopt dynamically to changing environmental

conditions towards a steady-state (Sutherland, 2001). Biopolymer-degrading enzymes are involved in degradation of water-soluble polymers including many polysaccharides, proteins and nucleic acids and water-insoluble compounds such as cellulose, chitin and lipids, as well as sorbed organic particles from the surrounding environment (Flemming & Wingender, 2010). Facilitated by these enzymes, biofilms carry out self-purification processes in soils, sediments and water, which are e.g. crucial for the biological treatment of drinking water and waste water by freeing the water of pollutants and organic substances (Flemming & Wingender, 2010).

EPS-modifying enzymes provide assembly and degradation of components in the EPS matrix. With their assistance, EPS polysaccharides and proteins are fragmented and rearranged to ensure biofilm formation (Flemming & Wingender, 2010). For example, EPS-modifying enzymes are activated during cellular detachment to partially degrade EPS, what allows biofilm dispersion (Picioreanu et al., 2001; Percival et al., 2000; Rendueles & Ghigo, 2012).

Structural proteins in the EPS matrix provide formation and stability by cross-linking bacterial surfaces and extracellular proteins. Lectins, which are carbohydrate-binding proteins seem to be involved in biofilm formation, which has been reported for lotic biofilms (e.g. Neu & Lawrence, 1999; Neu et al., 2001) and for the galactose-specific lectin LecA and fucosespecific lectin LecB of *P. aeruginosa* (Tielker et al., 2005; Diggle et al., 2006; Flemming & Wingender, 2010). Other proteins required for biofilm formation are the biofilm-associated surface protein (Bap) from *S. aureus* and the Bap-like proteins, which are high-molecular-mass proteins on the bacterial cell surface (Lasa & Penadés, 2006; Flemming & Wingender, 2010). Furthermore, proteinaceous appendages of the biofilm cells, i.e. pili, fimbriae or flagella, can interact with other EPS components such as DNA to strengthen the stability of the matrix (Flemming & Wingender, 2010).

Lastly, some structural proteins specifically bind to exopolysaccharides inducing a stable, cross-linked EPS matrix (Flemming & Wingender, 2010).

Exopolysaccharides represent another large integral part in the EPS matrix. Most of these exopolysaccharides are long, linear or branched molecules, which can be homo- or

heteropolysaccharides. Homopolysaccharides consist of repeated uniform sugar units stringed together as glucans, fructans or cellulose and are mostly neutral (Flemming & Wingender, 2010). By contrast, heteropolysaccharides such as kefiran, gellan, alginate, and xanthan are more commonly present in the EPS matrix and are constructed of a mixture of neutral and charged sugar residues (Wingender & Winkler, 1984; Flemming & Wingender, 2010; Gerbersdorf & Wieprecht, 2014). The charged sugar units can be of polyanionic or polycationic nature by means of uronic acids, ketal-linked pyruvate, phosphate, and sulfate or acetylated/deacetylated amine moieties, respectively. These polar functional groups feature high surface energies that lead to physicochemical interactions, which are involved in the structural integrity and the mechanical stability of the EPS matrix. The physicochemical interactions includes van der Waals, ionic attractive forces, electrostatic attractive forces, and electrostatic attractive forces due to interactions between polyanionic groups and cations, in particular Ca^{2+} (Mayer et al., 1999; Flemming & Wingender, 2010). Mostly combined with these interactions, a complex network of predominantly branched exopolysaccharides is involved in the development of microcolonies, the establishment and maintenance of the biofilm architecture, and in the adherence to surfaces, to name a few (Flemming & Wingender, 2010).

More recently, it became evident that **extracellular DNA (eDNA)** is an essential component in the EPS matrix, which is involved in various functions including quorum sensing, structural integrity of the biofilm, and adhesion (Dominiak et al., 2011; Gerbersdorf & Wiebrecht, 2014; Flemming & Wingender, 2010). Besides the liberation of eDNA from lysed cells, eDNA is often actively secreted or released from vesicles from living cells (Dominiak et al., 2011).

Overall, the described EPS can greatly differ in terms of quality and quantity between various biofilms and even between biofilms of the same species. The EPS matrix composition is modulated in response to exposed shear forces, ambient temperatures, and the availability of nutrients and also depends on the inhabited biofilm cells (Gerbersdorf & Wiebrecht, 2014; Flemming & Wingender, 2010). Owing to polar functional groups (anionic and cationic), apolar compounds, and groups with hydrogen-bonding potential, the EPS matrix is very sticky (Flemming & Wingender, 2010). Thus, a range of different

substances from the water phase can be absorbed including cations, anions, and apolar compounds. Even heavy metals (e.g. Zn^{2+}, Cd^{2+}, and Ni^{2+}) and nanoparticles like Ag NPs accumulate in the biofilms and can be enriched. Moreover, the multitude of binding sites correlated with intermolecular chemicophysical interactions between the biopolymers, which accounts for mechanical stability in biofilms is strongly connected to sediment stabilization, namely biostabilization in riverine and marine systems (Gerbersdorf & Wiebrecht, 2014).

1.4.2.3. Mechanical stability of biofilms

The mechanical stability, which is characterized by both cohesiveness of the biofilm matrix and adhesion to surfaces, seems to be mainly attributed to the exopolysaccharide in the EPS matrix (Flemming & Wingender, 2010). Biostabilization heavily depends on the mechanical stability of the biofilms and is crucial for the cohesion of sediments in aquatic environments (Lubarsky et al., 2010; Gerbersdorf & Wieprecht, 2014). Furthermore, flocculation, settling, and dewatering during treatment of waste water in STPs relies on the mechanical stability of flocs and biofilms (Klausen et al., 2004). Some biofilms developed a genetically modulated defence in response to external shear stress such as turbulent flows present in streams and industrial pipelines by increasing EPS production, though it has been speculated that this strategy might be too energy-consuming for tackling stress (Shaw et al., 2004). Indeed, due to the viscoelastic properties of the biofilm slime, biofilms can withstand a certain shear stress and are e.g. consequently well adapted for hydrodynamic forces during strong currents. The viscoelastic biofilms exhibit elastic solid-like response to short time-scale stress and viscous fluid-like response to long time-scale stress (Shaw et al., 2004). During this dual behaviour the elastic part of the biofilm structure enables significant buffering of pressure loads by reversible deformation. When a certain break point is reached where the applied stress has been too high or has been last too long, the biofilm responds with viscous behavior to ease sustained internal stress by non-reversible deformation (Shaw et al., 2004). As a result, physicochemical interactions between EPS components including hydrogen bonds, van der Waals forces, and electrostatic interactions as well as entanglements of biopolymers might be rearranged to withstand the external shear stress (Shaw et

al., 2004; Flemming & Wingender, 2010). Furthermore, the presence and concentration of cations can influence the mechanical stability of biofilms. It has been reported that the presence of Ca^{2+} enhanced the mechanical stability of biofilms, which has been attributed to the Ca^{2+}-mediated crosslinking of polyanionic alginate molecules (Flemming & Wingender, 2010; Körstgens et al., 2001). In contrast, other cations including Ag^{+} have shown to impair the cohesiveness, which has been explained with their affinity to bind to functional groups of exopolysaccharides and proteins in the EPS matrix (Chen & Stewart, 2002; Chaw et al., 2005). Thus, the network of physicochemical interactions, which is necessary for cohesiveness, might be disturbed and/or interrupted. However, data about the cohesive strength of biofilms differ among the literature, which seems to be attributed to the measurement methodology (Flemming & Wingender, 2010). Generally, from a quantitative perspective, biofilm properties individually differ according to the parameters elasticity modulus and viscosity (Flemming & Wingender, 2010; Klausen et al., 2004). Nevertheless, from a qualitative perspective, all biofilms show viscoelastic properties in response to the exposure of shear stress (Stoodley et al., 2002). Hence, stress relaxation time has been demonstrated to be approximately the same with about 18 min over a wide sample of biofilms (Shaw et al., 2004), though some biofilms show even shorter stress relaxation times (Fleming & Wingender, 2010). Collectively, biofilm structure and function i.e. the viscoelastic properties of the biofilm matrix interact in mutual relationship with hydrodynamic forces exerted by the flow of water in streams and rivers.

1.5. Stream biofilms

Streams and rivers represent a significant part of the Earth's total freshwater systems with an estimated surface area of 662,041 km^2 (Downing et al., 2012). Therefore, streams and rivers provide an immensely large surface area for microbial colonization (Manz et al., 1999; Romaní, 2010). Biofilms are the dominate life form of microorganisms in streams and rivers forming complex aggregates of bacteria, archaea, algae, protozoa, fungi and even metazoan (Manz et al., 1999; Romaní, 2010; Baschien et al, 2008). These diverse biological "consortium" develops complex biofilms with varying physical structures in dependence to dynamic flows and turbulences of the stream water overlying the

biofilms that are closely linked to microbial functioning and ecosystem processes (Wimpenny et al., 2000). Stream biofilms are key sites of enzymatic activity (Romaní et al., 2008), including organic matter cycling, ecosystem respiration and primary production and, as such, being the base of the food chain, transferring carbon to higher trophic levels (Wimpenny et al., 2000). In biofilms, close interactions between microbial phototrophs and heterotrophs for energy fluxes are facilitated (Wimpenny et al., 2000). Stream biofilms as the main sites of self-purification of organic compounds and biodegrading of anthropogenic pollutants provide crucial ecosystem processes that are of substantial importance for global biogeochemical fluxes (Battin et al., 2016). The biofilm matrix i.e. the EPS surrounding the microorganisms features extracellular enzyme activity and adsorption of dissolved organic matter (DOM) (Lock et al., 1984; Haack & McFeters, 1982). Furthermore, as streams can be subject to substantial fluctuations in water level and contained solutes and particles, the matrix protects embedded cells from erosion (Flemming & Wingender, 2010). Moreover, the EPS and also supracellular ropes produced by cyanobacteria (Vignaga et al., 2013; Garcia-Pichel & Wojciechowski, 2009) are involved in biostabilization of the streams' sediments. Overall, biofilms adjust their structural properties in response to abiotic conditions prevailing in the streambed such as hydrodynamic forces and chemical gradients. In return, the structure of the streambed is shaped by biofilms with respect to hydrodynamics (Battin et al., 2001; Drescher et al., 2013; Pintelon et al., 2012) and physicochemical characteristics (Boano et al., 2014). This mutual relationship between the sedimentary environment of the streambed and the overlying and even permeating biofilm might be considered as a "co-evolutionary" relationship (Battin et al., 2016). This relationship has consequences not only for the hydromorphology of the streambed, e.g. biostabilization, but also for the ecology and biodiversity of the biofilms therein (Battin et al., 2016). Because the organisms which construct and occupy stream biofilms span across the entire tree of life, these biofilms can be regarded as "jungles" of biodiversity (Battin et al., 2016). More precisely, in the benthic zone, eukaryotic algae (such as diatoms, green algae, chrysophytes, red algae and cryptophytes) and cyanobacteria, in the company of other members of the *Bacteria* and to some extent also archaea, form biofilms mainly in dependence of light

availability (Battin et al., 2016). Alternatively, bacteria and archaea are the predominant inhabitants in the darker layers of sediments due to limitation of phototrophic life (Battin et al., 2016).

Though much less poorly studied, fungi are also an integral part of stream biofilms (Baschien et al., 2008; Barlocher & Murdoch, 1989).

Within the food web, the grazing activity of primary consumers such as ciliates, flagellates, nematodes and even young-instar insects (such as midges) (Hakenkamp & Morin, 2000; Dopheide et al., 2008) can change the physical structure (Lawrence et al., 2002; Böhme et al., 2009), community composition (Wey et al., 2012), and carbon cycling of stream biofilms (Risse-Buhl et al., 2012). Moreover, because it has been shown that viruses are of particular relevance in marine ecosystems (Jacquet et al., 2010), this might be assignable also for viruses in stream biofilms, but still remains clarified. More precisely, in marine bacterial biofilms, bacterial viruses (or phages) play a role in regulating the dynamics and diversity of bacterial communities by infecting bacterial cells (Sutherland et al., 2004).

The application of molecular tools, especially FISH (fluorescence in situ hybridization) with 16S or 23S rRNA-directed oligonucleotide probes, was a starting point to elucidate the molecular microbial ecology of stream biofilms (Manz et al., 1999; Brümmer et al., 2000; Wimpenny et al., 2000). In situ hybridization enables the detection of specific nucleic acid sequences in eukaryotic and prokaryotic cells by binding of oligonucleotide probes to their complementary target sequences (Manz et al., 1999; Brümmer et al., 2000; Wimpenny et al., 2000; Böckelmann et al., 2000). By application of further microbial typing methods based on rRNA sequences such as amplified ribosomal DNA restriction analysis (ARDRA), terminal restriction fragment length polymorphism (t-RFLP), 16S rDNA sequencing composition, and more recently next-generation sequencing data of 16S rRNA on samples from stream biofilms, diversity patterns of bacterial freshwater biofilms is becoming increasingly clarified (Böckelmann et al., 2000; Zeglin, 2015; Romaní et al., 2014; Timoner et al., 2014). These data reveal that stream biofilm communities are highly diverse with hundreds to thousands of operational taxonomic units (OTUs) that span over major parts of bacterial phyla. (The classification of microorganisms based on an operational definition for species distinction that applies a percentage

similarity threshold to 16S rRNA sequences is commonly termed OTU (Battin et al., 2016)). These complex communities found in various habits in streams are predominantly colonized by the phyla Proteobacteria and Bacteroidetes. As subdivided into lower taxonomical classes, Betaproteobacteria and Alphaproteobacteria are commonly the numerically dominant classes of Proteobacteria in benthic and hyporheic biofilms (Manz et al., 1999; Brümmer et al., 2000; Zwart et al., 1998, Zwart et al., 2002). Some members of the Alphaproteobacteria are capable to utilize complex organic compounds of DOM in stream water such as humic substances (Battin et al., 2016). In addition, they possess the ability to be resistant to grazing by forming filamentous morphologies (Newton et al., 2011). Altogether, these qualities may instrumental in doing this class to be the dominant bacterial class in stream biofilms.

The classes Flavobacteriia and Sphingobacteriia are characteristic within the Bacteroidetes phylum, which might explainable due to characteristics that are perfectly adapted to the specific conditions of riverine ecosystems. Some members of Flavobacteriia and Sphingobacteriia posses the ability to degrade the biopolymers cellulose and chitin, which are also part of DOM in streams. Flavobacteriia are remarkable in adhering to surfaces under elevated flow velocities und thus promote colonization and subsequent formation of biofilms in streams (Newton et al., 2011; Kirchman, 2002; Zhang et al., 2013; Battin et al., 2016). Furthermore, Sphingobacteriia represent a key taxonomic group in stream biofilms (Widder et al., 2014) and seem to be likewise involved in biofilm colonization and formation in streams as they are characterized by these abilities in other aquatic systems (Kirchman, 2002; Battin et al, 2016).

In lower abundances, stream biofilms frequently are also composed of Gammaproteobacteria, Deltaproteobacteria, Actinobacteria, Firmicutes, Gemmatimonadetes, Verrucomicrobia, Planctomycetes and Deinococcus–Thermus (Manz et al., 1999; Brümmer et al., 2000; Zwart et al., 1998, Zwart et al., 2002). In contrast, archaea seem to be specialized in certain niches such as anoxic, methan-rich pockets in the hyporheic zone or ammonium-rich areas (Merbt et al., 2011; Buriánková et al., 2013) and thus stream biofilms host them to an rather insignificant extent (Romaní et al., 2014; Wilhelm et al., 2013; Besemer et al., 2012).

The bacterial diversity and community composition can be influenced by certain environmental conditions such as pH, temperature, and electrical conductivity (Battin et al., 2016). More precisely, it has been found that electrical conductivity shaped the presence of the major phyla Actinobacteria, Nitrospira and Verrucomicrobia in biofilms of glacier-fed streams (Wilhelm et al., 2013). Alternatively, shifts in pH affected the presence of Acidobacteria, Gemmatimonadetes and Proteobacteria in these biofilms (Wilhelm et al., 2013).

Furthermore, stability of the bacterial biofilm community is influenced by dispersal dynamics and hydrology (Widder et al., 2014). As changes in hydrology can be caused by climate change and/or anthropogenic activities such as damming or inter-basin diversion, these processes are becoming increasingly prevalent with potential negative effects on the biofilm community (Battin et al., 2016). Moreover, also change in land use such as destruction of native vegetation has shown to affect community composition and diversity in streams in New Zealand (Lear et al., 2013). Against this background, it can be assumed that community composition and diversity dynamics of stream biofilms are not random but driven by environmental factors (Battin et al., 2016). Generally, diversity of biological communities, e.g. species richness, is often positively related to ecosystem functions, which are processes driven by the communities (Zha et al., 2015; Frossard et al., 2012). Moreover, functions can be indirectly affected by an altered community structure (Frossard et al., 2012). Thus, shifts in the composition and/or diversity of the bacterial biofilm community may affect the functioning of biofilms and even ecosystem processes (Battin et al., 2016).

However, due to the tremendous diversity and complex communities of bacteria in stream biofilms it seems to be challenging to directly correlate bacterial community diversity (richness and evenness) and biofilm function (Battin et al., 2016). More precisely, the high abundance, extensive diversity, high dispersal capacity, physiological versatility and horizontal gene transfer of bacterial communities are often assumed to be responsible for functional redundancy of complex biofilm communities (Peter et al., 2011). Thus, the functional redundancy may blur the relationship between microbial diversity and function in bacterial communities (Battin et al., 2016; Langenheder et al., 2006; Comte et al., 2013).

This conclusion has been drawn by numerous studies which are partly inconsistent (Freimann et al., 2013; Hector& Bagchi, 2007; Peter et al., 2011; Wilhelm et al., 2015; Battin et al., 2016). For instance, to gain insights into ecosystem functions, the activity of a range of extracellular enzymes as well as the degradation of DOC was measured in biofilms, which have been grown in laboratory-scale bioreactors mimicking the hyporheic zone (Peter et al., 2011). In these biofilms decreasing microbial diversity was correlated with decreased enzymatic multifunctionality. This indicated a relatively low functional redundancy (Peter et al., 2011). By contrast, in another study, metabolic multifunctionality was not associated with the degree of bacterial diversity, which indicated metabolic redundancy regardless of the community composition. This was proven by reconstruction of artificial metagenomes from 140 orthologues being involved in metabolism of DOM compounds in benthic biofilms from alpine streams (Wilhelm et al., 2015). Therefore, it is still under discussion whether phylogenetic and/or functional diversity may be better predictors for ecosystem functioning than species richness *per se* (Eisenhauer et al., 2012).

In summary, diverse bacterial communities have well-developed and manifold strategies that facilitate different responds to environmental perturbations leading to e.g. resistance against perturbations and invasions (Eisenhauer et al., 2012), temporal and spatial variability (Battin et al., 2016), resilience (Gerbersdorf & Wieprecht, 2014), and reliability (Eisenhauer et al., 2012). Nevertheless, changing abiotic and biotic conditions influence biodiversity and/or phylogenetic composition of bacterial stream biofilms, which can affect biofilms' overall metabolic and structural performance. This may have further implications on both the biofilm and the ecosystem functioning, because stream biofilms represent the functional backbone of watercourses (Eisenhauer et al., 2012). Furthermore, as these biofilms absorb and accumulates contaminants and pollutants such as Ag NPs from wastewater streams, effects of Ag NPs on biodiversity as well as on community patterns und phylogenetic composition of bacterial stream biofilms need very close consideration.

1.6. Effects of Ag NPs on bacteria and bacterial biofilms

1.6.1. Bioavailability of Ag NPs

Generally, among the literature, reported effects of Ag NPs on bacteria and bacterial biofilms are as manifold as bacterial diversity as well as the characteristics and concentrations of the applied Ag NPs and the nature of the environment. A large and growing number of scientific papers describe toxicity tests for Ag NPs towards a wide range of pure or mixed cultures of bacteria and bacterial biofilms in either growth media or biological matrices (De Leersnyder et al., 2018; Schaumann et al., 2015; Fabrega et al. 2011). Nevertheless, because methodologies to assess Ag NP's toxicity differ among the literature (Moreno-Garrido et al. 2015), results are partly inconsistent and even hard to compare (Table 1). Results are varying pertaining to the reported inhibitory concentrations and are partly even contrary with bacteria and bacterial biofilms being sensitive or resistant to exposure of Ag NPs (Table 1) (Eymard-Vernain et al., 2018; De Leersnyder et al., 2018; Schaumann et al., 2015; Fabrega et al. 2011). Besides different methodologies, more general, this can be attributed to the bioavailability of Ag NPs i.e. the uptake rate of Ag NPs by organisms. Bioavailability defines whether Ag NPs are available and ultimately toxic for organisms by e.g. penetrating into the cells or by being retained at cell walls and/or external surfaces (Fabrega et al., 2011). The processes which, in turn, influence bioavailability and ultimately toxicity are combinations of

1) the concentration of the Ag NPs,

2) the nature of the Ag NPs,

3) environmental conditions,

4) the route of exposure, and

5) the biology and functional ecology of the organism involved (Fabrega et al., 2011; Luoma & Rainbow, 2005), which in this context are the involved bacteria.

Table 1. Effects of Ag NPs on bacteria and bacterial biofilms. ND: not determined. LC: lethal concentration. MIC: minimal inhibitory concentration. Modified and supplemented from Fabrega et al., 2011.

Organism	Size [nm]	Surface coating	Shape	Synthesis	Nominal conc./ LC50/ MIC	Dosis re-gime	Sample preparation	Major NP effects	Refer-ence
Bacteria spp	26	PVP360, PEGs, Tween	Spheri-cal	Modified Tollens process. chemical reduction of $[Ag(NH_3)_2]^+$ with D-maltose	MIC range of 1.69 to 6.75 µg mL^{-1}	24 h	Aqueous dispersions	SDS, Tween 80 and PVP360 increased particle stabilisation as well as antibacterial activity of Ag NPs	Kvitek et al., 2008
Gram-negative Bacteria spp	16 ± 8	ND	Cuboc-tahe-dral. Multiple-twinned icosa-hedral deca-hedral	Pur-chased NPs powder in a carbon matrix	100 µg ml^{-1}	30 min	Aqueous dispersion and homo-genate with ultrasonic cleaner	Ag NP <10 nm attached to bacterial cells. NPs with a high reactivity facet {111} were the most toxic	Morones et al., 2005
E. coli *S. typhi* *S. aureus*	10 – 15	ND	Spher-ical or poly-hedral	Chemical reduction by D-glucose and hydrazine	5 – 100 µg ml^{-1}	1 h, 20 h, 24 h	Aqueous media	Ag NPs are more effective in Gram-negative than in Gram-positive bacteria	Shrivas-tava et al., 2007
E. coli	10	ND	Spheri-cal and trun-cated	Chemical reduction with formalde-hyde, hydrazine and sodium formalde-hyde-sulfoxy-late	0.1 – 1 mg l^{-1}	24 h	Dispersed in culture media	Cell membrane/ protein damage. ROS production	Hwang et al., 2008
E. coli	>39	Citrate	Spherical and trun-cated train-gular plates, rods, poly-hedral plates	*Spherical:* chemical synth. by boro-hydride reduction *Trunc. triang NPs*: solution phase method	0.1 – 10 µg mL^{-1}	0 - 26 h	Aqueous suspension	Truncated triangular have strongest biocidal action	Pal et al., 2007

Organism	Size [nm]	Surface coating	Shape	Synthesis	Nominal conc./ LC50/ MIC	Dosis re-gime	Sample preparation	Major NP effects	Refer-ence
E. coli	9.2 ± 2.8 62 ± 18 nm	Citrate	Spheri-cal	Chemical synthesis by boro-hydride reduction	0 - 100 µg ml^{-1}	0 - 24 h	ND	Smaller Ag NPs were more toxic	Lok et al., 2007
Nitrifying bacteria	9 – 21	PVA	ND	Chemical reduction with boro-hydride	0.05 - 1 mg l^{-1}	180 days	Freshly synthesised suspensions	Ag NPs < 5 nm growth inhibition due to ROS pro-duction	Choi & Hu, 2008
Natural bacterial assem-blages in estuarine sediments	58.6 ± 18.6	ND	Sphero-idal	Pur-chased powder	25/ 1000 µg l^{-1}	20/ 30 days	Millipore water, sonicated for 30 min prior experi-ments	Ag NPs accumu-lated in the top 3 mm but did not affect the bacterial abundance nor diversity either in the sediment nor water column	Bradford et al., 2009
P. fluores-cens	65± 30	Citrate	Spheri-cal/ triangu-lar	Pur-chased powder	0 – 2000 ppb (~0 - 2000 µg l^{1-})	24 h	Dispersion in 0.025 mM citrate solution, sonicated for 30 min for 4 days. Diluted in biological media and stirred for 24 h prior exposure	Toxicity of Ag NPs was pH dependent. The presence of organic matter mitigated Ag NP toxicity	Fabrega et al., 2009a

Organism	Size [nm]	Surface coating	Shape	Synthesis	Nominal conc./ LC50/ MIC	Dosis re-gime	Sample preparation	Major NP effects	Refer-ence
P. putida biofilms	65± 30	Citrate	Spheri-cal/ triangu-lar	Pur-chased powder	0 – 2000 ppb (~0 - 2000 µg l^{1-})	24 h	Dispersion in 0.025 mM citrate solution, sonicated for 30 min for 4 days. Diluted in biological media and stirred for 24 h prior exposure	The presence of organic matter increased uptake of Ag NPs by biofilms. Toxicity of Ag NP was mitigated with org. matter	Fabrega et al., 2009b
E. coli	~30 to 80, 25–30	PVP	ND	Pur-chased powder, chemical reduction with PVP and ethylene glycol	50 mg l^{-1}	30, 60, 120, 360 min, 12 h	Redisper-sed and aged in sulfide solutions of 0, 0.1, 0.5, 2, 8, or 15 mM, at a AgNP concentra-tion of 800 mg l^{-1}	Sulfida-tion of Ag NPs decreases growth inhibition	Reinsch et al., 2012
P. putida	3-8, 10, 20, 40, 50,	Un-coated, citrate, tannic acid	Spheri-cal	Pur-chased powder/ aqueous solutions, diluted in Milli-Q-water and/or sonifica-ted	EC50 0.25 – 11.6 µg l^{-1}	16 h	Diluted in Milli-Q-water and/or sonificated	Growth inhibitions driven by Ag ion release	Matzke et al., 2014
Nitroso-monas europaea	20 and 80	Phos-phate	Spheri-cal	Aqueous reduction by Nano-Compo-six, Inc. (San Diego, CA)	0 – 5 ppm (~ 0 - 5 mg l^{-1})	3 h	Dispersed in deionized and distilled water and/or in 2.5 mM $(NH4)2SO4$ and 30 mM HEPES buffer (pH 7.8)	Smaller NPs were more toxic than bigger as measured by nitrification activity due to more released Ag ions due to a greater surface area to volume ratio, toxicity mediated by Ag ions	Rad-niecki et al., 2011

Organism	Size [nm]	Surface coating	Shape	Synthesis	Nominal conc./ LC50/ MIC	Dosis re-gime	Sample preparation	Major NP effects	Refer-ence
Planc-tonic cells vs. waste-water biofilms	~15	PVP	ND	Pur-chased Nano-powder	1, 50, 200 mg l^{-1}	24 h	Dispersed in 1% PBS and mixed by vortex	Wastewater biofilms are highly tolerant whereas planktonic cells are vulnerable to Ag NPs; microbial suscepti-bility to Ag NPs is different for each micro-organism	Sheng & Liu, 2011
P. aerugino-sa (planctо-nic and biofilm)	45 - 53	SDS	Spheri-cal	UV photo-reduction of 1 mM silver nitrate and 1.6 mM sodium dodecyl sulfate (SDS) and 0.85 M ethanol	0.01 – 5 mg l^{-1}	18, 24, 30 h	In Nutrient broth, center well on agar plates,	antibacterial and antibiofilm activity; NPs show higher antibiofilm activity than Ag^{+} ions; ROS production; NPs attached to the surface of bacterial cell wall, permeated the cell membrane and entered into the cell interior	Kora & Aruna-chalam, 2011
E.. coli	2.8 ± 0.47, 4.7 ± 0.20, 10.5 ± 0.59/ 18, 51, 72	Glycol-thiol-coated; PVP	Spherical	Reduction of silver acetate with oleylamin; purchased	0- 9 mg l^{-1};	6 h	Minimal medium ($NaHCO_3$ buffer solution, 2 mM) under aerobic and anaerobic conditions	Under anaerobic conditions no measurable effects; stimulatory effect at sublethal exposures, Ag^{+} is the definitive molecular toxicant	Xiu et al., 2012

Organism	Size [nm]	Surface coating	Shape	Synthesis	Nominal conc./ LC50/ MIC	Dosis re-gime	Sample preparation	Major NP effects	Refer-ence
Activated sludge bacterial commu-nity	5 and 35	ND	ND	ND	0.05 – 40 ppm (~ 0.05 – 40 mg l^{-1})	7 days	ND	Lower Ag NPs have a more pronounced effect on bacterial community structure compared to Ag ions; Ag NPs decreased the abundance of nitrifying bacteria	Yang et al., 2014
Aquatic bacterio-plankton (natural waters)	1 - 10	Carboxy-function-alized	ND	Pur-chased aqueous suspen-sion	0.01 – 1 mg l^{-1}	5 days	ND	Changes in bacterial commu-nity structure	Das et al., 2012
E. coli proteins	~ 30	Bare and carbo-nate-coated	ND		10/100 ppm (~10/ 100 mg l^{-1})	Over-night	Deionized water, soluble proteins/ protein fragments	Proteins bind specifically to bare or carbonate-coated Ag NPs. Strong binding of Ag NPs to TNase resulted in the significant reduction of the enzymatic activity	Wiggin-ton et al., 2010
Stream periphy-ton	35 ± 1 – 116 ± 1	Citrate-coated	ND	Purchased from Nanosys GmbH	12.5 - 200 µM (~ 1.35 mg l^{-1} – 21.58 mg l^{-1})	2 h in the dark	Exposure medium modified from Le Faucheur et al., 2005; ± DMPS	Inhibition of respiration, photosyn-thesis and extracellular enzymes; toxicity in most cases caused by Ag ions, but also NP specific effects independent from Ag ions	Gil-Allué et al., 2015

Organism	Size [nm]	Surface coating	Shape	Synthesis	Nominal conc./ LC50/ MIC	Dosis re-gime	Sample preparation	Major NP effects	Refer-ence
Nitrosomo-nas europaea	7 ± 3 and 40 ± 14 nm)	polyvinyl alcohol and adeno-sine triphos-phate diso-dium	ND	Chemical reduction with boro-hydride	0 – 10 mg l^{-1}	3 h	Defined mineral salt medium	Size- and coating-dependent inhibition of ammonia oxidation by Ag NPs; distinct Ag NP effect distinguish-able from Ag ions; damage of cell wall of *N. europaea;* inhibition of important protein functions	Yuan et al., 2013
E. coli	10 - 30	ND	ND	Produced from Ag+ in the presence of *E. coli* cells or aqueous EPS; sugar compo-nents in EPS reduced Ag^+ to Ag NPs	0 – 0.25 mg l^{-1} as Ag NO_3	16 h	Milli-Q water	Microbial extracellular polymeric substances reduce Ag^+ to Ag NPs and antagonize bactericidal activity; sugar components in EPS reduced Ag^+ to Ag NPs	Kang et al., 2014
Native planktonic commu-nity from freshwater pond	6 ± 0.33	No coating	Spheri-cal	Laser gene-rated	5 µg l^{-1}	24 h	Pond water	Ionic silver rather than silver nanopar-ticles are responsible for silver toxicity; no differences in samples treated with Ag NPs	Boenigk et al., 2014
Benthic microbial commu-nities (peri-phyton)	25	PVP	ND	Wet precipi-tation from $AgNO_3$ in the presence of PVP	2 and 20 µg l^{-1}	18 days	Modified Borgmann medium	Decreased biofilm volume; 3D structure and EPS indicated effects related to the silver species applied	Kroll et al., 2016

Organism	Size [nm]	Surface coating	Shape	Synthesis	Nominal conc./ LC50/ MIC	Dosis re-gime	Sample preparation	Major NP effects	Refer-ence
Bacillus subtilis	<100	PVP	ND	Pur-chased	1 mg l^{-1}	5 h; 72 h	LB medium; LB agar plate paper disks	Unaffected viability and metabolism; Poly-gamma-glutamate (PGA) physically interacts with the Ag NPs with a decrease in toxicity	Eymard-Vernain et al., 2018
K. pneumo-nias, P. aeru-ginosa	20	No coating	Spheri-cal	Reduction of silver nitrate solution with *Trigonella foenum graecum* extract	10, 20, 30, 40 mg l^{-1}	24 h	Agar plate	Damage of cell membrane by the formation of pits like structure, Stressing of the cell structure	Senthil et al., 2018
Aqua-bacterium citrati-philum biofilms	30 ± 3 and 70 (97 ± 3)	Citrate	Spheri-cal	Citrate reduction method; Pur-chased laser-genera-ted	600, 1200, 2400 µg l^{-1}	20 h	R2A medium	Viability, protein contents, biofilm architecture remained unaffected; Biofilms serve as sinks for Ag NPs, stability decreased	Grün et al., 2016
Aqua-bacterium citrati-philum biofilms	30 ± 3	Citrate	Spheri-cal	Citrate reduction method	600, 2400 µg l^{-1}	24 h	R2A medium	Production and composition of the EPS, the structure of the biofilm and overall biofilm adhesive-ness were significantly impacted	Schmidt et al., 2017

Organism	Size [nm]	Surface coating	Shape	Synthesis	Nominal conc./ LC50/ MIC	Dosis re-gime	Sample preparation	Major NP effects	Refer-ence
Bacterial freshwater biofilms	30 ± 3 and 70 (97 ± 3)	Citrate	Spheri-cal	Citrate reduction method	600 µg l^{-1}	14 days	Stream water	Thickness, density, and chlorophyll *a* and protein content were not significantly changed; altered biofilm community by Ag NPs	Grün et al, 2018

1.6.1.1. The concentrations of the Ag NPs

It has to be noted that the majority of ecotoxicological studies observing effects of Ag NPs on bacteria and bacterial biofilms applied Ag NPs at concentrations that are much higher than the expected environmental concentrations (Fabrega et al., 2011). Environmentally relevant concentrations are predicted to range from 0.1 pg l^{-1} to a few ng l^{-1} in the surfaces of fresh water and up to ~750 µg kg^{-1} in the sediments of fresh water (Giese et al., 2018). Concentrations of 100 ng l^{-1} Ag NPs have been determined by single particle ICP-MS in the effluents of a sewage treatment plant in Boulder, Colorado (USA) (Mitrano et al., 2012). Though, much higher silver concentrations in the range of µg l^{-1} have been actually proven for particularly exposed locations such as Ghanaian estuaries near silver mining areas with 100 µg l^{-1} (Essumang & Nortsu, 2008) and a heavily industrialized area in Bangladesh with concentrations from 5.23 to 14.9 µg l^{-1} (Ahmed et al., 2012). Surprisingly, in silver monitoring data from German rivers similarly high values of silver concentrations have been detected with average concentrations between 0.05 – 1.17 µg l^{-1} in various German federal counties during the years 2000 - 2007 and up to 65 µg l^{-1} silver in Bavaria in 2006 (Matzke et al., 2012). Nevertheless, applied concentrations of Ag NPs in ecotoxicity studies range from 0.1 µg l^{-1} – 200 mg l^{-1} with most frequently used concentrations in the range of mg l^{-1} (Table 1). Moreover, monitored time periods with average dose regimes of ~24 h (30 min - 18 days) (Table 1) might be too short to mirror an environmental relevant scenario (Fabrega et al., 2011). Taken together, in many cases, applied concentrations of Ag

NPs as well as monitored time periods of Ag NP exposure in ecotoxicity studies among the literature do not represent environmental conditions (Table 1).

1.6.1.2. The nature of the Ag NPs

Generally, the bioavailability of Ag NPs by bacteria and the resulting bactericidal effects seem to be dependent on the size, shape, surface coating, surface charge, surface structure and area, solubility, Ag^+ release, solution chemistry, Ag NP mediated ROS production and aggregation state of the Ag NPs (Fabrega et al., 2011, Xiu et al., 2012). Nevertheless, individual studies have been drawn different conclusions about which of these properties is the distinct Ag NP toxicity determinant (Table 2). For example, it has been reported that the antibacterial properties of Ag NPs are supposed to be attributed to their nanospecific properties e.g. to their small size and large surface area, which enables interaction with bacteria (Rai & Gade, 2009) such as membrane disruption due to physical contact (Sondi & Salopek-Sondi, 2004). In this context, smaller particles have been claimed to be more toxic than bigger ones (Lok et al., 2007; Pal et al., 2007; Radniecki et al, 2011). This is likely also dependent on the high surface area to volume ratio of the Ag NPs with the resulting higher release of silver ions compared to bigger particles (Rai & Gade, 2009). However, the intensely discussed topic among the literature is whether the toxicity is due to the release of Ag^+ ions from Ag NPs or due to a "particle-specific" toxicity by the Ag NP itself (Xiu et al., 2012). Consequently, it remains an open question whether the released Ag^+ ions contribute to the toxicity of the Ag NPs (Xiu et al., 2012) or *vice versa* because Ag NPs represent a continuous source for Ag^+ ion release (Boenigk et al., 2014) or both alternatives. For example, some studies have shown that Ag NPs often were more toxic than equivalent concentrations of silver salts (Yuan et al., 2013; Yang et al., 2014; Kora & Aruna-chalam, 2011). Contrary, in other studies, Ag^+ ions were identified as the main toxicant (e.g. Boenigk et al., 2014; Xiu et al, 2013). Xiu and co-workers (2013) postulate that Ag^+ ions are the definite toxicant because equivalent concentrations of Ag NPs showed no toxic effects to *E. coli* under strictly anaerobic conditions that preclude Ag(0) oxidation and Ag^+ ion release.

Furthermore, as noted earlier in chapter 1.3.3., Ag NPs are frequently coated with organic compounds to prevent aggregation and agglomeration such as (sodium) citrate, BSA, tannic acid, polyvinyl pyrrolidone (PVP) or starch. These stabilizing agents modify the physicochemical and morphological characteristics of manufactured Ag NPs and thus may interfere with their properties, behavior, and toxicity (Tolaymat et al., 2010). For example, the application of sodium citrate as a reducing agent produces a negatively charged Ag NP which may show different behaviour and interaction than a positively charged Ag NP produced with polyethyleneimine (Tan et al., 2007; Tolaymat et al., 2010). Furthermore, it has been observed that citrate-stabilized Ag NPs impacted dissolution pertaining to a lesser release of Ag^+ ions compared to Ag NPs stabilized with PVP or tannic acid (Yang et al., 2011; Dobias & Bernier-Latmani, 2013). This has been attributed to several mechanisms resulting in a lowering in solubility. More precisely, Ag^+ ions may bind to the carboxylic groups of the organic acid leading to the retention of the ions and/or citrate may serve as a reductant, which reduces the oxide layer at the surface of the Ag NPs back to Ag^0 (Dobias & Bernier-Latmani, 2013, Liu & Hurt, 2010; Li et al., 2011). For instance, Ag NPs stabilized with citrate exhibited reduced toxicity to S*taphylococcus aureus* compared to BSA-stabilized Ag NPs, which was explained by citrate antagonizing the dissolution of the Ag^+ ions from AgNPs (Grade et al., 2014).

Table 2. Proposed Ag NPs toxicity determinants. Modified and supplemented from Xiu et al., 2012.

Property	Target organism	Reference
Size	*E. coli, Nitrosomonas europaea*	Morones et al., 2005; Yuan et al., 2013
Shape	*E.coli*	Pal et al., 2007
Surface coating	*Staphylococcus aureus, Nitrosomonas europaea*	Grade et al., 2014; Yuan et al., 2013
Property	**Target organism**	**Reference**
Solution chemistry	Nitrifying bacteria	Choi et al., 2009
Surface charge	*B. subtilis*	El Badawy et al., 2010
Ag^+ release	*E. coli*, native planktonic community from freshwater pond	Xiu et al., 2012; Xiu et al., 2011; Boenigk et al., 2014
ROS Production	Nitrifying bacteria	Choi & Hu, 2008

Moreover, Ag NPs with different surface coatings have exhibited different extents of aggregation of the Ag NPs in aqueous media (Yuan et al., 2013). Yuan and co-workers (2013) have shown that polyvinyl alcohol (PVA) as surface coating preserve dispersion of these PVA-coated Ag NPs compared to adenosine triphosphate disodium (ATP) as surface coating, which prevented aggregation of these ATP-coated Ag NPs less efficient. A reduced toxicity to nitrifying bacteria was determined for these ATP-coated Ag NPs compared to the PVA-coated Ag NPs. This was explained by the higher surface area of the well-dispersed PVA-coated Ag NPs leading to the generation of dissolved Ag^+ ions and the production of ROS. The production of reactive oxygen species by Ag NPs is mediated in the oxidation

process and has also been reported to cause negative effects on other microorganisms such as *E. coli* (Hwang et al., 2008).

1.6.1.3. Environmental conditions

1.6.1.4. The route of exposure

As described in detail in chapter 1.3.5., Ag NPs undergo complex and highly dynamic physical, chemical, and biological transformations in aqueous solutions, which additionally may differ between the different environmental compartments beginning from biological/ecotoxicological media in laboratory studies over the waste stream, in particular with regards to sewage treatment plants (STPs), to the natural aquatic environment. The water chemistry, the presence of NOM, temperature, and nanoparticle concentration itself can be responsible for aggregation, dissolution, or stabilization of Ag NPs (Chambers et al., 2013; Fabrega et al., 2011). For instance, dissolution of Ag NPs is influenced by the presence of dissolved oxygen (Liu and Hurt 2010; Lowry et al. 2012), pH value and natural organic matter (Liu et al. 2007). Aggregation processes are caused by pH, ionic strength, divalent counter-ions (Ca^{2+} and Mg^{2+}), monovalent anions (Cl^-), polysaccharides, and proteins (Metreveli et al. 2015; Bradford et al., 2009; Fabrega et al., 2009a; Metreveli et al., 2016; Fabrega et al; 2011). All these modifications can affect the bioavailability of Ag NPs resulting in a decrease or increase of toxicity (Gil-Allué et al., 2014). For instance, in the sludge of STPs, Ag NPs are expected to be sulfidized, what has been reported to decrease the toxicity to bacteria (Levard et al., 2013). Moreover, Ag_2S NPs have been detected in the sludge and in the efflux of STPs which might have formed from Ag NPs and sulfide in the waste water (Kaegi et al., 2013) and for which the toxicity has been shown to be lower in lab studies (Choi et al., 2013; Reintsch et al., 2013; Levard et al., 2012). Additionally, the presence of salts in the medium can lead to aggregation of the Ag NPs, for which the toxicity for bacteria has been reported to be mitigated (Matzke et al., 2012; Lok et al., 2007). Due to the resulting lower surface-to-volume-ratio of the aggregated Ag NPs, a lesser extent of Ag^+ ions might be released into the medium and thus lowering the toxicity (Matzke et al., 2012; Lok et al., 2007). Consequently, dissolution of Ag NPs can substantially increase toxicity (Levard et al., 2013, Xiu et al., 2012). However, it has been proposed that ligands in

the exposure medium or in the environment such as chloride, sulfide, phosphate, or organic acids can bind to dissolved silver (Xiu et al., 2012). This, in turn, could lead to a reduction of bioavailability and thus toxicity of the Ag^+ ions to a greater extent than that of Ag NPs (Xiu et al., 2012).

Overall, the applied concentrations as well as the distinct properties of the Ag NPs and the chemistry of the environment affect the bioavailability and ultimately toxicity of the Ag NPs for bacteria. Consequently, all these factors have to be taken into account at every stage of Ag NPs' lifecycle and potential routes of exposure pertaining to the complex transformations the Ag NPs have been undergone, particularly in the form of Ag NPs or Ag^+ ions. Though some processes that influence the bioavailability have been elaborated, a clear and comprehensive picture has not yet fully established. Thus, further investigation is required to support any broad conclusions (Tolaymat et al., 2010; Fabrega et al., 2011). In addition, the large diversity of bacterial strains pertaining to their physiology, their cell wall type and their life form further complicates the elucidation of the Ag NPs' mode of action (Fabrega et al., 2011).

1.6.1.5. The involved bacteria

For centuries, silver, and more recently Ag NPs in particular have been shown to exhibit a strong toxicity to a wide range of bacterial species. Apart from factors pertaining to the properties of the Ag NPs, the antibacterial activities of Ag NPs further seem to depend on the type of bacteria investigated (Hajipour et al., 2012). Physiological and morphological diversity among bacterial species hampers to determine the mode of action of the Ag NPs (Fabrega et al., 2011), which again is reflected by various contradicting reports on adverse effects (Schaumann et al., 2015). However, bringing together the findings from various studies, Gram-negative and Gram-positive bacteria are equally affected by Ag NPs, which suggests the toxicity appearing to be independent of the cell wall composition (e.g. Table 1) (Fabrega et al., 2011). Furthermore, it can be assumed that a unique MIC or lethal concentration of AgNPs can be assigned to each bacterial species (Table 1). This assumption is corroborated by studies investigating bacterial community patterns in response to exposure of Ag NPs (Das et al., 2012; Sheng & Liu, 2011; Yang et al., 2014; Grün et al., 2017). In these studies, the community pattern was altered

towards Ag NP-sensitive and Ag NP-tolerant species by a given concentration of Ag NPs, respectively (Das et al., 2012; Sheng & Liu, 2011; Yang et al., 2014; Grün et al., 2017). Ag NP-tolerant strains may withstand Ag NPs by common metal resistance mechanisms such as extrusion of heavy metal ions by efflux systems, segregation by thiol-containing molecules, and reduction into less toxic oxidative states (Yang et al., 2014). Ag NP-sensitive strains may be affected by Ag NPs due to structural changes in their cell wall and intracellular membranes as well as DNA and RNA denaturation, which led to the inhibition of replication, because these effects have been observed to be responsible for the Ag NP mediated toxicity towards bacteria (Reidy et al., 2013). Other observed effects include overall inhibition of enzyme activity due to complex formation of Ag NPs and/or released Ag^+ ions with the sulfur-, nitrogen- or oxygen-containing functional groups of these enzymes, suppression of bacterial respiration, and oxidative damage due the production of ROS by Ag NPs (e.g. Choi et al., 2008; Dror-Ehre et al., 2009; Hwang et al., 2008; Reinsch et al., 2012; Morones et al., 2005; Reidy et al., 2013; Tolaymat et al., 2010). The underlying bactericidal mechanisms for these effects have been proposed as follows, (Reidy et al., 2013; Tolaymat et al., 2010):

1.) Adhesion of Ag NPs to the bacterial surface. The adsorbed Ag NPs lead to altered membrane properties, possibly degradation of lipopolysaccharides, and accumulation inside the membranes by forming pits, and a large increase of membrane permeability.

2.) Intrusion of Ag NPs inside the cell causing DNA damage.

3.) Dissolution of Ag NPs with releases of antimicrobial Ag^+ ions.

The mode of action of Ag NPs is even more complex in bacterial biofilms. It has been reported that biofilms reveal a high uptake capacity for Ag NPs (Fabrega et al., 2009b; Grün et al., 2018), which indicates cumulative enrichment (Grün et al, 2018). The Ag NPs have been found to penetrate 40 µm thick *E. coli* biofilms (Eymard-Vernain et al., 2018) and up to 3 mm thick marine biofilms (Bradford et al., 2009). Nevertheless, bacteria in biofilms are more resistant to Ag NPs than living as planktonic cells (Sheng & Liu, 2011; Fabrega et al., 2009b; Eymard-Vernain et al., 2018). For instance, *E. coli* biofilms were four times more resistant to Ag NP exposure than planktonic *E. coli* cells (Eymard-Vernain et al.,

2018). Some biofilm building bacteria showed an increased production of extracellular polymeric substances (EPS) in response to Ag NP exposure (Joshi et al., 2012; Zhang et al., 2014). In biofilms, the EPS secreted by the imbedded bacteria protect them by trapping the Ag NPs (Fabrega et al., 2009b; Eymard-Vernain et al., 2018). Furthermore, reducing sugars within the EPS (Kang et al., 2014) and Ag^+ chelating compounds as functional groups of the EPS can antagonize the antibacterial activity of Ag NPs and the release of Ag^+ ions (Wirth et al. 2012). Biopolymer fractions i.e. β-D-glucose and N-acetyl-D-glucosamine, which represent a large integral part in the EPS are capable of reducing Ag^+ ions to Ag NPs in stream biofilms (Sambalova et al., 2018).

Consequently, wastewater and marine biofilms have been found to be highly tolerant to Ag NP exposure or negligible impacted (Sheng & Liu, 2011; Bradford et al., 2009). However, a reduction in biomass (Kroll et al., 2016, Fabrega et al., 2011a) and alterations in bacterial community composition as a consequence of Ag NP exposure to bacterial biofilms have also been reported (Fabrega et al., 2011a, Yang et al., 2014, Grün et al., 2018). Putatively Ag NP-sensitive bacterial taxa such as Actinobacteria, Chloroflexi, and Cyanobacteria have been shown to be displaced by the taxa Acidobacteria, Sphingomonadales, and Comamonadaceae in stream biofilms (Grün et al., 2018).

In addition, reduction of mechanical stability and adhesiveness *of Aquabacterium citratiphilum* biofilms have been described after exposure to sublethal concentrations of Ag NPs (Grün et al., 2016). It has been proposed that Ag NPs and/or released Ag^+ ions may impair the cohesiveness due to their affinity to bind to functional groups of exopolysaccharides and proteins in the EPS matrix (Chen & Stewart, 2002; Chaw et al., 2005). This observed Ag NP-mediated decrease in biofilm stability might have negative implications for biostabilization in streams (Grün et al., 2016). Furthermore, shifts in the community composition of bacterial freshwater biofilms may lead to an impairment of ecosystem functions pertaining to biodegradation with respect to nutrient loads, transformation and/or degradation of pollutants, and biostabilization (Grün et al., 2018). Moreover, the cumulative enrichment of Ag NPs in biofilms might lead to “poisoned” nutrients for grazers and detritus feeders inside the food web (Schaumann et al., 2015). This enrichment clearly underlines the importance of

biofilms and organic matter in general for the retention of metals (or nanoparticles) in the environment (Harrison et al. 2007).

1.7. Aims and objectives

The general objective of this thesis was to perform effect assessment of Ag NP exposure on bacterial biofilms with ambient Ag NPs concentrations and under environmentally relevant conditions.

Even though for the past several years, the number of studies pertaining to the physical, chemical and biological properties of nanoparticles as well as the understanding of nanotoxicity has increased significantly (Fatisson et al., 2013), the knowledge on the environmental and ecological impact of nanomaterials, in particular with respect to the prevalently applied Ag NPs, is still lagging behind the industrial growth of nanotechnology (Holden et al., 2014). Though titanium dioxide, silicon dioxide, and zinc oxide are the most manufactured nanomaterials worldwide (on a mass basis), in terms of quantity Ag NPs are most widely inserted in 24% of the products (Vance et al., 2015). Consequently, there is a pressing need to study the effects of Ag NPs on the environment and thus, in this work, Ag NPs were chosen as that nanomaterial to be investigated.

Next, because citrate is the most commonly used stabilizing agent for Ag NPs accounting for 27% and 50% of the stabilizing agents used in general and specific applications, respectively, among the literature (Tolaymat et al., 2010), citrate-stabilized Ag NPs were used in this work.

Furthermore, bioavailability of Ag NPs by bacteria and the resulting bactericidal effects seem to be dependent on the size, shape, surface coating, surface charge, surface structure and area, solubility, Ag^+ release, solution chemistry, Ag NP mediated ROS production and aggregation state of the Ag NPs (Fabrega et al., 2011, Xiu et al., 2012). These distinct physicochemical properties can be determined by the type of NP syntheses (Fabrega et al., 2011). Consequently, to strive for a broader picture about toxicity mechanisms, Ag NPs synthesized both by top-down technique i.e. laser ablation (particular GmbH, Hannover, Germany) and bottom-up approach i.e. citrate reduction method (Metreveli et al.,2015) were applied in this work.

In this context, because smaller particles have been claimed to be more toxic than bigger ones (Lok et al., 2007; Pal et al., 2007; Radniecki et al, 2011), citrate-stabilized AgNPs with two different sizes (30 nm and 70 nm) were selected.

Applied concentrations of Ag NPs in ecotoxicological studies which range from 0.1 µg l^{-1} – 200 mg l^{-1} with most frequently used concentrations in the range of mg l^{-1} do not represent environmental conditions (Table 1). Therefore, in order to approximate environmental relevant conditions, concentrations from 600 - 2400 µg l^{-1} Ag NPs were chosen in this work. This would be still at the higher end of the concentration range estimated for German rivers, where silver concentrations between 0.05 – 65 µg l^{-1} have been detected (Matzke et al., 2012). Nevertheless, because up to ~750 µg kg^{-1} Ag NPs in the sediments of fresh water are estimated (Giese et al., 2018), 600 - 2400 µg l^{-1} Ag NPs would mimic a worst case scenario, such as the resuspension of sediments by flooding events or production plant outfalls.

Bacterial biofilms are regarded as the dominant lifestyle of microorganisms in the environment (Flemming & Wingender, 2010; Kolter & Greenberg, 2006). Moreover, biofilms are the dominate life form of microorganisms in streams and rivers and are key sites of enzymatic activity (Romaní et al., 2008), including organic matter cycling, ecosystem respiration and primary production and, as such, being the base of the food chain, transferring carbon to higher trophic levels (Battin et al., 2016). Thus, due to the ecological relevance of bacterial biofilms and because Ag NPs are expected to accumulate within these biofilms in the environment, it was an inevitable methodological consequence to address bacterial biofilms as the object of investigation.

The toxicity mechanisms of Ag NPs are not entirely clarified (Eymard-Vernain et al., 2018), but Ag NPs are proposed to e.g. disrupt bacterial cell walls and intracellular membranes as well as act bacteriostatic by interfering with replication (Reidy et al., 2013), reduce the biomass of biofilms (Kroll et al., 2016, Fabrega et al., 2011a), and provoke alterations in bacterial community composition (Fabrega et al., 2011a, Yang et al., 2014). Consequently, a comprehensive set of methods was applied

in this work to elucidate potential toxicity mechanisms of Ag NPs. Important parameters of the biofilms have been ascertained in this work which (i) have been found and/or claimed to be affected by Ag NPs among the literature, (ii) are essential for the structural integrity and/or (iii) are relevant for biofilm ecological services in streams.

Consequently, aim of this work was to study if and how Ag NPs environmentally relevant affect bacterial biofilms i.e. both *monospecies* biofilms and freshwater biofilms.

The main focus of **chapter 2** was to test the hypothesis that Ag NPs affect the mechanical stability of *monospecies* biofilms of the freshwater model bacterium *Aquabacterium citratiphilum,* because investigating the mechanical stability of bacterial biofilms in response to Ag NPs had been neglected among the literature. The mechanical stability of biofilms has implications for biostabilization (Gerbersdorf & Wieprecht, 2014). Biostabilization is crucial for the cohesion of sediments in aquatic environments (Gerbersdorf & Wieprecht, 2014). Consequently, an Ag NP-mediated decrease in biofilm stability would indicate that not only the ability of biofilms to withstand potentially harsh hydrodynamic conditions in flowing waters was diminished, but also detrimental effects on sediments in river beds would occur.

Furthermore, the hypothesis was tested that Ag NPs affect further important parameters of these biofilms. Important parameters have been ascertained in this chapter which **(i)** have been found and/or claimed to be affected by Ag NPs among the literature, **(ii)** are essential for the structural integrity and/or **(iii)** are relevant for biofilm ecological services in streams. The application of a comprehensive set of methods under defined laboratory conditions have been chosen to draw clearer conclusions about cause and effect relationships, which might then be transferred to the more complex mesocosm-system in the next step of this work.

Within **chapter 3** the aim was to broaden the knowledge of the impact of Ag NPs on freshwater biofilms of rivers.

Streams and rivers represent a significant part of the Earth's total freshwater systems with an estimated surface area of 662,041 km^2 (Downing et al., 2012). Therefore, streams and rivers provide an immensely large surface area for microbial colonization (Manz et al., 1999; Romaní, 2010). However, only a few studies have addressed natural habitats when investigating effects of Ag NP exposure. In this area there is a critical knowledge gap, which needs to be filled up because of the relevance of freshwater biofilms in important ecosystem functions. Hence, the impact of Ag NPs on freshwater biofilms of rivers with respect to biofilm structure and bacterial community composition was studied in this work. As it is still challenging to monitor the ecotoxicity of Ag NPs in natural freshwater environments, there is a need for suitable experiments to facilitate this task.

Therefore, a mesocosm study was performed in this work to provide the possibility for the detailed investigation of effects of Ag NPs on freshwater biofilms under realistic environmental conditions.

Because shifts in the composition and/or diversity of the bacterial biofilm community may affect the functioning of biofilms and even ecosystem processes (Battin et al., 2016), the main focus of this chapter was to test the hypothesis that the biodiversity and community composition of bacterial freshwater biofilms in response to Ag NP treatment was shifted with detrimental effects on the functioning of biofilms and even ecosystem processes.

1.8. Thesis outline

Sublethal concentrations of silver nanoparticles affect the mechanical stability of biofilms.

Chapter 2 describes in detail experiments that were performed under defined laboratory conditions with *monospecies* biofilms of the model bacterium *Aquabacterium citratiphilum* with concentrations of Ag NPs from 600 - 2400 µg l^{-1} and a monitored time period of 20 h. In order to depict the effects of Ag NPs environmentally relevant, *monospecies* biofilms of the model bacterium *A. citratiphilum* were investigated. *A. citratiphilum* was chosen as the representative of the Comamonadaceae within the phylogenetic lineage Betaproteobacteria, a numerically dominant group of bacteria in various freshwater habitats.

Descriptive biofilm parameters were quantified with respect to protein content as well as bacterial cell membrane integrity. In addition, structural parameters were assessed by obtaining quantitative data for biofilm thickness, density, and dry mass as well as of EPS protein content.

Furthermore, the mechanical stability of these biofilms was investigated by the application of a newly developed stability assay. The rational of developing a stability assay in this work, which tried to imitate hydrodynamic forces that biofilms have to withstand in riverine systems, was to test the impact of Ag NPs on mechanical stability of riverine biofilms. The applied flow velocity of 0.4 m s^{-1} in the stability assay reflects flow velocities in natural riverine systems which are in the range of 0.1 - 6 m s^{-1}.

The biofilms of *A. citratiphilum* were cultivated and exposed to Ag NPs in R2A medium with values of pH and ionic strength in the same range as are those of water from the river Rhine. Therefore, measured effects in this work such as toxicity of Ag NPs to the biofilms as well as characterization of Ag NPs in the medium can be extrapolated to naturally occurring environmental conditions in streams. To identify nano related effects and to assess the potential harm of Ag NPs in aquatic environments, characterization of Ag NPs in R2A medium with respect to dissolution of Ag^{+} ions, size distribution and aggregation of Ag NPs, and measurement of silver content in the biofilms was performed with state-of-the art methods including ICP-OES, DLS, and AAS, respectively.

Effects of low dose silver nanoparticle treatment on the structure and community composition of bacterial freshwater biofilms.

Chapter 3 describes mesocosm experiments that were performed with more natural like multispecies freshwater biofilms in mesocosms with 600 µg l^{-1} Ag NPs and a monitored time period of 14 days. Freshwater biofilms were cultivated and exposed to Ag NPs in mesocosms, which were filled with natural water and sediment from the river Rhine. Methods to assess descriptive and structural biofilm parameters were applied, which mainly have been established in chapter 2. Thus, protein content and chlorophyll α contents were investigated as well as data for biofilm thickness, density, and dry mass were collected.

The main focus of this chapter was to elucidate the biodiversity and community composition of these freshwater biofilms in response to Ag NP treatment. This approach was assessed with t-RFLP as well as ARDRA and phylogenetic studies based on the 16S gene, respectively.

Final conclusions

In **chapter 4** the outcomes of the previous chapters are critically summarized and further improvements and potential future applications are discussed.

1.9. References

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2. Sublethal concentrations of silver nanoparticles affect the mechanical stability of biofilms

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Environmental Science and Pollution Research (2016) 23: pp 24277–24288.

https://doi.org/10.1007/s11356-016-7691-0

3. Effects of low dose silver nanoparticle treatment on the structure and community composition of bacterial freshwater biofilms

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PLoS ONE (2018) 13(6): e0199132

https://doi.org/10.1371/journal.pone.0199132

4. Final conclusions

In this thesis, effects of environmentally relevant concentrations of Ag NPs on the mechanical stability of bacterial biofilms as well as on the structure and community composition of bacterial freshwater biofilms have been described. The experimental approaches used in this study allowed the characterization of descriptive, structural, and functional characteristics of bacterial biofilms, i.e. both *monospecies* and freshwater biofilms, in response to environmentally relevant concentrations of citrate-stabilized Ag NPs with two different sizes (30 and 70 nm).

Hence, based on these results, 4 general effect mechanisms of Ag NP treatment have been identified, which can be extrapolated to natural freshwater biofilms (Figure 1):

(i) Comamonadaceae are Ag NP-tolerant,

(ii) biofilms show a particular resilient behaviour i.e. the maintenance of structural properties of the biofilms whereas functional traits can be impoverished in response to Ag NP treatment,

(iii) the two applied size fractions of Ag NPs exhibited similar effects independent of their sizes and their synthesis method, and

(iv) *monospecies*-biofilms of *A. citratiphilum* revealed a high uptake capacity for Ag NPs, which indicates cumulative enrichment. The cumulative enrichment of Ag NPs in biofilms may be a general response and may therefore expected for more complex, native multispecies biofilms.

(i) Descriptive and structural parameters of the biofilms of *A. citratiphilum* remained unaffected by Ag NPs of 30 and 70 nm after 20-h exposure within the concentration range of 600–2400 µg l^{-1}. Hence, *A. citratiphilum* as a representative of the Comamonadaceae may belong to the more Ag NP-tolerant species with enhanced adaptability towards Ag NP stress. This assumptions has been substantiated in the mesocosm experiments, where Comamonadaceae have been identified to be silver-tolerant, because the family of Comamonadaceae was a major component of biofilms treated with 600 µg l^{-1} Ag NPs of 30 and 70 nm for 14 days, respectively, which had been proven by sequence analysis of cloned 16S rRNA genes.

(ii) though descriptive and structural parameters of *A. citratiphilum* biofilms remained unimpaired by Ag NPs and *A. citratiphilum* itself may belong to the silver-tolerant species, functional characteristics of the biofilms i.e. the mechanical stability were affected. Consequently, functional characteristics can be impoverished while maintaining structural properties of the biofilm, which indicates a particular resilient behaviour. By applying descriptive and structural approaches to analyze biofilms as a whole in response to Ag NP treatment in the mesocosm experiments, which had been mainly established in the first part of this work, further evidence was provided for this particular resilient behaviour i.e. the maintenance of structural properties of the biofilms whereas functional traits can be impoverished. The bacterial community composition was clearly affected and changed due to Ag NP exposure although neither the diversity as well as the species abundance of the bacterial community nor the biomass parameters were impacted negatively. Nevertheless, the shift in the bacterial community composition, where Ag NP-sensitive bacteria such as Actinobacteria, Chloroflexi, Cyanobacteria, and Alphaproteobacteria seem to be replaced by more Ag NP-tolerant species with enhanced adaptability towards Ag NP stress may be associated with potential detrimental effects on the functioning of biofilms and even ecosystem processes (e.g. transformation and/or degradation of pollutants and biostabilization of cohesive sediments in aquatic habitats). Furthermore, even the unchanged abundance of Betaproteobacteria with their family of Comamonadaceae as a major component of Ag NP-treated biofilms may be affected by Ag NP treatment with respect to functional biofilm traits such as the mechanical stability as demonstrated for the mechanical stability of biofilms of *A. citratiphilum*. Overall, it is still under discussion whether phylogenetic and/or functional diversity may be better predictors for ecosystem functioning than species richness *per se* (Eisenhauer et al., 2012) as discussed in detail in chapter 1.5.. Against this background, the current findings in this work provide support for phylogenetic and functional diversity being better predictors for ecosystem functioning than species richness *per se.*

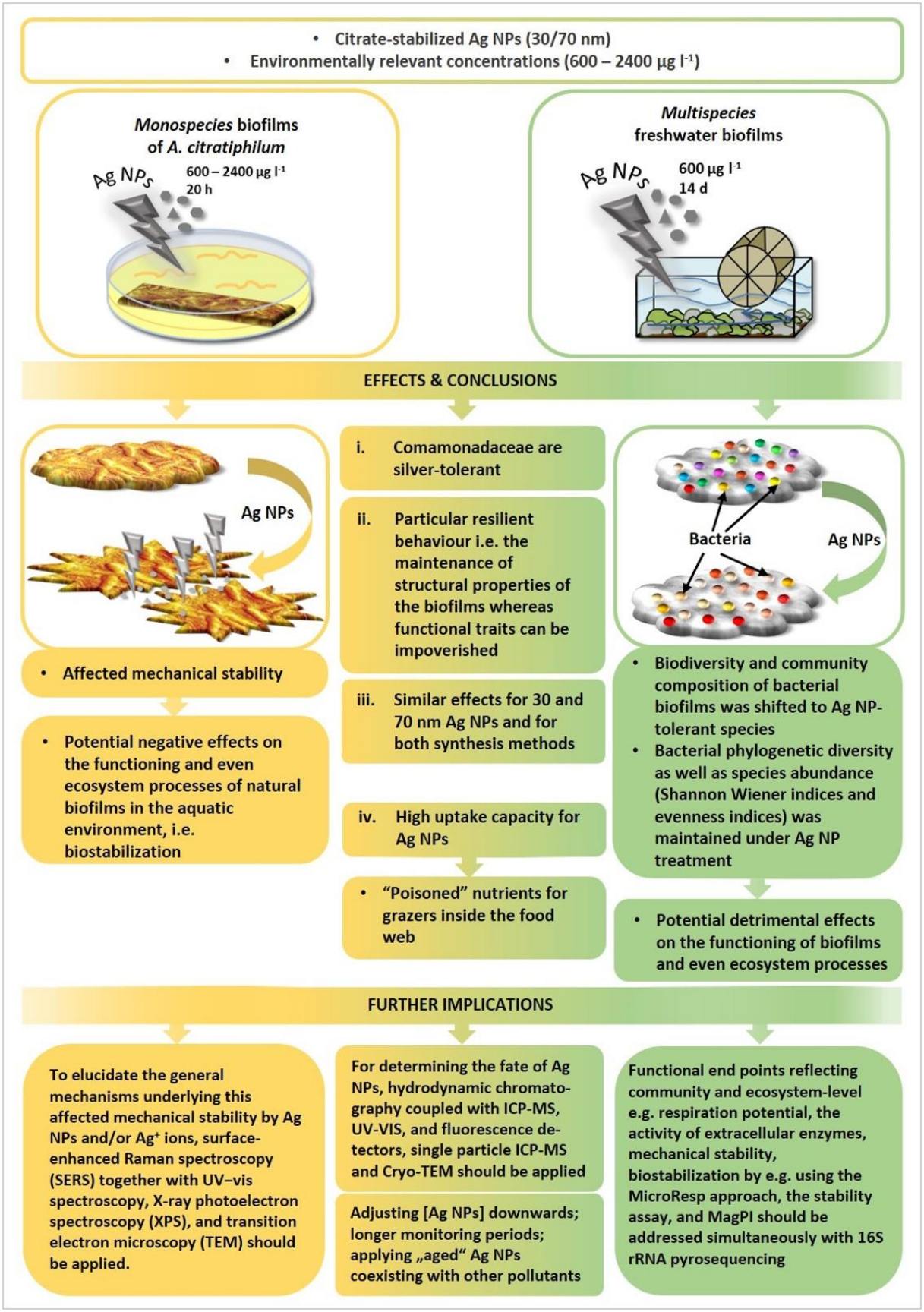

Figure 1: Effects of environmentally relevant concentrations of Ag NPs on the mechanical stability of bacterial biofilms as well as on the structure and community composition of bacterial freshwater biofilms: Conclusions and further implications.

To collect further evidence for negative impacts of Ag NPs on functional traits and ecosystem processes of *multispecies* freshwater biofilms, functional end points reflecting community and ecosystem-level processes in biofilms such as respiration potential, the activity of extracellular enzymes (e.g. β-Glucosidase, alkaline phosphatase, leucine aminopeptidase) mechanical stability, and biostabilization should be addressed by e.g. using the MicroResp approach, the stability assay, and MagPI in further studies. Simultaneously, in order to obtain a deeper and more holistic picture about the association between metabolic multifunctionality and community composition in response to Ag NP treatment, a higher number of OTUs should been captured by combining 16S rRNA gene sequencing with a high-throughput method, such as 16S rRNA pyrosequencing.

The development of the stability assay enabled to investigate the impact of Ag NPs on the mechanical stability of bacterial biofilms in their natural condition. Even though this stability assay was applied on *monospecies* biofilms of *A. citratiphilum* until now, in principle, this method is adaptable to other *mono*-or *multispecies* biofilms to test their mechanical stability. The applied flow velocity of 0.4 m s^{-1} in the stability assay reflects flow velocities in natural riverine systems which are in the range of 0.1 - 6 m s^{-1}. The *A. citratiphilum* biofilms were cultivated and exposed to Ag NPs in R2A medium with values of pH and ionic strength in the same range as are those of water from the river Rhine. Consequently, the affected mechanical stability as proven in this work as well as the characterization of Ag NPs in the medium can be extrapolated to naturally occurring environmental conditions in streams. Thus, conclusion can be drawn about negative effects on the functioning and even ecosystem processes of natural biofilms in the aquatic environment, e.g. biostabilization. This assumption was confirmed in a later study, which applied a method precisely targeted to assess biostabilization, i.e. MagPI (Schmidt et al., 2017).

Nevertheless, to what extent the release of Ag^{+} ions by Ag NPs, and to what extent physical effects, i.e., the nanoparticle size and shape, are more relevant to the impact of Ag NPs on the mechanical stability of biofilms, needs to be clarified in further studies. Therefore, the mechanical stability of

biofilms from *A. citratiphilum* should be determined after treatment with concentrations of Ag^+ ions, which were released from the Ag NPs in this study.

Furthermore, to elucidate the general mechanisms underlying this proven impairment in mechanical stability by the Ag NPs and/or Ag^+ ions, the involved functional groups in the EPS of biofilms of *A. citratiphilum*, which are hypothesized to interact with Ag NPs and/or Ag^+ ions should be characterized. Therefore, surface-enhanced Raman spectroscopy (SERS) together with UV−vis spectroscopy, X-ray photoelectron spectroscopy (XPS), and transition electron microscopy (TEM) should be applied (Sambalova et al., 2018).

(iii) The two applied size fractions of Ag NPs exhibited similar effects independent of their sizes and their synthesis method. This can be explained by an increase of the initial size of the 30 nm sized Ag NPs during exposure time of 20 h in R2A medium. Consequently, the surface area to volume ratio might became less relevant, what might be accompanied by a decrease in toxicity potential. Furthermore, Ag NPs underwent rapid aggregation in water from river Rhine due to the presence of Ca^{2+} ions. Hence, aggregation masks the differences between primary particle sizes of the two applied size fractions of Ag NPs.

(iv) The *monospecies*-biofilms of *A. citratiphilum* revealed a high uptake capacity for Ag NPs, which indicates cumulative enrichment. The cumulative enrichment of Ag NPs in biofilms may be a general response and may therefore expected for more complex, native multispecies biofilms such as the mesocosm biofilms in this work and might lead to “poisoned” nutrients for grazers inside the food web. Moreover, due to the high uptake capacity of the biofilms for Ag NPs, continuously released Ag^+ ions from the accumulated Ag NPs might be relevant because of their antibacterial activity. Consequently, it is an important issue that future studies define the speciation of the Ag in the biofilms and distinguish between the effects on biofilms mediated by Ag NPs and those mediated by Ag^+ ions.

Within the mesocosms, a rather negligible dissolution of Ag NPs was anticipated as also shown for the release of Ag^+ ions by oxidative dissolution in the culture medium of *A. citratiphilum* with ranges of pH and ionic strength equivalent to those of water from the river Rhine. Nevertheless, it cannot be fully

excluded that the observed Ag NP-mediated toxicity was attributed to antibacterial properties of released Ag^+ ions besides particle-specific effects.

Hence, to determine in detail the fate of Ag NPs in natural environments to better understand the detailed toxicity mechanisms, appropriate techniques should be applied and refined, such as hydrodynamic chromatography coupled with ICP-MS, UV-VIS, and fluorescence detectors, as well as single particle ICP-MS and Cryo-TEM.

To sum up, further work should closely interlink all these techniques mentioned above in this chapter with the experimental design proven to be appropriate to mirror a freshwater biofilm ecosystem close to natural conditions in this work. Moreover, in principle, this design is adaptable to other ecotoxicological studies as long as limitations of detection techniques complicate the investigation of the fate and toxicity of engineered nanoparticles in natural freshwater environments (Yin et al. 2017). In this work, a number of conditions were met to perform effect assessment of Ag NP exposure on bacterial biofilms environmentally relevant such as Ag NP concentrations in the range of $\mu g\ l^{-1}$, periods of exposure from 20 h to 14 days, and natural water or nutrient medium with values of pH and ionic strength in the same range of water from the river Rhine, to name a few. Therefore, conclusions can be drawn about the negative impacts of Ag NPs on the functioning and even ecosystem processes of natural biofilms in the aquatic environment.

Nevertheless, in future studies, concentrations of Ag NPs in toxicity studies should be adjusted downwards with concentration in the range of $ng\ l^{-1}$ and their toxic effects should be monitored over longer periods (months to years). Moreover, Ag NPs undergo complex and highly dynamic physical, chemical, and biological transformations in aqueous solutions along their journey beginning from the consumer product or biological/ecotoxicological media in laboratory studies over the waste stream, in particular with regards to STPs, to the natural aquatic environment in natural freshwater environments. Hence, to gain insights into the toxicity mechanisms of Ag NPs as they occur in the natural environment, transformed or “aged” Ag NPs should be applied in ecotoxicity studies such as

Ag_2S NPs and/or Ag NPs coexisting with other compounds (such as ZnO, TiO_2, and CuO NPs) or persistent organic pollutants (such as polychlorinated biphenyls) (Zhang et al., 2019).

Overall, the broad insights about negative effects of environmental relevant concentrations of Ag NPs on bacterial biofilms, i.e. both *monospecies* and freshwater biofilms, with a special focus on the consequences of Ag NP contamination for major biofilm ecological services, which have been obtained in this work are helpful to shape further work for assessing the environmental fate and risk of Ag NPs.

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TAGUNGSBEITRÄGE

Poster

A. Grün, M. Madzgalla, W. Manz (2012) Interaction of engineered anorganic nanoparticles with bacterial biofilms. VAAM Tübingen, 2012

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A. Grün, C. App, A. Breidenbach, J. Meier, W. Manz (2014) Interaction of silver nanoparticles with bacterial biofilms. Monospecies vs. natural biofilms. International Workshop, Nanoparticles in Soils and Waters: Fate, Transport and Effects, Landau, 2014

Danksagung

An erster Stelle gilt mein besonderer Dank dem Betreuer meiner Promotion Herrn Prof. Dr. Werner Manz für seine intensive wissenschaftliche und methodische Unterstützung während der gesamten Bearbeitungsphase meiner Dissertation. Von ganzem Herzen möchte ich mich zudem für die großmütige Chance bedanken, dass ich die Promotion unter seiner Betreuung wieder habe aufnehmen können.

Außerdem gilt mein herzlicher Dank Frau Prof. Dr. Gabriele Schaumann, die mich darin bestärkt hat, diese Arbeit fortzusetzen und mich bei der Bearbeitung stets durch zielführende Anmerkungen und anhaltende Hilfestellung begleitet und unterstützt hat.

Ich danke der Deutschen Forschungsgemeinschaft (DFG, INTERNANO: FOR 1536, subproject BIOFILM MA3273/3-1 (WM)) für die Förderung sowie der Universität Koblenz-Landau für die Bewilligung des Promotionsabschlussstipendiums für Doktorandinnen.

Frau Dr. Jutta Meier danke ich von Herzen für ihren Glauben an mich und meine Fähigkeiten, die zahlreichen und mit viel geteilter Freude geführten, unermüdlichen fachlichen Gespräche und für ihre Ratschläge und Anmerkungen, die mich auf dem Weg zur fertigen Arbeit immer wieder neue Aspekte und Ansätze entdecken ließen. Auch die vielen nicht-wissenschaftlichen, motivierenden und persönlichen Gespräche haben meine Arbeit maßgeblich unterstützt.

Allen Mitarbeitern der AG Mikrobiologie, insbesondere Frau Kerstin Hoffmann, Herrn Christian Sodemann, Herrn Constantin App und Frau Jessica Tepper sowie allen Beteiligten meiner Publikationen, insbesondere Herrn Dr. George Metreveli bin ich sehr dankbar für die großartige und zahlreiche Unterstützung sowie die konstruktive und äußerst angenehme Zusammenarbeit.

Ganz besonders möchte ich an dieser Stelle auch meinen Eltern, meinem Sohn und meinen Freunden, Frau Jennifer Ernst, Frau Katharina Busch, Frau Dr. Nicole Leib, Frau Susanne Saidel, Herrn Paul Pützfeld und Herrn Dr. Béla Bajan für die unermüdliche Stärkung und Motivation danken sowie für das stets offene Ohr für meine Gedanken.