Toxicity of silver nanoparticles to selected Australian freshwater biota: influences of coating and age of nanoparticles

A thesis submitted in fulfilment of the requirements for the degree of Doctor of Philosophy

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Declaration

I certify that except where due acknowledgement has been made, the work is that of the author alone; the work has not been submitted previously, in whole or in part, to qualify for any other academic award; the content of the thesis is the result of work which has been carried out since the official commencement date of the approved research program; any editorial work, paid or unpaid, carried out by a third party is acknowledged; and, ethics procedures and guidelines have been followed.

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<td>AChE</td>
<td>Acetylcholinesterase</td>
</tr>
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<td>AgNP</td>
<td>Silver nanoparticle</td>
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<tr>
<td>ASTM</td>
<td>American society for testing and materials</td>
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<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
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<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
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<td>BMF</td>
<td>Biomagnification factor</td>
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<td>C-AgNP</td>
<td>Curcumin coated nanoparticles</td>
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<td>CAT</td>
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<tr>
<td>CNT</td>
<td>Carbon nanotubes</td>
</tr>
<tr>
<td>COD</td>
<td>Chemical oxygen demand</td>
</tr>
<tr>
<td>DGV</td>
<td>Default guideline value</td>
</tr>
<tr>
<td>DLS</td>
<td>Dynamic light scattering</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>DO</td>
<td>Dissolved oxygen</td>
</tr>
<tr>
<td>DOC</td>
<td>Dissolved organic carbon</td>
</tr>
<tr>
<td>DOM</td>
<td>Dissolved organic matter</td>
</tr>
<tr>
<td>E-AgNP</td>
<td>Epigallocatechin-gallate coated nanoparticles</td>
</tr>
<tr>
<td>EC&lt;sub&gt;50&lt;/sub&gt;/LC&lt;sub&gt;50&lt;/sub&gt;</td>
<td>median lethal concentration of the substance that affects/kills 50% of test organisms</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EGCG</td>
<td>Epigallocatechin-gallate</td>
</tr>
<tr>
<td>ENP/ENM</td>
<td>Engineered nanoparticle/Engineered nanomaterial</td>
</tr>
<tr>
<td>EPS</td>
<td>Extracellular polymeric substances</td>
</tr>
<tr>
<td>GST</td>
<td>Glutathione S-transferase</td>
</tr>
<tr>
<td>HC</td>
<td>Hazardous concentration</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
</tr>
<tr>
<td>---------</td>
<td>-----------------------------------------------------------------------------</td>
</tr>
<tr>
<td>HDD</td>
<td>Hydrodynamic diameter</td>
</tr>
<tr>
<td>ICP-MS</td>
<td>Inductively coupled plasma mass spectrometry</td>
</tr>
<tr>
<td>LPO</td>
<td>Lipid peroxidation</td>
</tr>
<tr>
<td>MDA</td>
<td>Malondialdehyde</td>
</tr>
<tr>
<td>MWCNT</td>
<td>Multi-walled carbon nanotube</td>
</tr>
<tr>
<td>MWCO</td>
<td>Molecular weight cut-off</td>
</tr>
<tr>
<td>NOM</td>
<td>Natural organic matter</td>
</tr>
<tr>
<td>NP</td>
<td>Nanoparticle</td>
</tr>
<tr>
<td>NW</td>
<td>Nanowire</td>
</tr>
<tr>
<td>nZVI</td>
<td>Nano zerovalent ion</td>
</tr>
<tr>
<td>OECD</td>
<td>Organisation for economic co-operation and development</td>
</tr>
<tr>
<td>pdi</td>
<td>Polydispersity index</td>
</tr>
<tr>
<td>PEG</td>
<td>Polyethylene glycol</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>POD</td>
<td>Peroxidase</td>
</tr>
<tr>
<td>PVP</td>
<td>polyvinylpyrrolidone</td>
</tr>
<tr>
<td>QD</td>
<td>Quantum dot</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>SOD</td>
<td>Superoxide dismutase</td>
</tr>
<tr>
<td>SPR</td>
<td>Surface plasmon resonance</td>
</tr>
<tr>
<td>SSD</td>
<td>Species sensitivity distribution</td>
</tr>
<tr>
<td>T-AgNPs</td>
<td>Tyrosine coated nanoparticles</td>
</tr>
<tr>
<td>TBARS</td>
<td>Thiobarbituric acid reactive substances</td>
</tr>
<tr>
<td>TEM</td>
<td>Transmission electron microscopy</td>
</tr>
<tr>
<td>UV-vis</td>
<td>Ultra violet-visible spectroscopy</td>
</tr>
<tr>
<td>WWTP</td>
<td>Waste water treatment plant</td>
</tr>
</tbody>
</table>
Abstract

Nanotechnology is a fast-growing industry yielding many benefits to society with various types of nanoparticles (NPs) used in numerous applications. Intensive manufacture of NPs started in 1980s and these materials are being increasingly used in consumer products. However, there is also increasing concern regarding their potential impacts on biota in the environment since the release of NPs to the ecosystems is increasing. Aquatic environments are particularly at risk since considerable amounts of NPs may eventually end up in water bodies causing direct and indirect effects on aquatic organisms and could transfer up trophic chains. However, currently there are very few techniques that can measure the quantity of NPs released into aquatic and terrestrial environments which makes it difficult to predict their effects on organisms. Though NP size remains central to determining toxicity, studies suggest that other inherent factors like coating agents should be considered in toxicity studies. Little is known about the transformation and stability of NPs in the environment and the effects of aged NPs on organisms compared with their pristine forms. In the current PhD project, how surface coatings influence the toxicity of NPs to selected aquatic organisms, their uptake through the trophic chain and their toxicity with ageing were investigated using standard toxicity tests, bioassays and fate and behaviour assessment of NPs in media. Four freshwater organisms; *Hydra vulgaris*, *Raphidocelis subcapitata*, *Daphnia carinata* and *Paratya australiensis* which represent different trophic levels were used to evaluate the toxicity of silver NPs (AgNPs) coated with three different ligands; Tyrosine-coated (T-AgNP), Epigallocatechin gallate-coated (E-AgNP) and Curcumin-coated (C-AgNP) AgNPs. These AgNPs were produced, characterized, and their stability was assessed in relevant media as representative of acute toxicity and ageing experiments. Organisms cultured in the laboratory or collected from the field were exposed to the AgNPs and their effects were assessed using several endpoints. The results generated from this research are presented and compared with published data from literature. From the study, it was found that the effects of AgNPs were dependent on several factors; the surrounding medium, ageing and type of coating all influenced the transformation and stability of NPs. Coating and dose-dependent morphological, behavioural, acute and sub-lethal effects on the organisms were observed. Differences in sensitivity and response among different organisms in the end-points tested were observed. Organisms also responded differently when exposed to aged AgNPs; compared with exposure to their pristine forms. The findings from this study will contribute to a better understanding of the potential risks of differently coated AgNPs to organisms in the freshwater environment.
**Chapter 1. General introduction**

**1.1 Nanotechnology**

Nanostructures or nanoparticles (NPs) have been used for various purposes since ancient times. A variety of colourful glass windows in medieval cathedrals are attributed to the presence of metal oxide NPs. The Lycurgus Cup, a 4th Century Roman glass cage cup is made up of soda-lime glass that contains gold and silver NPs (AgNPs) distributed in a specific arrangement (Poole Jr and Owens, 2003). The Damascus-steel swords, made and used between the 3rd and 17th Century contains oriented cementite nanowires and carbon nanotubes (Reibold et al., 2006).

Increased interest in nanoscience and nanotechnology developed at the beginning of the 21st Century. Richard Feynman, a Nobel Prize Laureate in physics is considered as the pioneer of modern nanotechnology. He introduced the concept of manipulating matter at the atomic level at his revolutionary lecture titled, “There’s Plenty of Room at the Bottom” during the 1959 American Physical Society meeting at Caltech, USA. In 1974, Norio Taniguchi from the Tokyo University of Science first used the term “nanotechnology” to describe semiconductor processes such as thin film deposition and ion beam milling that occurred on the nanometer scale. The golden era of nanotechnology began with the discovery of the buckminsterfullerenes in 1985 by Harry Croto, Rick Smalley and Robert Curl. The first book on nanotechnology, Engines of Creation: The Coming Era of Nanotechnology was published by Eric Drexler, which laid out the foundations for practically materializing “molecular machines”. Since then, several landmarks and milestones have been achieved in the nanotechnology sector impacting several fields (Pande and Bhaskarwar, 2016, Hulla et al., 2015). Currently, nanotechnology is a trillion-dollar industry which is increasing exponentially (Larsson et al., 2019).

**1.1.1. Nanomaterials**

The prefix “nano”, increasingly used in the scientific literature is derived from the Greek “nanos”, meaning “dwarf”. Popularly, nano is used as an adjective to describe objects, systems, or phenomena with characteristics arising from nanometer-scale structure. The nanometer is a metric unit of length and denotes one-billionth of a meter or $10^{-9}$ m. Nanomaterials include NPs, nanofibres and nanotubes, composite materials and nano-
structured surfaces. The size of NPs is limited to 100 nm as the choice while this upper limit is justified because of physical properties of NPs approach those of their bulk counterparts when the size exceeds this value. Therefore, NPs are a subset of nanomaterials, that have structural components smaller than 100 nm in at least one dimension (Kreyling et al., 2010). Size of agglomerates can be larger than 100 nm but are included in the discussion since they may break down on weak mechanical forces or in solvents. NPs are considered a distinct state of matter compared with the solid, liquid, gaseous, and plasma states, due to their distinct properties. Two primary factors which cause NPs to behave significantly differently than bulk materials are the surface effects and quantum effects. The chemical reactivity of NPs as well as their mechanical, optical, electric, and magnetic properties are affected by these factors (Borm et al., 2006, Buzea et al., 2007, Cattaneo et al., 2009a).

1.2 Ecotoxicology

French Professor Réné Truhaut who coined the term, “ecotoxicology” describes it as the “branch of toxicology concerned with the study of toxic effects, caused by natural or synthetic pollutants, to the constituents of ecosystems, animal (including human), vegetable and microbial, in an integral context” (Truhaut, 1977). Ecotoxicological studies comprise; the study of the emission, entry, distribution and fate of pollutants in the abiotic environment; the study of the entry into and fate of pollutants in the biosphere, and the qualitative and quantitative study of the toxic effects of chemical pollutants to ecosystems, with investigation of the impact on humans (Truhaut, 1977). It draws from knowledge and techniques in the fields of ecology and toxicology and the goal of this approach is to predict the effects of pollution so that the most effective action to prevent or remediate any harmful effect can be identified. Also, ecotoxicological studies can inform as to the best course of action to restore the ecosystems that are already impacted by pollution.

1.2.1 Nanoeotoxicology

It can be assumed that naturally produced NPs have been in a form of equilibrium in nature, but engineered NPs are a growing concern among institutions and the public due to their possible negative consequences on living organisms (Moore, 2006, Tiede et al., 2009). Research on nanotoxicology started in the early 1990s and was highlighted by the work of Oberdörster et al. (2005b) on the effects of ultrafine particles, while studies on nanoeotoxicology started around a decade ago (Moore, 2006, Hund-Rinke and Simon, 2006).
The work carried out by the nanotoxicology community over recent years is commendable. The toxicity of NPs to organisms has been the subject of a number of studies (Klaine et al., 2008, Moore, 2006, Levard et al., 2012); however, coherent, consistent and well-founded data is still lacking (Selck et al., 2016b, Giese et al., 2018). To date the knowledge that the scientific world has acquired is inadequate to draw conclusions on the actual release and fate of NPs in the natural environment, actual environmental exposure to NPs and the magnitude of harm they incur to living beings (Gottschalk and Nowack, 2011, Bäuerlein et al., 2017).

Establishing the safety of nanomaterials is important to protect the environment and health of organisms. The effects of NPs depend on many factors including their intrinsic properties, fate and bioavailability in the respective environment and response of the receptor organisms (Lapresta-Fernández et al., 2012). Currently available data on exposure to NPs and effects on organisms are insufficient to conclude on the risks involved (Ma et al., 2013, Holden et al., 2014, Skjolding et al., 2016, Hjorth et al., 2017a, Hjorth et al., 2017c). One of the main factors inhibiting progress in this area is around the difficulty in the detection and quantification of NPs in the environment (Bundschuh et al., 2016b).

1.3 Why be concerned about NPs?

Although, as previously discussed engineered NPs (ENPs) have been around for some time, due to their increased use, concerns about the risks associated with their use arose a few years ago. Despite huge concerns, due to a lack of sample-related certified standards, analytical procedures and reliable units of measure (Mottier et al., 2017) the presence of NPs in water sources and receiving bodies including waste effluents, surface or ground waters and sediments has not been well studied and documented (Mirzajani et al., 2013, Mitrano et al., 2012b). However, the presence of NPs in the environment is a proven phenomenon as per recent studies, as discussed earlier.

Bulk materials are usually defined in terms of properties like density, resistivity, magnetism and dielectric constant which are averaged for the whole unit. Compared to their bulk form, NPs possess unusual and different properties which cannot be explained with Newtonian mechanics, only with quantum mechanics (Throbäck et al., 2007, Bhushan, 2010, Poole Jr and Owens, 2003). In comparison to naturally available NPs, engineered NPs may have different physical and chemical characteristics (Handy et al., 2008).
Unpredictable consequences due to their colloidal nature and the dynamics of NPs in receiving environments represent a huge challenge in assessing their toxicity (Service, 2004, Nowack and Bucheli, 2007b, Blaser et al., 2008, Diegoli et al., 2008, Hassellöv et al., 2008, Tiede et al., 2008). Chemical and physical properties like zeta potential and metal binding capacity are determined by the size of the particles (Madden et al., 2006) which varies significantly in NPs. In addition, due to their small size, the behaviour of NPs in the environment and effects on organisms are different to those of conventional xenobiotics (Scown et al., 2010, Klaine et al., 2012). High surface to volume ratio and abundant reactive sites on the surface are some unique characteristics of NPs, and these along with their mobility could result in unexpected health hazards (Maynard et al., 2006, Wiesner et al., 2006). Also, physicochemical characteristics of both NPs and their surrounding environment and modalities of the suspension decide the attributes of the dispersed nano-phase (Ko et al., 2018).

Once released into the environment, NPs may transform due to several reasons. The pristine organic coating can be degraded or substituted by NOM which strongly affects the surface charge of NPs, their aggregation, and toxicity (Omar et al., 2014). The aggregation of NPs may impact on the transportation of NPs. The metallic core of the NPs may transform after exposure to different organic and inorganic compounds at different environmental scenarios (Gottschalk et al., 2009). Reaction of NPs with media constituents such as sulfur affect the bioavailability and toxicity of NPs (Bianchini and Wood, 2008). In addition to their own toxicity, NPs also influence the toxicity of other contaminants which are harmful to aquatic organisms (Tan and Wang, 2014, Fan et al., 2016).

It was reported that creation of free radicals and oxidative damage is the main cause of adverse effects in cells (Auffan et al., 2009). Since NPs have a very large surface area in relation to volume, they may cause direct generation of oxyradicals which can attack DNA, proteins and membranes (Brown et al., 2001). Once in the cell, NPs may embed within the functional machinery of a cell resulting in different toxicological responses compared to conventional toxicants (Moore, 2006).

Risk assessment of exposure to NPs is still at the research and development level. A number of authors have proposed approaches to NP risk assessment (Dekkers et al., 2016, Domercq et al., 2018, Garner et al., 2017, Hristozov et al., 2016). However, a comprehensive risk assessment of NPs, data requirements, models and advancement related to NP production, release, exposure, fate and behaviour, risk characterization etc. (Scott-Fordsmand et al., 2017)
have not yet been developed. The models that are proposed each have their own limitations and the tools used in characterization of NPs in exposure assessment and toxicology tests are not sufficient for risk assessment (Garner et al., 2017, Mattsson and Simkó, 2017).

Several parameters influence NP toxicity tests, but there exists a lack of scientific understanding of the importance of each parameter or the interactions between them for the toxicity endpoints in current test guideline (Hjorth et al., 2017c). The available test guidelines are not sufficient to analyse the behaviour of NPs in test media. For example, Wasmuth et al. (2016) concluded that the available organisation for economic co-operation and development (OECD) guidance document No. 29 which was designed to determine the rate and extent of ion release from metals does not cover analytical methods for NPs. Also, the development of toxicity test guidelines is still at the early stage of development with only a few guidelines available, published recently (OECD, 2017c, OECD, 2017a, OECD, 2017b). Accordingly, the lack of NP toxicity test data which are suited for regulatory decision-making is still a pressing issue (Hjorth et al., 2017c).

An additional factor that must be considered is the fact that currently available remediation or purification technologies appear ineffective in reducing the concentration of NPs to environmental permissible levels. Furthermore, increasing NP release may cause further issues in wastewater and sewage sludge treatment plants which may pose a risk to microbes in the digestion systems (Wang and Chen, 2016).

Overall, although numerous studies have been performed in the field of nanocotoxicology, it is still critical that further research continues to explore the consequences of the interactions of NPs with biota. The emphasis should be placed on studying the ecological effects of pristine and aged NPs, specifically addressing the characterisation and transformation of NPs in the environment and the influence of exposure scenarios and coatings on the bioavailability, uptake, toxicity and trophic transfer of NPs (Selck et al., 2016a, Lee et al., 2015).

1.4 Test substances

1.4.1. Silver NPs

Silver is popular due to its antibacterial potential and has been used since ancient times for several purposes (Nowack et al., 2011, Reidy et al., 2013, Amato et al., 2011, Alexander,
Due to its long history of use in varied applications, a significant number of studies have been carried out relating to AgNPs (Nowack et al., 2011). The synthesis of citrate-stabilized AgNPs was reported over 120 years ago (Lea, 1889); The production of AgNPs stabilized with protein was reported in 1902 and gelatine-stabilized AgNPs were produced in 1953 (Nowack et al., 2011). In terms of the “nanotoxicity of AgNP”, their toxicity was reported back in 1924 (Nowack et al., 2011, Drake and Hazelwood, 2005). The use of products containing AgNPs has significantly increased in the last three decades (Nowack et al., 2011, Amde et al., 2017) while more than 800 new products have been introduced to the market in the last 3 years (Nanodatabase, 2018).

1.4.2 Coating materials

Coatings are used to selectively change or influence particle properties of NPs. A wide variety of substances are used to cover the core of the particles with single or multi-layered coatings. The surface properties of particles determine the physicochemical behaviour of NPs, determine the level of interaction between the particles and other molecules and influence the biological properties of particles (Daima et al., 2014, Nune et al., 2009, Hoet et al., 2004). Currently many different types of compounds are used as capping agents in for commercial NP production (Table 1.1) (Lekamge et al., 2018a).

Table 1.1: Different types of capping agents of NPs - Source: Lekamge et al. (2018a)

<table>
<thead>
<tr>
<th>Category</th>
<th>Compound</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carboxylic acids</td>
<td>Citrate, Oleic acid, Mercaptosuccinic acid</td>
</tr>
<tr>
<td>Polymers</td>
<td>Polyvinylpyrrolidone, Polyacrylate, Polyvinylalcohol, Polyacrylamide, Polylactic acid, Poly vinyl chloride, Polystyrene, Dodecanothiol</td>
</tr>
<tr>
<td>Polysaccharides</td>
<td>Gum Arabic, Sophorolipids, Chitosan, Heparin, Hyaluronic acid, Cellulose, Starch, Alginic acid, Dextran, Maltose</td>
</tr>
<tr>
<td>Biological molecules</td>
<td>Bovine Serum Albumin, Fatty acids, Tyrosine</td>
</tr>
<tr>
<td>Inorganic coatings</td>
<td>Silver carbonate</td>
</tr>
<tr>
<td>Surfactants</td>
<td>Sodium dodecyl sulphate, Cetyltrimethylammonium bromide, Polyoxyethylenesorbitane monooleate</td>
</tr>
<tr>
<td>Organic coatings</td>
<td>Plant extracts, Whole plant extracts, Food sources from plant origin, Triethanolamine, Thioglycerol, Hexamine, Sodium dodecylbenzenesulfonates</td>
</tr>
</tbody>
</table>
1.5 Test species

Since no single species shows uniform sensitivity, a battery of bioassays with different sensitivity profiles are recommended for ecotoxicological studies. The risk assessment is more informative if the battery involves organisms from different trophic levels (Kahru and Dubourguier, 2010). Therefore, hydra, algae, daphnids and shrimps which represent different trophic levels were chosen for this study.

1.5.1 Hydra vulgaris

Hydra (Medusozoa: Anthomedusae: Hydridae) (Fig. 1.1) is a freshwater coelenterate native to tropical and temperate regions. It is a diploblastic animal (i.e. contains an inner endoderm and an outer ectoderm) with a tubular body terminated by an oral apparatus with a crown of tentacles, and a basal disc at opposite extremities. The body is covered by a cuticle, a thick multilayered extracellular matrix secreted by ectodermal cells that forms the distal interface with the environment. Their diploblastic body nature, recognizable morphological changes, ubiquitous presence in freshwater environments, easy maintenance in the laboratory, cost effectiveness and the ability to reproduce asexually resulting in large number of genetically similar individuals make them an ideal organism for acute toxicity tests (Beach and Pascoe, 1998, Trottier et al., 1997).

Fig. 1.1: Hydra vulgaris – Source: https://en.wikipedia.org/wiki/Hydra_vulgaris

1.5.2 Raphidocelis subcapitata

R. subcapitata (Fig. 1.2) is a sickle-shaped, unicellular freshwater green microalga. The cells are presented in a solitary form and are 8 to 14 µm in length and 2 to 3 µm in width. It is
the most frequently used and recommended algal species for freshwater ecotoxicity testing due to its high growth rate, sensitivity to toxicants, good reproducibility and ubiquitous distribution compared to other algae (Yamagishi et al., 2017, Moreira-Santos et al., 2004). The responses of *R. subcapitata* upon exposure to a variety of contaminants are available in a large database (Radix et al., 2000, Weyers et al., 2000).

![Raphidocelis subcapitata cells](image)

**Fig. 1.2: Raphidocelis subcapitata**

### 1.5.3 Daphnia carinata

*Daphnia sp.* (Crustacea: Cladocera: Daphniidae) is a small crustacean which lives in the pelagic zone of most freshwater habitats. Their bodies are enclosed by an uncalcified shell known as the carapace which is largely made of chitin. The double wall makes the body cavity and hemolymph flows inbetween. They have up to 10 pairs of appendages while the limbs form an apparatus for feeding and respiration. The body length of daphnids ranges from less than 0.5 mm to more than 6 mm. Compared to the females, males are smaller in size, have larger antennules, modified post-abdomen and the first legs are armed with a hook for clasping. Daphnids are filter feeders ingesting mainly unicellular algae and various other sorts of detritus. *Daphnia spp.* is widely used as a model organism in aquatic toxicology tests since they are one of the most sensitive organisms and their ecology, phylogeny, toxicology, and physiology are relatively well understood and a great number of toxicants have been evaluated using daphnids (Von der Ohe and Liess, 2004, Altschuler et al., 2011, Edwards and Pascoe, 2018). Being primary consumers, *Daphnia spp.* are at a bottom level of the food chain in aquatic freshwater ecosystems and thus, a subtle change in the population affects other populations of aquatic organisms, resulting in major environmental effects (Becaro et al., 2015, Martins et al., 2007). There are several species within the genus *Daphnia*; the North American *D. magna* is the most
readily available and represents the preferred species in ecotoxicological studies. *D. carinata* (Fig. 1.3) is distributed in the Southern Hemisphere where it is considered a suitable alternative species to *D. magna* for toxicological studies (Zalizniak and Nugegoda, 2006, Cáceres et al., 2007, Cooper et al., 2009).

Fig. 1.3: *Daphnia carinata*

1.5.4 *Paratya australiensis*

The freshwater atyid shrimp, *P. australiensis* (Crustacea: Decapoda: Atyidae) (Fig. 1.4), commonly called “Glass shrimp” inhabits streams and other freshwater and estuarine habitats along the south and east coast of Australia. It is the most widespread, common freshwater shrimp in Australia (Hughes et al., 1995, Cook et al., 2006). The adult shrimps are about 50 mm long from head to tail and the body is transparent. They are prolific breeders, breeding all year around. *P. australiensis* is an important food source for native biota, plays an important role in ecosystem processes via detrital decomposition and influence the composition of algal and benthic invertebrate communities (Richardson et al., 2004, Walsh and Mitchell, 1995, March et al., 2001, March et al., 2002, Pringle, 1996). *P. australiensis* is increasingly used as a model organism in ecotoxicological studies (Kumar et al., 2010a, Kumar et al., 2010b, Daly et al., 1990); however, no studies have been published regarding their response to NP (Lekamge et al., 2018b).

Fig. 1.4: *Paratya australiensis*
1.6 Toxicity studies

In ecotoxicology, acute and chronic toxicity studies often involve individual species and endpoints. The primary route of exposure is assumed to be aqueous and thus, the standard toxicity assessments are routinely based on the concentrations of contaminants in the external medium. However, other assessment methods such as quantification of associated contaminants within the biological tissues are recommended for much better predictions (Chapman, 2002, Chapman, 2000).

Gathering data related to dose-response characteristics of a contaminant is the primary objective of toxicological studies (Murphy, 1979). Analysis of data provide estimates of the risk to a particular population of organisms from the contaminant while the research or testing approach depends on several factors. Oberdörster et al. (2005a) outlined physicochemical characterisation, in vitro studies and in vivo studies as three key elements in toxicity testing of NPs. Lethal Concentration (LC$_{50}$) and Median Effective Concentration (EC$_{50}$) are commonly used toxicity endpoints in acute toxicity studies. The EC$_{50}$ is the concentration at which 50% of the population exhibit a response while the LC$_{50}$ is the concentration at which 50% of organisms are expected to die (Calow and Forbes, 2003, OECD, 2004a, OECD, 2011a). The European Directive 67/548/EEC classifies substances as “harmful”, “toxic” or “very toxic” as per the EC$_{50}$ and LC$_{50}$ values to fish, daphnids and algae. If the LC$_{50}$/EC$_{50}$ values of the substance was < 1 mg L$^{-1}$, the substance was classified as “very toxic” to aquatic organisms, if between 1 and 10 mg L$^{-1}$, the substance was classified as “toxic to aquatic organisms” and if the values were between 10 and 100 mg L$^{-1}$, the substance was classified as “harmful to aquatic organisms” (Kahru and Dubourguier, 2010).

1.7 Aims of the research

1. To evaluate and compare the toxicity of silver nanoparticles coated with ligands (Tyrosine, Epigallocatechin gallate (EGCG) and Curcumin) and ionic silver to Hydra vulgaris, Raphidocelis subcapitata, Daphnia carinata and Paratya australiensis.
2. To determine bioaccumulation and trophic transfer of silver nanoparticles coated with Curcumin, EGCG and Tyrosine from R. subcapitata to D. carinata.
3. To identify sub-lethal effects of silver nanoparticles coated with Curcumin, EGCG and Tyrosine in R. subcapitata and P. australiensis and use biomarkers as tools to differentiate sub-lethal effects of NPs with different coatings.
4. To study the influence of ageing of silver nanoparticles coated with Curcumin, EGCG and Tyrosine in the aquatic environment on their toxicity to *R. subcapitata* and *P. australiensis*. 
Chapter 2. Literature review

This chapter has been published in the peer-reviewed literature and is presented here with only minor modifications to adjust formatting to the requirements of the thesis.


NPs are released into all ecosystems including freshwater, marine water, soil and air. However, the behaviour of NPs in the freshwater environment is likely to differ in each due to their unique environmental characteristics. For example, high salinity in the marine environment causes increased agglomeration, aggregation and precipitation of NPs which affects the bioavailability of NPs (Keller et al., 2010b, Gambardella et al., 2015, Buffet et al., 2013). High surface area increases the potentiality of ion release from NPs (Mudunkotuwa and Grassian, 2011), while higher aggregation reduces surface area for dissolution and any metal cations released from NPs are likely to be complexed by free chloride (Cl⁻) ions present in salt waters (Baker et al., 2014). Moreno-Garrido et al. (2015) report that the EC₅₀ values of NPs for marine algae species are two-fold higher than for freshwater species as per the published literature. Also, they claim that organisation for economic co-operation and development (OECD) documents on safety and toxicity tests for NPs do not have any specific references to marine water. The major source of NPs to soil is through the disposal of wastewater treatment plant (WWTP) sewage sludge, and NPs are unlikely to enter the soil in their original form due to organically rich reactive environments in the WWTPs. The attachment of NPs to soil colloids is rapid, and therefore, the mobility of NPs away from the point of source could be limited in soils (El Hadri et al., 2018). Also, the assessment of the form of NPs in soil matrices is hampered by the relative lack of procedures for their characterization compared to aqueous media (Tourinho et al., 2012, Kraas et al., 2017). Due to the variations in the fate and behaviour of NPs, mode of organism exposure in different spheres and considering the relevance to this study, this literature review was restricted to the freshwater environment only. However, this does not undermine the importance for more research related to the nanotoxicity to organisms in other environments which attract comparatively less nanecotoxicology studies (Minetto et al., 2016).
Metal oxide NPs, metal NPs and carbon nanotubes (CNT) are the most relevant materials in terms of worldwide production volumes and exposure (Bundschuh et al., 2018, Tiede et al., 2016), while the OECD has highlighted silver (Ag), zinc oxide (ZnO), titanium dioxide (TiO$_2$) and cerium dioxide (CeO$_2$) NPs as high interest due to their widespread use, commercial importance and inherent properties (Baker et al., 2014). Rocha et al. (2015) reported that 85% of toxicological studies on marine bivalves are based on inorganic NPs and only 15% are on organic NPs. Also, more than 70% of inorganic NPs examined in salt water are metal oxides and metals that mainly consist of TiO$_2$, Ag, Au, ZnO and CuO NPs (Minetto et al., 2016, Rocha et al., 2015). A large number of studies (~80%) on the effects of inorganic NPs on the organisms considered in this review also reflect the production, release and exposure risk concerns of inorganic NPs in the freshwater environment. However, a large proportion of those inorganic NPs are coated with organic capping agents. As an example, citrate and carboxylic acids are the most used reductant and capping agents in the synthesis of AgNPs (Sharma et al., 2014).

Low-throughput tests such as microcosms, mesocosms, or field scale studies are more representative of actual environmental conditions in comparison with high-throughput tests such as in vitro tests which lack environmental complexity. Therefore, using widely accepted key environmental organisms in ecotoxicology with corresponding in vivo tests is still highly regarded in environmental risk assessment of NPs. The European Chemical Agency mentions that in vitro data are relevant information for aquatic toxicity assessments, but also note that there are no EU/OECD guidelines for in vitro tests at the moment (Hjorth et al., 2017b). Therefore, the authors have restricted this review to in vivo studies. In vitro assays have a role in hazard identification, but their usefulness in environmental risk assessment is limited (SCENIHR, 2009, Mattsson and Simkó, 2017). There is a limited correlation between in vitro and in vivo toxicity results (Sharifi et al., 2012). These reasons may have influenced the large number of in vivo studies published compared to in vitro studies as reflected in this review. However, this does not undermine the importance of alternative testing strategies in nanoeffectotoxicology risk assessment since there is an ongoing discussion and proposals to use those effectively (Hjorth et al., 2017b). Also, certain aspects of NP toxicity studies such as shape-dependent toxicity are based on in vitro experiments (Sharifi et al., 2012, Forest et al., 2017).
2.1 Nanoparticles (NPs) in the environment

Several types of NPs are present in the environment and exposure to those particles is a reality. Therefore, it is important to understand the flow of NPs to the environment and exposure to assess the risks (Scown et al., 2010). Release of NPs to the environment may occur from the manufacturer through to the end user who consumes NP-enabled products (Sun et al., 2014). The majority of the products containing NPs belong to cosmetics and personal care products with sunscreen representing the dominant application (Boxall et al., 2007). A significant fraction of the NPs released to soil and air would end up in water bodies as well with cosmetics, coatings, paints and pigments contribute 89-97% of total NP emissions to water (Keller et al., 2013a). The data related to NP production, release volumes in the literature have large variations and Giese et al. (2018) provides a comprehensive summary based on data from literature, their own surveys and modelling. About 250,000 metric tonnes are released to landfills, soil and air every year (Keller and Lazareva, 2013) and it is predicted that about 69,000 metric tonnes of NPs are released globally to surface waters directly. This amount is increasing since the predicted NP production in 2019 is close to 600,000 metric tonnes with an annual growth rate of 21.1% of NP production (Vale et al., 2016). It is estimated that around 10-30% of NPs released into the environment would end up in water bodies in Asia while it is 3-17% in Europe and 4-19% in North America (Keller and Lazareva, 2013).

Usage of NPs are increasingly popular in several consumer and industrial sectors including health and fitness, home and garden, automotive, electronics, contaminant remediation and food and beverage (Vance et al., 2015, Cecchin et al., 2016). The number of inventories with catalogue products which contain NPs is rising globally (Hansen et al., 2016, Vance et al., 2015, McGillicuddy et al., 2017). Hansen et al. (2016) summarised the number of products listed yearly until 2015 in the Consumer Product Inventory (CPI, 2018) and Nanodatabase (Nanodatabase, 2018) which list products containing NPs; or are based on nanotechnology available to the European market. The number of products listed in the Nanodatabase has increased from 2231 to 3038 from 2015 to date. There are several classes of engineered NPs based on chemical composition and morphology (Kümmerer et al., 2011). Though metal oxide NPs, SiO$_2$ NPs and CNTs are the most produced worldwide (Figure 2.1), AgNPs are the most used in consumer products representing 25% of the products containing NPs (Bondarenko et al., 2013, Vance et al., 2015, Bundschuh et al., 2018). Due to their excellent antimicrobial action, Ag NPs are increasingly popular in medicines, cosmetics,
Manufacturers are not required to report the use of NPs in products except for a few NPs in some countries (eg. carbon nanotubes in USA). Also, manufacturers are not legally bound to label products that contain NPs (Kessler, 2011) or may be ignorant with respect to specific information (Giese et al., 2018). A survey conducted by Piccinno et al. (2012), found that the manufacturers are reluctant to provide production amounts with respect to NPs. As per the listed NP containing products in the Nanodatabase for 2018, the constituent NPs in 64% of the consumer products have not been disclosed (Nanodatabase, 2018) while metallic NPs (19.5%), metal oxide NPs (6.5%) and other types including organic NPs (9.5%) constitute rest of the products. The majority of organic NPs are constituted of carbon (present in 2% of products), Carbon nanotubes (2.1%), Bamboo charcoal (1.4%), graphite (0.6%), carbon black (0.5%) and fullerene (0.3%).

Figure 2.1: Annual production volumes of nanoparticles – Source: Lekamge et al. (2018a).

Emission and environmental concentration levels are mainly estimated by using material flow models following the NP life cycle (Mueller and Nowack, 2008, Boxall et al., 2007, Gottschalk et al., 2013b, Sun et al., 2014, Piccinno et al., 2012, Markus et al., 2017, Markus et al., 2016, Jiménez et al., 2016, Sun et al., 2017) and analytical methods (Gottschalk et al., 2013b, Chang et al., 2017, Aznar et al., 2017, Laborda et al., 2016a, Laborda et al., 2016b,
Majedi and Lee, 2016, Venkatesan et al., 2018, Vidmar et al., 2017, Yang et al., 2016, Gondikas et al., 2018, Folens et al., 2018, Markus et al., 2018, Hartmann et al., 2013, Gondikas et al., 2014, Chen and Ding, 2012, Astefanei et al., 2014). However, there are number of known incorrect assumptions in all the models (Giese et al., 2018). Measured field data are essential to validate predicted environmental concentrations of NPs (Bäuerlein et al., 2017). Most NP analytical studies have so far concentrated on method development, but a rise of efforts to apply these methods to measure actual concentrations in the environment is observed (Aznar et al., 2017, Bäuerlein et al., 2017, Venkatesan et al., 2018, Peters et al., 2018). However, limitations in analytical methods in discriminating engineered NPs from naturally occurring NPs have caused results of models difficult to validate (Giese et al., 2018, Gondikas et al., 2014, Wagner et al., 2014). Also, factors such as transformation of NPs in the environment, aggregation and the co-presence of dissolved ions may cause measurement of NP properties and concentrations less accurate (Majedi and Lee, 2016). The physicochemical characteristics of the surrounding environment and NP properties such as coating agent and size have a huge impact on their fate and behaviour in the environment which demands careful attention in both modelling and analytical efforts (Ellis et al., 2016a, Pu et al., 2016, Luo et al., 2018). Once released into the environment, NPs undergo transformation and change their characteristics such as size compared to their pristine form (Nowack et al., 2012). For example, NPs may agglomerate in the environment and the modelling considers agglomerates larger than 100 nm as well and thus targets the complete NP pool. In contrast, analytical methods may consider only the nanofraction and therefore, the measured concentrations may indeed be smaller than the actual concentrations. However, this was true for certain types of NPs that were measured in a recent study conducted by Bäuerlein et al. (2017) in the Dutch environment, but the measured concentrations of AgNPs were higher than the predicted concentrations in sewage treatment plant effluent.

From various sources, Gottschalk et al. (2013b) summarised predicted environmental concentrations of TiO$_2$ ($10^{-3} - 10^1$ µg L$^{-1}$), Ag ($10^{-5} - 1$ µg L$^{-1}$), ZnO ($10^{-4} - 10^{-3}$ µg L$^{-1}$), CNT ($10^{-6} - 10^{-3}$ µg L$^{-1}$), Fullerines ($10^{-5} - 10^{-4}$ µg L$^{-1}$), and CeO$_2$ NPs ($10^{-3} - 10^{-1}$ µg L$^{-1}$) in freshwater. In the year 2017, the global average predicted environmental concentrations of SiO$_2$, CeO$_2$ and Ag NPs in the freshwater were 5300.0, 7.0 and 0.3 ng L$^{-1}$, respectively and predicted to increase up to 25300.0, 46.7 and 2.1 ng L$^{-1}$ in 2050, respectively. These increased concentrations correlate with the predicted increased release of NPs into the environment (Giese et al., 2018). Based on the per capita contributions from the households, Markus et al.
(2018) estimated the concentrations of ZnO, TiO$_2$ and AgNPs in the river Dommel in Netherlands to be 1.4 µg L$^{-1}$, 1.0 µg L$^{-1}$ and 13.0 ng L$^{-1}$, respectively. Relatively few reports are available on actual application of analytical methods to determine the presence, properties and concentrations of NPs in freshwater samples collected from the environment. The concentration of nC$_{60}$ was found up to 98 ng L$^{-1}$ by using Liquid Chromatography–Mass Spectrometry (LC-MS) in surface water samples collected from a creek in Hsinchu Science Park, Taiwan (Chen and Ding, 2012). Comparatively, C$_{60}$ and C$_{70}$ concentrations were reported in using an Ultra Performance Liquid Chromatography-Tandem Mass Spectrometry (UPLC-MS) method with concentrations between 25 and 330 pg L$^{-1}$ in freshwater samples collected from several ponds located around Barcelona’s airport (Asteñaei et al., 2014). Folens et al. (2018) reported Pt NP concentrations in the range of 0.05 to 0.9 ng L$^{-1}$ by measuring with Single Particle Inductively Coupled Plasma–Mass Spectrometry (spICP-MS) in the road dust leachate of Ghent, Belgium and Gothenburg, Sweden which might be released into aquatic environment. Peters et al. (2018) reported actual environmental concentrations in the range of 0.3 – 2.5 ng L$^{-1}$ for Ag NPs, 1.3 – 5.2 ng L$^{-1}$ for CeO$_2$ NPs and 0.2 – 8.1 µg L$^{-1}$ for TiO$_2$ NPs measured using spICP-MS in the samples collected from the rivers Ijssel and river Meuse in the Netherlands. Venkatesan et al. (2018) found TiO$_2$ particle concentration in the range of 260 to 659 ng L$^{-1}$ in the Salt river, Arizona, USA by measuring with spICP-MS. The morphological features of those particles were similar to the NPs present in sunscreens. In the last decade, predicting environmental concentrations of NPs by modelling has received considerable attention, but determining actual concentrations is critical for validating those estimates, reliable risk assessment and for regulating NP industry. However, the development of real-world parameters of NPs and concentrations remain scarce due to several limitations such as lack of appropriate analytical methods and complexity of the real sample matrices (Gondikas et al., 2018).

2.2 Physicochemical characteristics of NPs on ecotoxicity

NPs exhibit unique physicochemical properties compared to their bulk counterparts. Several researchers have tried to investigate the influence of physicochemical characteristics of NPs such as size, shape, surface properties and charge that could change their toxicity to organisms. Though the results seem to be inconsistent and conflicting, they suggest that the physicochemical properties of NPs could affect the toxicity to organisms. Therefore, these
parameters need to be considered in environmental risk assessments and demand further research.

2.2.1 Size

The uptake and toxicity of NPs depend on the inherent properties of NPs and also the chemistry of the surrounding environment (Park et al., 2015). The behaviour of NPs depends on factors like size, shape, surface chemistry, surface area, functional groups, coatings, charge, aggregation, solubility, photochemistry, crystallinity and the presence of other compounds (Scown et al., 2010, Albanese et al., 2012, Shang et al., 2014, Fröhlich, 2012, Clément et al., 2013b, Hund-Rinke and Simon, 2006, Barbero and Yslas, 2016, Garner and Keller, 2014). The size of NPs determines the physicochemical properties, adsorption, distribution, metabolism and excretion in the biological systems (Qu et al., 2017). The toxicity of AgNPs is size dependent with smaller particles which are more active (Lok et al., 2007). Choi and Hu (2008) observed that the inhibition of nitrifying organisms correlated with a fraction of AgNPs less than 5 nm in size. This was achieved through examining the correlation between nanoparticle size distribution, photocatalytic reactive oxygen species (ROS) generation, intracellular ROS accumulation, and nitrification inhibition. They concluded that NPs of this size could be more toxic to bacteria than any other fractions of NPs.

Choi et al. (2008) saw no indication of internalisation of AgNPs (14 nm in size) into the bacteria Escherichia coli since internalization of NPs into bacteria cells depends on the size of the NPs, and only smaller NPs (< 10 nm in size) could enter (Morones et al., 2005). Jiang et al. (2008) found that binding and activation of membrane receptors and subsequent protein expression in mammalian cells depend on nanoparticle size. Hoecke et al. (2009) reported that the toxicity of CeO$_2$ NPs to Raphidocelis subcapitata increased with decreasing particle size. Lei et al. (2016b) observed increased toxicity of zerovalent iron (nZVI) NPs to Chlorella pyrenoidosa with decreasing particle size. Hartmann et al. (2010) studied the ecotoxicity of three TiO$_2$ NPs with different sizes, to the alga Raphidocelis subcapitata. They found that the smallest particle type (< 10 nm) resulted in higher inhibition than the other two types (3 - 300 nm). Kim et al. (2010) investigated the effects of particle size of TiO$_2$ NPs on Daphnia magna. They showed that the particle fraction in between 400 nm and 800 nm increased antioxidant enzyme activities in comparison with the NPs which were less than 400 nm in size. Cui et al. (2017) reported that the longer Ag nanowires (NWs) (20 µm) were more toxic to Daphnia magna and Daphnia galeata than those that were shorter (10 µm). Similarly, Chae and An
(2016) showed that larger Ag nanowires (AgNW) (20 µm) were more toxic to aquatic organisms than smaller ones (10 µm) by exposing *Chlamydomonas reinhardtii* and *Daphnia magna* and to Ag NWs.

However, Matzke et al. (2014) observed no clear cut relationship between NP toxicity and size of NPs when the bacterium *Pseudomonas putida* was exposed to AgNPs. Li et al. (2010a) studied the effects of three different sized (36, 52 and 66 nm) Ag NPs but concluded that the toxicity was not a function of size possibly due to the large degree of aggregation of NPs in synthetic freshwater. Also, Lopes et al. (2014) studied the effects of ZnO NPs with two different particle sizes (30 and 80 - 100 nm) and ionic Zn. They found that the acute toxicity of ZnO NPs did not depend on particle size. Iswarya et al. (2017) exposed the alga to polyvinylpyrrolidone (PVP)-coated Au NPs in different sizes but observed no size-dependent toxicity. However, the toxicity of citrate-coated Au NPs depended on the size with the smaller particles being less toxic. The smaller-sized NPs reacted rapidly with the substances in the solution causing aggregation which may have caused less toxicity. Wiench et al. (2009) reported that the acute toxicity to *Daphnia magna* was independent of particle size, type of coating, aggregation of particles or the type of medium for TiO$_2$ and ZnO NPs.

2.2.2. Shape

Peng et al. (2011) observed that rod-shaped zinc oxide NPs (ZnO NPs) were more toxic to the alga *Phaeodactylum tricornutum* than sphere-shaped NPs. Bacchetta et al. (2018) observed higher internalization of spherical- and tube-shaped CNTs into *Daphnia magna* compared to the cubic NPs. They also reported that NP shape influenced the severity of pathogenesis with cubic NPs being more effective in terms of physical damage and cellular degeneration. Liu et al. (2018b) observed that star-shaped Au NPs were more toxic to the fungus *Aspergillus niger, Mucor hiemalis* and *Penicillium chrysogenum* compared to the toxicity of spherical-shaped ones. Also, the toxicity of star-shaped NPs increased with smaller sizes. Nasser et al. (2016) suggested that shape and charge played an important role in the toxicity and uptake of Au NPs to *Daphnia magna*. Abramenko et al. (2018) observed higher toxicity of spherical shaped Ag NPs to *Danio rerio* embryos compared with Ag nano-plates. In contrast Dai et al. (2015) saw no effect of NP form or shape on the toxicity of CuO NPs to *Capitella teleta*. Also, Silva et al. (2014a) claimed that particle shape did not contribute to the toxicity of organo-coated Ag NPs to *Escherichia coli* and *Daphnia magna*. Chauhan et al. (2011) claimed that the rod-shaped CdSe/CdS NPs penetrated tumour cells more rapidly than
spherical NPs. Truong et al. (2015) suggested that nonspherically-shaped, such as filamentous, discs or wormlike NPs were better as drug delivery carriers. However, Chithrani et al. (2006) observed higher uptake of spherical-shaped Au NPs into mammalian cells than the rod-shaped Au NPs.

2.2.3 Surface properties

Though NP size still remains central in determining toxicity, studies suggest that other inherent factors like coating agents should be considered in toxicity studies (Silva et al., 2014b). The role of the surface properties of NPs is poorly understood and cannot be generalized to determine the risks (Baumann et al., 2014, Saei et al., 2017). Surface properties of NPs are key factors in determining behaviour of NPs; multiple types of surface ligands pose new challenges in understanding the toxicity of NPs (Yu et al., 2013). NPs are highly reactive because of their large surface area. The surface chemistry and reactivity of NPs determine their interactions with the surface lining layers of biological tissues (Hoet et al., 2004) and transfer of NPs to higher levels through the food web (Geitner et al., 2016). Many NPs which are in development are complex and carry different coatings which can alter their surface properties (Daima et al., 2014). Currently many different types of compounds are being used as capping agents in commercial NP production (Lekamge et al., 2018a). The physicochemical characteristics of these different coatings lead NPs to behave differently in the environment. Different ligands impart different chemical properties and affect charge, particle size, surface area and aggregation of NPs (Elsaesser and Howard, 2012, Lapresta-Fernández et al., 2012, Rana and Kalaichelvan, 2013, Cupi et al., 2016b). NPs are stabilized against aggregation and other chemical reactions like oxidation and sulfidation through adsorption or covalent attachment of organic compounds which provide electrostatic, steric or electrosteric repulsive forces between particles (Phenrat et al., 2008, Hotze et al., 2010). However, the impacts of different coatings on toxicity have been scarcely explored (Dominguez et al., 2015). It was shown that fullerene can cause oxidative damage in mammalian cells, and their toxicity is related to lipophilicity; reduction of lipophilicity with modification of the surface of fullerene by introducing aliphatic and hydroxyl groups resulted in reduced toxicity (Colvin, 2003, Sayes et al., 2004). It has been reported that uncoated colloidal fullerenes may damage the brain of largemouth bass (Oberdörster, 2004). Iron oxide NPs coated with ascorbate and dextran have been shown to be more toxic to the freshwater cladoceran Daphnia magna in comparison with the same NPs coated with citrate and polyvinylpyrrolidone (Baumann et al., 2014). Bozich et al. (2014) found
that both the type of ligand and the charge of the NP surface affected the toxicity of Au NPs to *Daphnia magna* at acute and chronic level. Bone et al. (2012) found that the Ag speciation from AgNPs varied significantly by coating type (gum Arabic and polyvinylpyrrolidone) and the presence of plants (*Potamogeton diversifolius* and *Egeria densa*) in the medium, which reduced the toxicity of NPs to *Daphnia magna*. Interestingly, the fate and behaviour of NPs are changed by organisms as well. Adeleye and Keller (2016a) observed charge reversal and change of surface properties of TiO$_2$ NPs by the extracellular polymeric substances (EPS) produced by *Chlamydomonas reinhardtii*. The presence of EPS may affect the bioavailability of NPs, their interactions with organisms and overall effects. Therefore, the authors suggested that the fate and effects of NPs cannot be simply predicted by the physicochemical characteristics of NPs.

### 2.2.4 Charge

The surface charge of NPs measured as zeta potential contributes to the adhesion of NPs on cell surfaces and hence is important in the toxicity of NPs. The NPs with the highest positive charge are the most toxic to the algae cells. Algal cells, having a negative charge on their surface, attract positive NPs to neutralize the charge, and this causes surface alterations resulting in cell death (Karunakaran et al., 2015). El Badawy et al. (2010b) observed a surface charge-dependent toxicity of Ag NPs to bacteria (*Bacillus* sp.) when they were exposed to four different Ag NPs with different surface charges. Dominguez et al. (2015) showed that different types of coatings and the charge of NPs had an impact on ROS formation and gene expression in *Daphnia magna*. Nasser et al. (2016) suggested charge played an important role in toxicity and uptake of Au NPs to *Daphnia magna*.

### 2.3 Effects of the surrounding environment on NP toxicity

OECD (2014) highlights the importance in identifying transformation, degradation and dissolution in the characterization of NPs in toxicity tests (Cupi et al., 2016a). Transformation of Ag NPs affects their surface properties, transportation, reactivity and toxicity in the environment (Xiu et al., 2011, Liu et al., 2010, Levard et al., 2011b, Levard et al., 2011a). It is important to further assess the effects of the transformed NPs as well as fresh NPs to clearly understand how the transformation of NPs in the aquatic environment affects organisms (Levard et al., 2012). Biological systems have not evolved in the presence of ENPs which are produced today and hence, the lack of knowledge about transport and fluxes of such particles
present problems (Hoet et al., 2004, Dowling, 2004). Generally, abiotic factors like pH, salinity, hardness of water and chemical oxygen demand (COD) influence the aquatic toxicity of chemicals (Li et al., 2013a, Fabrega et al., 2011). The fate and toxicity of NPs in the aquatic environment are governed by physicochemical pathways which include aggregation and subsequent sedimentation, dissolution, adsorption to particulate and other solid surfaces, stabilization via surfactants and binding to natural organic matter (NOM) (Wang et al., 2016b, Boncel et al., 2015, Apul and Karanfil, 2015, Köser et al., 2017, Ellis et al., 2018). Biological degradation, abiotic degradation, oxidation and reduction could also be of concern in some aquatic environments (Batley and McLaughlin, 2007). It was reported that the surface coatings change or are replaced with new coatings during their transit in water (Jarvie and King, 2010).

Less is known about the comparative toxicity of metallic NPs and their ionic forms (Wang et al., 2016a). It has been found that NPs release ions into water over time and the rate and the degree of dissolution depend on their surface functionalization. Therefore, the biological toxicity of aged and freshly prepared NPs differ (Kittler et al., 2010). Strigul et al. (2009) studied the toxicity of boron NPs (B NPs) to Daphnia magna. Depending on the age of the test solution, the calculated 48 h LC$_{50}$ values for B NPs ranged from 56 to 66 mg L$^{-1}$, and the difference in toxicity was attributed to dissolution of NPs releasing free ions. Once released into the environment, the toxicity also depends on the oxidation state of the NPs (Conway et al., 2015). Lei et al. (2016b) found that the toxicity of nZVI to the alga Chlorella pyrenoidosa decreased after NPs was aged for 3 months in the medium, in comparison with the toxicity of fresh NPs. They attributed this to the surface oxidation of the NPs.

2.3.1 Media and exposure system

Abiotic factors like pH, salinity, water hardness, temperature, different organic ligands and the components in the media affect the ecotoxicity of NPs (Handy et al., 2008, Jin et al., 2010, Djurišić et al., 2015, Yung et al., 2017b). The fate and transport of NPs in aquatic systems largely depend on the chemical characteristics of water (Garner and Keller, 2014). Physicochemical factors in freshwater are different from brackish or sea water. Therefore, the behaviour and effects of NPs identified in one medium cannot readily be applied to other media.

Li et al. (2011a) assessed the toxicity of ZnO NPs to Escherichia coli in 5 different media (ultrapure water, 0.85% NaCl, phosphate-buffered saline (PBS), minimal Davis (MD) and Luria Bertani (LB)). They observed different toxicity levels in a range of media and recommended that attention be paid to the physicochemical characteristics of NPs and media.
in bacterial toxicity tests. Li et al. (2013) found that the toxicity of ZnO NPs to *Escherichia coli* depended on the dissolution of NPs. Interestingly, toxicity was reduced by the presence of Cu\(^{2+}\) and Mg\(^{2+}\) in the medium which could compete with toxic Zn\(^{+}\) ions for binding sites on the organisms. Lopes et al. (2012) observed higher bacterial toxicity in MilliQ water than in ASTM hard water which may be due to the interference of ions in ASTM hard water causing higher aggregation. von Moos et al. (2015) exposed *Chlamydomonas reinhardtii* to CuO NPs (10 mg L\(^{-1}\)) in five different exposure media. They observed that the media was decisive in determining toxicity regardless of the effects from NPs or ions. Similarly, Aravantinou et al. (2015) observed that the different sensitivity of the algae *Chlorococcum sp.* and *Scenedesmus rubescens* to ZnO NPs strongly depended on the algae medium. Zhang et al. (2016b) observed that media chemistry had profound effects on aggregation, dissolution and toxicity of TiO\(_2\), ZnO and Ag NPs and CNTs to *Chlorella pyrenoidosa*. Seo et al. (2014) observed different toxicity levels in different media (ISO and moderately hard water (MHW)) when *Daphnia magna* was exposed to Ag, Cu and Zn NPs. Though the dissolution rate of NPs was higher in ISO medium, the toxicity was highest in MHW. Muna et al. (2017) found increased total Cu body burden from Cu NPs after exposing *Daphnia magna* in natural freshwater compared with OECD202 artificial medium. The Cu body burden in daphnids in natural freshwater bodies may be higher than laboratory predictions carried out using artificial media. Also, the total Cu body burden was higher in daphnids exposed to Cu NPs than Cu salt. Hu et al. (2017) reported higher toxicity of AgNPs and AgNO\(_3\) to *Daphnia magna* in M4 medium in comparison with the surface water. For both forms of Ag, daphnids took up less and depurated more in the surface water. The authors suggested a reduced toxicity for the observation. However, Salieri et al. (2015) did not observe any significant influence of test media on toxicity of TiO\(_2\) NPs to *Daphnia magna*. They believe this may be due to fast and strong agglomeration of NPs in all media, creating secondary particle size in the micrometre range. They did however report that the exposure volume of the medium had a significant influence on toxicity.

Nicolas et al. (2016) conducted standard algal growth inhibition tests (OECD, 2011a) with *Raphidocelis subcapitata* to test how the exposure system (24-well microplate, cylindrical vials and Erlenmeyer flasks) influenced the toxicity of TiO\(_2\) NPs. They found that the exposure system significantly affected the results and recommended attention be paid during the algal growth inhibition test. Sørensen and Baun (2015) exposed the alga *Raphidocelis subcapitata* to AgNPs and AgNO\(_3\) for 2 h and 48 h in standard algal toxicity tests. Similar toxicity levels were observed for Ag\(^{+}\) ions in the two tests, whereas the toxicity of AgNPs was less toxic in 2
Interestingly, ageing AgNPs in the medium for 48 h before performing the 2 h test increased the toxicity while ageing beyond 48 h prior to testing reduced the toxicity. Xiao et al. (2018) observed higher toxicity in a dynamic exposure system with a vibration speed of 140 rpm in comparison with static exposure when *Daphnia magna* was exposed to CuO NPs. The aggregation of NPs in the dynamic system was less, and therefore, they hypothesized that the reduced toxicity may be due to the lower hydrodynamic diameter (HDD) of NPs. Sørensen et al. (2016b) claimed that the acute toxicity of AgNPs and CuO NPs to *Daphnia magna* after pulse exposure (1 – 2 h) was comparable to the effects levels of 24 h continuous exposure. They attributed this to rapid toxicokinetic and toxicodynamic features of NPs causing the same level of toxicity following a few hours of exposure, concluding that the dissolved fractions of NPs are responsible for the toxicity. With this, they suggested that the use of pulse exposure was more environmentally relevant for NP toxicity assessments than standard continuous exposure tests.

Media critically influence the toxicity of NPs; this is due to several reasons. The physicochemical characteristics of the medium affect the fate and behaviour of NPs. The constituents of the medium may react with dissolved ions from NPs causing complexation and aggregation or compete with them for binding sites on the organisms. In addition, an organisms’ sensitivity and response to NP exposure also depends on the medium. Due to these reasons, the toxicity of NPs in one medium cannot be readily applied to other media. Other than the media, the outcome of toxicity testing of NPs is highly influenced by the test duration, the time from the moment NPs are added to the test medium, dynamic vs static exposure, and pulse vs continuous exposure.

### 2.3.2. Natural organic matter

Studies suggest that the presence of some organic and inorganic substances in the medium could change the properties of NPs which contribute to determining the fate and toxicity of NPs (Metreveli et al., 2016, Gunsolus et al., 2015, Wang et al., 2018, Luoma et al., 2016). NPs may be more stable in natural waters than in synthetic waters where no NOM is present (Batley and McLaughlin, 2007). NOM present in media could form a layer on NPs and increase the stability of NPs (Baalousha and Lead, 2013, Omar et al., 2014). Once released to the aquatic environment, NOM coated on NPs changes the reactivity and bioavailability of NPs to organisms (Aiken et al., 2011). Also, DOM may promote the mobility of the NPs in the aquatic environment (Ren et al., 2017). Liberation of ions from NPs is influenced by the
presence of NOM in water (Wang et al., 2015). Jiang et al. (2015) observed that NOM affected the dissolution kinetics of ZnO NPs and found that the dissolution rate constants and dissolved Zn concentrations increased with increased NOM concentration. In addition, they found that the aromatic carbon content of NOM played a key role in promoting dissolution. Li et al. (2016) studied the effects of DOM in the medium on the generation of ROS and the acute toxicity of metal oxide NPs to *Escherichia coli*. They observed that different photo-reactivity of humic and fulvic acids resulted in different effects on ROS generation and acute toxicity of NPs. Seitz et al. (2015b) found that the pH and dissolved organic matter (DOM) in water considerably influenced the acute and chronic toxicity of Ag NPs to *Daphnia magna*. Xiao et al. (2018) observed that the toxicity of CuO NPs to *Daphnia magna* was mitigated in the presence of DOM. There are different views about the effects of NOM on the toxicity of NPs. However, NOM have been shown to influence the stability, dissolution, reactivity, bioavailability and mobility of NPs which directly or indirectly affected the toxicity.

2.3.3. Sulfidation

In natural waters, AgNPs will preferentially transform to Ag$_2$S or AgCl as per the thermodynamic constraints. Also, the transformation will depend on pH and redox potential (Eh) and the composition of natural waters; by knowing those values, it is possible to predict the speciation of Ag in simple systems. Under aerobic conditions, formation of AgCl species is predicted, but under anaerobic conditions, sulfidation is predicted (Levard et al., 2012). Bioavailability and toxicity of ions change with sulfidation and it was found that the toxicity of Ag$^+$ ions to *Daphnia magna* decreased by about 5-fold in the presence of environmentally relevant levels of sulphide (Bianchini and Wood, 2008, Bianchini et al., 2002). Guo et al. (2017) observed that the toxicity depended on sulfidation rate when the bacteria *Escherichia coli* was exposed to Ag NPs. Reinsch et al. (2012) observed decreasing toxicity of Ag NPs to *Escherichia coli* with increasing sulfidation (Ag$_2$S:Ag$^0$ ratio).

2.3.4 Other factors

The interactions between NPs and bacteria can be affected by several other factors such as the pH and ionic strength of the medium and the photocatalytic activity of NPs under different irradiation conditions (Djurišić et al., 2015). Pagnout et al. (2012) observed toxicity changed with different electrolytes (NaCl, CaCl$_2$, Na$_2$SO$_4$) in the medium when *Escherichia coli* was exposed to TiO$_2$ NPs. Also, they observed that the toxicity changed with pH which
may cause changes in the surface charge of NPs resulting in different interactions between bacteria and NPs. Bhuvaneshwari et al. (2015) observed increased toxicity of ZnO NPs to *Scenedesmus obliquus* under UV-C irradiation compared with that under visible light. They ascribed this to increased ROS production in UV-C irradiated algal cells compared with cells under other irradiation conditions. Sendra et al. (2017a) reported a significant increase in the toxicity of TiO$_2$ NPs and bulk TiO$_2$ under UV-A irradiation with comparison to that observed under other irradiation conditions. Ratti et al. (2016) observed light enhanced antimicrobial activity of NPs when *Escherichia coli* and *Bacillus subtilis* were exposed to AgNPs. Lee and An (2013) exposed *Raphidocelis subcapitata* to ZnO and TiO$_2$ NPs under visible, UV-A and UV-B irradiation conditions. Though the growth of algae was inhibited under all conditions, there was no significant toxicity difference among the light conditions.

Physicochemical characteristics of NPs alter upon environmental release with time under the influence of the surrounding environment, thereby affecting their impact on organisms. Several environmental factors such as media composition, exposure scenario, sulfidation, irradiation, pH and ionic strength of media influence the toxicity of NPs. Most ecotoxicological studies to date have focused on the effects of as-prepared NPs on organisms (Lekamge et al., 2018a); few studies have evaluated the effects of the transformation of NPs on toxicity. More studies focusing on this aspect which are biologically and environmentally relevant are warranted.

### 2.4 NP stability and aggregation

Aggregation, sulfidation and oxidation are examples of changes that could happen to varying degrees (Fortner et al., 2005, Brant et al., 2005b, Teeguarden et al., 2007, Garner and Keller, 2014, Conway et al., 2015). Size and aggregation are the crucial factors in determining the ecotoxicity of carbon NPs, while solubility and speciation determine the toxicity of metal oxide NPs (Blinova et al., 2010). The degree, kinetics and size range of aggregates depend on the characteristics of the particles, the characteristics of the environment and the concentrations of the particles (Phenrat et al., 2007, Hyung et al., 2007). Negatively charged NPs are electrostatically stabilized when the negative charge is strong enough to repel NPs from each other to overcome attractive forces. However, the presence of counterions in the solution will reduce the repulsive forces resulting in decreased stability. Several researchers provided supportive evidence for this phenomenon claiming that the different ionic strengths of the environment affect the aggregation and stability of NPs (El Badawy et al., 2010b, Li et al.,
2010b, Liu et al., 2011, Delay et al., 2011). Even a slight increase in salinity decreases colloids by particle aggregation and precipitation (Stolpe and Hassellöv, 2010). The ionic strength of freshwater systems ranges from 1 to 10 mM and that of seawater is about 700 mM (Levard et al., 2012). However, there is a tendency for less aggregation when NPs are stabilized sterically other than solely by surface charge. Attachment of certain polymers causes steric stabilization and several researchers demonstrated that adsorption of compounds in natural waters induced steric forces that resist aggregation (Fabrega et al., 2009, Delay et al., 2011, Chinnapongse et al., 2011, Cumberland and Lead, 2009). Polyelectrolytes exhibit additional electrosteric forces in addition to steric stabilization which makes them excellent in stabilizing NPs (Badawy et al., 2010).

Aggregation is a crucial factor in determining NP toxicity. In general, the majority of studies support the idea that the aggregation of NPs reduces the toxicity to organisms though some researchers have claimed otherwise. Several researchers reported a correlation between aggregation of NPs and their toxicity to isolated strains of bacteria (Kvitek et al., 2008, Lok et al., 2007, Bradford et al., 2009). They demonstrated that aggregation mitigates the potential toxicity of NPs. It is generally accepted that aggregation reduces toxicity to aquatic organisms. Low environmental concentrations lead to less aggregation, and hence, unlike traditional toxicants, it is possible that low concentrations are more toxic than higher concentrations with time (Tiede et al., 2009). Lok et al. (2007) observed higher aggregation in high salt media resulting in loss of antibacterial activities of AgNPs to *Escherichia coli*. Fernandes et al. (2006) suggested that NPs would disaggregate in the presence of household or industrial detergents. Limbach et al. (2008b) found that protein breakdown products and surfactants in wastewater change the zeta potential of NPs causing stabilization. Oleszczuk et al. (2015) found that certain surfactants decrease the toxicity of TiO$_2$ NPs to *Daphnia magna*, and they hypothesized that the surfactants increase the aggregation of NP, reducing the bioavailability to daphnids. In contrast, there are instances where higher toxicity was observed with the aggregation of NPs. In one such study, Kashiwada (2006) found increased salinity caused higher toxicity by NPs to medaka eggs though NPs showed higher aggregation in saline media.

### 2.5 Influence of NPs on other contaminant effects

There is also evidence that NPs influence the toxicity of other contaminants (Deng et al., 2017) and that influence is mitigated by the characteristics of the aquatic environment, such as the presence of organic matter. Fan et al. (2016) observed a reduction in Cu accumulation
in *Daphnia magna* in the presence of TiO$_2$ NPs, but humic acids decreased that reducing effect. In a similar study, Rosenfeldt et al. (2015) observed a two-fold decrease in Cu toxicity to *Daphnia magna* in the presence of TiO$_2$ NPs in the medium. They attributed this to the adsorption of Cu to NPs leading to a reduction in the bioavailability of Cu as the cause of toxicity reduction. In another study, Liu et al. (2015) found that TiO$_2$ NPs increased Cu accumulation in *Daphnia magna*, while TiO$_2$ nano-sheets decreased Cu accumulation. Interestingly, the presence of Cu$^{2+}$ ions in the medium caused agglomeration and sedimentation of TiO$_2$ NPs causing decreased NP bioaccumulation. Hartmann et al. (2010) investigated the toxicity of cadmium (Cd$^{2+}$) ions in the presence of TiO$_2$ NPs. Toxicity from Cd was reduced in the presence of TiO$_2$ NPs compared to Cd alone due to the decreased bioavailability of Cd resulting from the sorption of Cd to NPs. Kim et al. (2016) observed decreased bioaccumulation of Cu while both acute toxicity and bioaccumulation of Cd increased in the presence of citrate coated Ag NPs after 24 h exposure of *Daphnia magna*. Simon et al. (2015) observed a reduction in the toxicity of triclocarbon to *Daphnia magna* in the presence of multi-walled carbon nanotubes (MWCNT). In contrast, Wang et al. (2014) observed increased toxicity of Nickel (Ni) to *Daphnia magna* in the presence of hydroxylated MWCNTs. They found that this was due to the uptake of Ni-adsorbed NPs. NPs could also influence the multixenobiotic resistance (MXR) of aquatic organisms. Georgantzopoulou et al. (2016) reported similar findings when they exposed *Daphnia magna* to Ag NPs. Zhang et al. (2017) evaluated the joint toxicities of TiO$_2$ NPs with four different organochlorine contaminants (OC) towards the alga *Chlorella pyrenoidosa*. The results indicated that there were synergistic, antagonistic and additive effects between TiO$_2$ NPs and OCs on the alga. Similarly, Yu et al. (2018) reported synergistic and antagonistic effects of TiO$_2$, SiO$_2$ and AgNPs and CdTe/CdS QDs on Cd$^{2+}$ ion toxicity towards alga *Chlamydomonas reinhardtii*. Li et al. (2017a) also observed increased toxicity of Cd$^{2+}$ ions to *Daphnia magna* in the presence of TiO$_2$ NPs. These studies show that the NPs influence the effects of existing environmental contaminants on organisms and therefore highlight the importance of systematic studies of toxicological effects of NPs due to their own effects plus their influence on other contaminant effects in environmental risk assessment.

2.6 The toxicity of NPs to freshwater organisms

Human and industrial wastes enter waterways, and hence, it is inevitable that NPs also end up in water bodies due to the mass use of products containing NPs (Daughton, 2004,
Moore, 2002). Ingestion and inhalation are considered as the major routes of NP uptake by terrestrial organisms (Dowling, 2004, Warheit, 2004, Moore and Allen, 2002). In addition, there might be other routes of exposure in aquatic organisms, such as uptake through gills and surface epithelia (Moore, 2004, Oberdörster, 2004). Once internalized into invertebrates, the gut epithelium, the cellular immune system and the hepatopancreas are likely targets for reactive mechanisms, while the liver is a probable target in fish (Moore, 1990, Moore et al., 2004). Eukaryotes have developed advanced mechanisms, endocytosis (100 nm or less) and phagocytosis (100 nm – 10,000 nm) for cellular internalization of particles (Na et al., 2003, Pelkmans and Helenius, 2002, Moore, 2006). Contamination of waterways is not only harmful for aquatic biota but also to terrestrial organisms including humans by direct or indirect exposure to NPs via direct ingestion, inhalation of water aerosols, skin contact or food (Daughton, 2004). The ability of water treatment plants to treat NPs is still doubtful, and in particular, uncharged or anionic NPs could pass through into the sewage effluent. Also, some studies showed that there is a potential for NPs to harm important bacteria in sewage treatment plants (Choi et al., 2008, Kwak et al., 2001, Ghafari et al., 2008, Nyberg et al., 2008) which may put freshwater aqueous ecosystems under threat from other contaminants.

Metallic NPs have the potential to dissolve and release ions into the aquatic media. Some researchers claim that these liberated ions are the only cause of toxicity to aquatic organisms, while other studies indicate that NPs are the major cause of toxicity (Li et al., 2017b, Abramenko et al., 2018). This debate is still prevalent though the effects of metallic NPs have been intensively studied in the past decades (Wang et al., 2016a). In general, the toxicity of NPs is compared to the toxicity of the counterpart bulk material, usually metal salts to test this hypothesis (Djurišić et al., 2015). Evaluation of acute and chronic toxicity and mechanism of toxicity is crucial in environmental risk assessment of NPs in protecting the organisms and setting up guidelines. A considerable number of studies have been undertaken to date in assessing the toxicity of different NPs and the sensitivity of organisms to NPs and on the mechanisms of toxicity.

As primary producers, algae are a key functional group of organisms. Alterations of the composition of algal community due to environmental stressors may affect the structure and functioning of the whole ecosystem. Their fast growth rate and high sensitivity to toxicants make them a suitable model organism in ecotoxicological studies. Also, the algal tests are less expensive, simple and easy to perform (Nyholm and Källqvist, 1989). Invertebrates are the
most widely distributed organisms on the earth. Their presence in almost all ecological niches, fast and high rate of reproduction, short life span and relatively high sensitivity to pollutants make them excellent candidates for ecotoxicological studies. Small adult body size, transparent bodies and stereotyped behaviour with easily recognizable disruption are some other advantages. Tests with invertebrate are cost-effective, fast and easy to perform with reproducible results. Invertebrates are able to uptake NPs by different ways such as direct ingestion, from contaminated prays, water filtration, inhalation, and body surface contact.

2.6.1 The toxicity of NPs to hydra

_Hydra spp._ is a commonly used model organism for developmental and environmental studies (Karntanut and Pascoe, 2002, Ambrosone et al., 2012). Tests with Hydra are fast, reliable and less expensive. Also, they provide a wide repertoire of responses and therefore, a suitable organism for ecotoxicological studies (Ambrosone et al., 2012). The clubbing movements of tentacles are early signs of exposure to toxic agents, before reproductive changes or death, while differentiation into true hermaphrodite and sexual reproduction are determined by unfavourable conditions (Blaise and Kusui, 1997, Holdway, 2005). Ambrosone et al. (2012) observed clear signs of cytotoxicity to _Hydra vulgaris_ upon exposure to CdTe QDs and the effects were more potent than dissolved Cd$^{2+}$ ions. The reproduction, regeneration and cell proliferation were affected while genotoxic effects such as chromosomal fragmentation, alterations in gene expression profiles were also exerted. In a similar study, Ambrosone et al. (2014) observed morphology alterations, paralysis of the gastric region, disorganization and depletion of tentacle specialized cells, increase of apoptotic and collapsed cells and reduction of the epithelial cell proliferation rate in _Hydra vulgaris_ upon exposure to SiO$_2$ NPs. Interestingly, several genes, mostly involved in stress response and cuticle renovation were differentially expressed. The results showed that organisms were able to rebalance the animal homeostasis up to a relatively high dose of SiO$_2$ NPs, and that the physiological modifications are transduced to gene expression modulation. Exposure of _Hydra vulgaris_ to rod-shaped CdSe/CdS semiconductor NPs resulted in Ca$^{2+}$ dependent tentacle-writhing behaviour (Malvindi et al., 2008). However, spherical NPs with same composition, but without surface functionalization did not induce any behavioural changes suggesting the unique shape-tunable electrical properties of the surface-functionalized and rod-shaped NPs caused the neuronal stimulation. In a similar study, Tortiglione et al. (2009) observed positively charged, amino-PEG coated CdSe/CdS semiconductor NPs were actively internalized by the tentacle and body
ectodermal cells of *Hydra vulgaris*. However, negatively charged NPs were not uptaken. Marchesano et al. (2015) exposed *Hydra vulgaris* to pristine and chemically modified multi-layer fullerenes (0.05 to 0.1 mg L\(^{-1}\)) and observed no effects to behaviour, morphology, development and reproductive capability of the organism. Similarly, Yeo and Kang (2010) found no significant biological toxicity of Zn-doped TiO\(_2\) NPs to *Hydra magnipapillata*. However, morphological damage to the tentacles, deformity of the basal disc and a thin or dissolved gastric region were observed upon exposure to both the Zn-doped TiO\(_2\) or pure TiO\(_2\) NPs under UV-A photocatalysis conditions. The damages were not linked to the NPs, but rather due to the photocatalytic reaction.

2.6.2 The toxicity of NPs to algae

Freshwater microalgae are primary producers in the environment and hence carry out a pivotal role in the food chain. Therefore, any abnormal structural or population changes of the organism will affect higher organisms which directly or indirectly feed on them (Nyholm and Peterson, 1997). This highlights the importance of assessing any causes for such changes and the effects due to such causes. Their main habitats, freshwater bodies, are always under threat of chemicals released by households and industries. Also, toxicity tests with algae are recommended internationally by organizations as a source of basic information to understand environmental hazards (OECD, 2011a, ASTM, 2012).

Most acute toxicity tests have been performed over 72 h (> 40%), a time recommended for algae by the OECD; 18% and 15% of studies report 96 h and 48 h toxicity tests, respectively. Several methodologies have been used to measure the acute toxicity of NPs to algae, although growth inhibition has been predominantly used. Growth inhibition can be evaluated using several techniques and methodologies including cell counting, ATP measurement, optical density measurement and chlorophyll content. Other endpoints assessed include membrane damage, oxidative stress, uptake, accumulation, cell morphology, mitochondrial dysfunction, cellular growth and metabolic profiling (Lekamge et al., 2018a). *Raphidocelis subcapitata* (31%), *Chlamydomonas reinhardtii* (24%), *Chlorella pyrenoidosa* (12%), *Chlorella vulgaris* (8%) and *Euglena gracilis* (8%) have been the most preferred species in NP studies while most studies on algae have assessed the effects of AgNPs (24%) followed by TiO\(_2\) (23%), ZnO (11%) and CeO\(_2\) NPs (8%) (Lekamge et al., 2018a).
Lee and An (2013) exposed the alga *Raphidocelis subcapitata* to ZnO NPs and concluded that the observed toxicity was almost entirely caused by the dissolved free Zn$^{2+}$ ions. Li et al. (2015b) exposed the alga *Euglena gracilis* to AgNPs in the presence and absence of cysteine which is a strong Ag ligand. The effects of NPs on photosynthesis decreased in the presence of cysteine suggesting that the effects of AgNPs were mediated by the dissolved Ag$^+$ ions. Müller et al. (2016) exposed the alga *Chlamydomonas reinhardtii* to Cu NPs and corresponding dissolved fraction of Cu$^{2+}$ ions and observed that the toxicity was similar. Also, when the same experiments were performed in the presence of ethylenediaminetetraacetic acid (EDTA), which is a strong metal ion chelator, the toxicity of both NPs and Cu$^{2+}$ ions decreased. These results indicated that the toxicity of Cu NPs arises mostly from the dissolved fraction of Cu$^{2+}$ ions. Despite Zn$^{2+}$ ions being toxic, Iswarya et al. (2017) saw a reduction in toxicity of Au NPs to the alga *Scenedesmus obliquus* with the addition of Zn$^{2+}$ ions to the medium. Navarro et al. (2008b) examined the short-term toxicity of Ag$^+$ ions and AgNPs to photosynthesis in *Chlamydomonas reinhardtii*. They found that the toxicity of Ag$^+$ ions in terms of EC$_{50}$ was about 18 times higher compared to AgNPs. However, the observed toxicity by AgNPs could not be fully explained relative to the Ag$^+$ ions measured in the AgNP suspension, and the toxicity of Ag NPs appeared to be much higher when compared as a function of Ag$^+$ ion concentration. When the alga *Raphidocelis subcapitata* was exposed to AgNPs, the toxicity from 2 h to 48 h did not increase at the corresponding ionic release rate. Also, the addition of cysteine in equimolar concentrations to Ag did not eliminate toxicity. Therefore, Sørensen and Baun (2015) suggested that the dissolution cannot be the only process which contributes to the algal toxicity.

Angel et al. (2015) found that the presence of dissolved organic carbon (DOC) reduced the toxicity of NPs to *Raphidocelis subcapitata*. The presence of DOC substantially reduced the sorption of NPs to the algal cells, and therefore, they concluded that sorption was the cause of the toxic mechanism. However, though they stopped ROS generation by using UV filters, the toxicity observed was similar to the levels when ROS was present. They concluded, in contrast to many other findings that the toxicity was not caused by localized exposure to ROS. Rogers et al. (2010) assessed the effects of CeO$_2$ NPs and CeO$_2$ macro-particles ($<$ 5 µm) to *Raphidocelis subcapitata*. They concluded that the effects were due to membrane damage of cells by lipid peroxidation caused by the production of hydroxyl radicals. Sørensen et al. (2016a) observed growth inhibition in *Raphidocelis subcapitata* and *Chlamydomonas reinhardtii* following exposure to Pt NPs and attributed toxicity to oxidative stress caused by
ROS production. Higher body burden of NPs was found in *Raphidocelis subcapitata*, possibly due to favoured binding of NPs to the polysaccharide-rich cell wall. Interestingly, the accumulation of intracellular ROS levels was comparatively less in *Raphidocelis subcapitata* though it was the most sensitive species. Membrane damage was not observed in both algae species. Bhuvaneshwari et al. (2015) noted significant toxicity correlated with intracellular ROS generation in the alga *Scenedesmus obliquus* when exposed to ZnO NPs. Substantial membrane damage and a significantly enhanced lactate dehydrogenase enzyme release into the medium were also observed. Nogueira et al. (2015) exposed the alga *Raphidocelis subcapitata* to graphene oxide NPs and observed increased ROS production and membrane damage in algal cells which was suggested as the cause of observed growth inhibition. Oukarroum et al. (2017) suggested that several cellular alterations, such as the inhibition in cellular division processes, the deterioration of photosynthetic apparatus and the generation of ROS, caused the cell viability in alga *Chlorella vulgaris* to decrease when exposed to NiO NPs. Qian et al. (2016) saw increased ROS production and lipid peroxidation in the cyanobacterium *Microcystis aeruginosa* when exposed to AgNPs. They also showed that ROS inhibited Superoxide dismutase (SOD) and peroxidase (POD) transcription and expression. In contrast, ROS production was mediated by the induction of SOD and POD activity and the expression of the antioxidant enzyme glutamine synthetase in *Chlorella vulgaris* at same exposure scenario. Dauda et al. (2017) reported a significant increase in glutathione S-transferase (GST) and POD enzymes in *Chlorella vulgaris* upon exposure to TiO$_2$ NPs.

Miao et al. (2010a) studied the behaviour and toxicity of AgNPs to the freshwater alga *Ochromonas danica* to determine whether there were any other mechanisms in algal toxicity other than due to the Ag$^+$ ions liberated outside the cells. They demonstrated that the AgNPs were taken inside the cells where they exerted their toxic effects. However, they did not discuss how the NPs exerted toxic effects inside the cells. Dorobantu et al. (2015) observed that Ag NPs caused membrane damage in the alga *Euglena gracilis*, but not in *Chlorella protothecoides*. In addition, Ag NPs caused morphological changes in *Euglena gracilis* altering the shapes from spindle to round with the cells showing increased diameter. Ag$^+$ ions from AgNO$_3$ caused membrane damage in both algae causing intracellular material leaking out of the cells resulting in a depressed volume of cells. Hartmann et al. (2010) evaluated the toxicity of TiO$_2$ NPs to the alga *Raphidocelis subcapitata* and suggested that the observed decreased growth rate could be caused by the adhesion of NPs onto the algal cell surfaces. Ozkaleli and Erdem (2018) observed lipid peroxidation of the alga *Raphidocelis subcapitata* cell membrane.
upon exposure to TiO$_2$ NPs, resulting in the deformation of the membrane structure. Li et al. (2015b) reported a doubling of cell volume when the alga *Euglena gracilis* was exposed to AgNPs. They suggested that the enlargement was a result of unspecific interactions of Ag$^+$ ions released from AgNPs with the thiol groups of glycoproteins in the pellicle. However, they did not observe any internalization of NPs into the algal cells. Ji et al. (2011) excluded the effects of ions or shading for the observed toxicity of ZnO NPs to the alga *Chlorella sp.* but concluded that the toxicity was caused by entrapping and wrapping by the NPs.

Zhou et al. (2016) observed increased toxicity and cell internalization of AgNPs in the absence of EPS compared to the presence of EPS. EPS could bind to both NPs and Ag$^+$ ions reducing the internalization and toxicity. Stevenson et al. (2013) investigated the toxicity of AgNPs to the populations of the alga *Chlamydomonas reinhardtii* at different phases of batch culture and found that the toxicity was highest for the cultures at early phases in growth. Dynamic process modelling, incorporating algal growth rate, dissolution, bioaccumulation and extracellular DOC production revealed that the DOC was a strong factor mitigating the toxicity of NPs. Kadukova (2016) exposed the alga *Parachlorella kessleri* to AgNO$_3$ and noted that the Ag$^+$ ions were removed from the medium by biosorption by algae. Interestingly, the majority of Ag was released back into the medium in the next 14 days, while the algal cells had formed Ag NPs inside, within that period. Those NPs were comparatively less toxic against algal cells than Ag$^+$ ions at the same Ag concentrations. The importance of charge of the algal surfaces and the NPs is highlighted in causing toxicity. There is a high tendency for negatively charged NPs to bind on the positively charged algal surfaces. Also, once bound to the cells, the charge density of the NPs decreases which favours the adsorption of more NPs resulting in large clusters. It is widely accepted that the sorption of NPs on the algal surfaces facilitates the localized exposure to ROS resulting in oxidative damage to the cell membranes. However, sorption of NPs might cause toxicity even without the production of ROS. Certain other effects were also reported including adverse effects to morphology, cell division, gene expression and even physical effects. Also, algae have their own mechanisms to mitigate the NP toxicity into certain extent.

### 2.6.3 The toxicity of NPs to daphnia

Among invertebrates, *Daphnia sp.* is the first choice for standard toxicity tests among control agencies (Jonczyk and Gilron, 2005). Except in extreme environments, this organism is present in all aquatic habitats and possesses all the positive characteristics for standard tests.
Daphnids exerts strong grazing effects and support the aquatic food web. They feed on several sources like bacteria, algae, other invertebrates and plants and enter the trophic chain at intermediary level by being a preferential prey for larger organisms like fish, birds and humans. This also makes them a possible important linkage for passing contaminants through the food chain which should be studied for any such contaminants which are suspected of being capable of bioconcentration and bioaccumulation (Zhu et al., 2010b).

The *Daphnia sp.* 48 h acute test is one of the most widely used aquatic standardized tests, and this is reflected in NP toxicity studies. However, there are some suggestions to improve the sensitivity by prolonged exposure up to 72 h or the 48 h test duration followed by a 24 h recovery period (Novak et al., 2018). LC$_{50}$ and EC$_{50}$ are the most common endpoints used, while other effects such as uptake, accumulation, feeding rate, reproduction, enzyme activity, oxidative stress and morphological changes are reported (Lekamge et al., 2018a). More than 87% of studies have used *Daphnia magna* as the test species possibly as a result of its inclusion in regulatory chemical testing, guidelines and international standards (Baun et al., 2008) while majority of studies have tested against Ag NPs (27%) followed by TiO$_2$ (23%), Au NPs (11%) ZnO NPs (10%) and CuO NPs (10%) (Lekamge et al., 2018a).

There are different views on whether NPs or liberated ions from NPs cause the toxicity to *Daphnia sp.* Some evidence suggests that the ions are the cause and the NPs merely represent a source of ions, while several other studies suggest cumulative effects or more adverse effects from NPs. Li et al. (2015a) observed significant changes in the metabolomic profile of *Daphnia magna* after exposure to Ag NPs and Ag$^+$ ions for 48 h. The changes in metabolites of daphnids exposed to AgNPs were identical to those exposed to Ag$^+$ ions and therefore, they concluded that ionic silver is the dominant cause of toxicity. Sakamoto et al. (2015) observed higher toxicity from AgNPs than Ag$^+$ ions to *Daphnia magna*, *Daphnia galeata* and *Bosmina longirostris*. However, the 48 h EC$_{50}$ values of Ag NPs based on Ag$^+$ ion concentrations liberated from AgNPs were comparable with those of Ag$^+$ ions, and therefore, they concluded that the effects of AgNPs were due to liberated Ag$^+$ ions from AgNPs. Zhao and Wang (2011) observed no toxicity from AgNPs to *Daphnia magna* when the liberated Ag$^+$ ions were complexed by cysteine, suggesting that the toxicity was primarily caused by Ag$^+$ ions. Shen et al. (2015) exposed *Daphnia magna* to seven types of Ag NPs with different sizes and coatings in NaNO$_3$ medium for 8 hours to identify Ag species responsible for acute toxicity. The LC$_{50}$ values of the seven AgNPs as liberated Ag$^+$ ions from AgNPs agreed well with that of AgNO$_3$.
and therefore, they concluded that ionic silver is exclusively responsible for acute toxicity. Bacchetta et al. (2016b) noted the toxicity of ZnO NPs to *Daphnia magna* was similar to the toxicity from Zn\(^{2+}\) ions and therefore concluded that the toxicity was caused by released ions from NPs. Adam et al. (2014) found chronic effects of ZnO NPs (EC\(_{50}\): 0.112 mg L\(^{-1}\)) and ionic Zn (EC\(_{50}\): 0.082 mg L\(^{-1}\)) in *Daphnia magna* following exposure for 21 days. They studied the influence of free, dissolved and aggregated Zn fractions in the medium and concluded that the dissolved fraction was largely responsible for the chronic toxicity. Adam et al. (2015c) concluded that the ions from the dissolution of Cu NPs caused toxicity to *Daphnia magna* by exposing them to CuO and ZnO NPs and Cu and Zn salts for 21 days.

In contrast, there are reports by some researchers regarding the toxicity of NPs which cannot be explained by ionic effects (Navarro et al., 2008b, Fabrega et al., 2009, Yin et al., 2011). Allen et al. (2010) observed that coffee-coated AgNPs were more toxic to *Daphnia magna* than Ag\(^{+}\) ions. Pakrashi et al. (2017) observed that AgNPs significantly affected the reproduction process of the first two broods in comparison with AgNO\(_3\) which affected only the first brood. Based on this, they suggested that AgNPs may have longer adverse effects than Ag\(^{+}\) ions. Bhuvaneshwari et al. (2016) claimed that the relative contribution of dissolved ions from NPs towards acute toxicity to *Ceriodaphnia dubia* was less than that of ZnO NPs. When *Daphnia magna* was exposed to CuO NPs and CuSO\(_4\), Kim et al. (2017) observed that the dissolved Cu\(^{2+}\) ion concentration from CuO NPs after 72 h was much less than the 72 h median effective concentration of CuSO\(_4\). These authors therefore suggested that the observed median toxicity of CuO NPs at 72 h was caused by the particles rather than by the dissolved ions. Xiao et al. (2015) reported that the relative percentage contributions of dissolved ions from CuO and ZnO NPs were 26% and 31%, respectively, when *Daphnia magna* was exposed to NPs. Therefore, they concluded that the particles rather than the dissolved ions were the main source of toxicity.

Ingestion via active and passive diffusion is the most common way of NP uptake by daphnids. Many NPs are lipophilic, and the ingested NPs are highly likely to be found in storage cells which contain lipids such as triacylglycerol and glycogen (Goulden and Hornig, 1980, Moore, 2006). The size of the particles daphnids can uptake depends on their body size. *Daphnia magna* can ingest particles up to about 70 µm, and the minimum size depends on the distances between setulae on thoracic limbs, which do not depend on the age or size since the gap is constant throughout (Burns, 1968, Geller and Müller, 1981). Zhao and Wang (2011)
observed a linear and positive correlation between Ag concentration in the daphnids and the concentration in the medium after exposing *Daphnia magna* to Ag NPs. Also, at same Ag exposure concentration levels, the Ag body burden from Ag NPs was two to three orders of magnitude higher than that from AgNO₃, showing the potential of daphnids to accumulate Ag NPs due to ingestion of NPs into their gut environment. Zhao and Wang (2012b) demonstrated that the Ag NP influx rate of *Daphnia magna* decreased with increased NP size. Also, they found 60% of Ag distributed in the gut of daphnids and concluded that ingestion was the dominant uptake pathway. Similarly, Skjolding et al. (2014a) observed a higher uptake of smaller mercaptoundecanoic acid-coated Au NPs than bigger particles. However, no such correlation was observed for citrate-coated Au NPs. In contrast, Rosenkranz et al. (2009) reported a lower uptake of smaller carboxylated polystyrene NPs (20 nm) in terms of mass compared to larger particles (1000 nm). Tan et al. (2016b) observed that the uptake of polyacrylate-coated TiO₂ NPs by *Daphnia magna* depended on the calcium concentration in the medium. At low Ca concentrations, NPs were ingested via endocytosis and passive drinking and distributed throughout the body, with the highest NP concentration at the abdominal zone and gut. In contrast, NPs were actively ingested and concentrated only in the gut at high Ca concentration levels in the medium. Conine and Frost (2017) found that the presence of food reduced the toxicity of AgNPs in terms of the growth and survival of *Daphnia magna*. They also found that toxicity was greater for animals fed with P-rich algae compared to P-poor algae. The algal-bound AgNPs were not toxic at any tested concentrations and they suggested that the reduced toxicity in daphnids fed with P-rich algae was due to higher removal efficiency of Ag NPs by P-rich algae from the medium leaving less for uptake by daphnids. They also suggested that the algae may convert NPs to non-toxic form to daphnids, while the nutrition and overall health of daphnids also play a role in responding to NPs. Skjolding et al. (2014b) studied the influence of surface functionalization of ZnO NPs and observed fast uptake of ZnO NPs and ZnO-octyl NPs compared to ZnO-OH NPs. Daphnids ingest NPs via active and passive diffusion, while the body size of the daphnids and the concentration of NPs in the medium positively correlate with ingestion. The body burden of NPs may be higher than their bulk counterparts due to the higher NP accumulation in the guts. The size of the NPs influences the ingestion though there are conflicting views on the correlation of size and ingestion rate. Also, several other factors such as media composition, the presence of food and the surface functionalization of NPs influence the ingestion.
The widely accepted key toxic mechanism for acute toxicity from metals and metal NPs to invertebrates such as daphnids is the inhibition of Na\textsuperscript{+}/K\textsuperscript{+}-ATPase activity and the prevention of the absorption of Na\textsuperscript{+} ions which could induce ionoregulatory failure and finally cause the death of the organism (Bianchini and Wood, 2003, Kennedy et al., 2012, Rüdel et al., 2015). In addition to this, several other effects are reported at acute and chronic level. Bacchetta et al. (2016b) exposed Daphnia magna to ZnO NPs and noted morphological changes in the digestive epithelium. They attributed these effects to the dissolved Zn\textsuperscript{2+} ions from NPs. Zn\textsuperscript{2+} ions enter into the gut enterocyte cytoplasm and resulted in altered mitochondria membrane permeability causing ROS production, which stimulates the extensive autophagy process eventually causing cell and animal death. Chae and An (2016) reported structural damage to the digestive organs of Daphnia magna along with the production of lipid droplets and concluded that AgNPs adversely affected nutrient uptake leading to immobility and death. Das et al. (2013) suggested that the observed decreased reproduction, growth inhibition and erratic behaviour of Daphnia magna from chronic exposure to TiO\textsubscript{2} and Ag NPs could be due to the uptake of NPs in their gut plus decreased enzyme activity. Zhu et al. (2010a) observed growth retardation and reproductive defects in Daphnia magna upon exposure to TiO\textsubscript{2} NPs. A significant amount of NPs accumulated in the body interfered with food intake which could conceivably be the cause. Blinova et al. (2017) saw long-term effects on reproductive potential with decreased number of neonates hatched from ephippia when Daphnia magna was exposed to Fe\textsubscript{3}O\textsubscript{4} NPs. Lv et al. (2017) observed reduced digestive enzyme activities in Daphnia magna upon exposure to C\textsubscript{60} and Si NPs. They also reported a concentration-dependent increase in SOD and lipid peroxidation (LPO) levels. However, the SOD activity decreased at a higher dose of C\textsubscript{60} exposure after 72 h along with increased malondialdehyde (MDA) levels. They suggested this may be due to the breakdown of the antioxidant system at high concentrations over lengthy exposures. Ulm et al. (2015a) found increased glutathione (GSH), catalase (CAT) and acetylcholinesterase (AChE) activity levels in Daphnia magna upon exposure to TiO\textsubscript{2} NPs. When Dabrunz et al. (2011) exposed Daphnia magna to TiO\textsubscript{2} NPs for 96 h, they observed that the second moulting was disrupted due to the biological surface coating of NPs on the daphnids. Disruption to molting directly results in reduced reproduction rates. Vijayakumar et al. (2016) noted ingestion of ZnO NPs in Ceriodaphnia cornuta and Moina micrura which caused blackening of the intestine, rupture of intestinal wall, shrinkage of the abdomen and loss of carapace and antennae leading to structural deformities. Rainville et al. (2014) reported increased protein carbonylation indicating ROS, changed vitellogenin levels and higher haemoglobin levels indicating cellular
respiration from Ag NP exposure in *Daphnia magna*. NPs would have adverse effects on ionoregulatory processes, digestive system, growth, reproduction, behaviour, oxidative stress and moulting. *Daphnia* acute and chronic tests are widely used by regulatory regimes. However, the toxicity of NPs might not be reflected within the scope of the tests, and careful consideration of the mechanisms of toxicity is important to analyse effects. It is also reported that daphnids release certain proteins creating eco-corona around NPs (Nasser et al., 2016) resulting in heightened uptake and toxicity which warrants careful investigation of NP risks under environmentally relevant scenarios.

2.6.4 The toxicity of NPs to shrimps

Freshwater shrimps are important components of freshwater ecosystems, both in terms of their role in ecosystem functioning and their economic value (Daam and Rico, 2018). Freshwater shrimp species were generally found to be more sensitive to certain toxicants than *Daphnia magna*. Therefore, considering their high sensitivity, a combination of indigenous shrimp and cladoceran species is proposed to be used when evaluating the potential toxicity of compounds to the arthropod community (Daam and Rico, 2018). Freshwater shrimps were used in several ecotoxicological studies to evaluate the effects of pesticides and herbicides (Daam and Rico, 2018, Hose and Wilson, 2005, Kumar et al., 2010a, Kumar et al., 2010b, Olima et al., 1997, Phyu et al., 2005), and metals (Daly et al., 1990, Oulton et al., 2014). However, there are no studies on the effects of NPs on freshwater shrimps. Cordeiro et al. (2016) evaluated the effects of TiO$_2$ NPs on the accumulation, metabolism, and oxidative stress in the gills and hepatopancreas of the marine shrimp *Litopenaeus vannamei*. They found an increased glutamate-cysteine-ligase activity in the gills, while the total antioxidant capacity increased.

2.7 Bioaccumulation and trophic transfer of NPs

NPs in current use are expected to persist in the aquatic environment in different forms. Bioaccumulation of NPs is significant and calls for more research even though the emission of NPs to the aquatic environment is low, because of their limited degradability combined with the probability that they will be fed on by many invertebrates (Baun et al., 2008). To understand the trophic transfer of NPs through the food web, it is important to understand the mode of action at cellular and higher levels within individual organisms (Aschberger et al., 2011). Cells use different routes to internalize NPs, and a particular preferred route is chosen based on NP properties like size, shape and surface characteristics (Yameen et al., 2014). Any foreign
materials which the cell finds harmful are transported to the lysosomes where they are digested. Therefore, in medical nanotechnology, many NPs are designed to enter the target cell through the caveolae to avoid degradation (Na et al., 2003, Panyam and Labhasetwar, 2003). Once they are released into the environment after use, their non-degradative nature might negatively affect aquatic organisms. Bioaccumulation and biomagnification of NPs through the food webs are yet to be properly understood, with more research required on the influential physicochemical characteristics of NPs and trophic transfers (Zhu et al., 2009). The potential of accumulation and biomagnification of NPs may be higher in comparison with conventional contaminants, but the current testing paradigms do not emphasize the importance of evaluating the ecological impacts in this context (Wu et al., 2017a).

2.7.1 Bioaccumulation of NPs

Cellular uptake and accumulation of NPs may determine the toxicity (Taylor et al., 2016a). However, studies show contradictory results regarding the internalization mechanisms and where NPs accumulate inside the algae cells. Some reports claim that NPs enter into the cells, while others claim that they are just absorbed onto the cellular surface of algal cells or restricted to the outer region including the cell wall or periplasmic space. Taylor et al. (2016a) noted NPs in the periplasmic space of *Chlamydomonas reinhardtii* algal cells when they were exposed to Ag NPs. However, there were no Ag NPs accumulated inside the vesicle or the endosome around the cell, excluding the possibility of endocytosis or passive diffusion which is proposed to be the most feasible route (Von Moos et al., 2014, Behra et al., 2013) for cellular internalization. In contrast, they observed Ag$_2$S particles in the cytoplasm which they suggested were present as a result of sulfidation of Ag$^+$ ions from Ag NPs. Sulfidation is widely accepted as a mechanism for the complexation and sequestration of heavy metals in plants to mitigate the toxicity (Chen et al., 2013). Lee et al. (2015) found that the bioaccumulation of Au NPs in *Euglena gracilis* was higher than in *Chlamydomonas reinhardtii* and noted that the reason might be the difference in the physical structure of organisms and the surface area available for interaction with NPs. They also observed the transfer of NPs to *Daphnia magna* after feeding them with Au NP-treated algae. Zhao et al. (2016) observed internalization of CuO NPs into *Chlorella pyrenoidosa* cells by endocytosis followed by storage in the vacuole. Yue et al. (2017) observed cell-associated Ag in the alga *Euglena gracilis* when exposed to Ag NPs. However, Ag NPs did not enter the algal cells, only absorbed onto the algal surface.
Several studies have evaluated the bioaccumulation of NPs in daphnids. Waterborne exposure and diet are the major routes for uptake of NPs in daphnids. NPs may enter the body cavity, be retained in the attached to the body surface including the carapace. The concentration of NPs, media composition and physicochemical characteristics of NPs such as size and charge influence the uptake and retention of NPs in daphnids. Ribeiro et al. (2017) concluded that waterborne exposure to Ag NPs causes more accumulation of Ag than dietary exposure in *Daphnia magna*. However, more Ag from AgNO₃ was accumulated through the diet. Similarly, Wu et al. (2017a) observed a higher uptake, retention of NPs and attachment to the carapace surface of *Daphnia magna* upon waterborne dermal exposure to CuO NPs when compared to feeding exposure. Oral exposure was predominant in feeding exposure through NP-treated algae, and the ingested Cu was regulated within the body and transferred to other biological compartments such as neonates and carapaces which may have caused less toxicity. Botha et al. (2016) observed that the uptake of Au NPs into *Daphnia magna* was related to NP concentration in the medium and the charge of NPs. NPs were seen adsorbed to the surface of daphnids and in the gut, but there was no evidence of NP internalization into the body cavity. No effects on reproduction or molting patterns were observed. Wray and Klaine (2015) observed that the uptake and elimination of Au NPs by *Daphnia magna* were influenced by the size and surface charge of NPs, whereas shape of NPs was non-influential. However, they also found no evidence for NP internalization into the body with NPs restricted to the gut lumen and the carapace. Adam et al. (2014) found increased concentrations of Zn in *Daphnia magna* with increased ZnO NPs and Zn²⁺ ion concentrations in the media after exposing them for 21 days. In a similar study, Adam et al. (2015a) observed localization of CuO NPs in the gut of *Daphnia magna* when they were exposed to CuO NPs for 10 days. However, CuO were not internalized in the cells and were easily eliminated. Khan et al. (2014) observed accumulation of Au NPs in the gut lumen of *Daphnia magna*, but there was no internalization into the gut epithelial cells. Zhu et al. (2010a) found that significant amount of TiO₂ NPs accumulated in the body and *Daphnia magna* and had difficulty in eliminating these NPs. Tan et al. (2016a) reported that Ca concentration in the medium influenced NP uptake into *Daphnia magna*. They observed TiO₂ NPs distributed throughout the daphnid while NPs were concentrated in the gut at high Ca concentrations. Vijayakumar et al. (2016) observed the bioaccumulation of ZnO NPs in the gut region of *Ceriodyphnia cornuta* and *Moina micrura*. Pakrashi et al. (2017) exposed *Daphnia magna* to AgNPs and saw the NPs accumulated in the gut and non-gut tissues. Interestingly, a higher degree of positive correlation between the concentration of Ag in the non-gut tissue was found. Xiao et al. (2015) reported that the bioaccumulation of NPs or
dissolved ions from NPs were concentration dependent. At low concentrations, *Daphnia magna* accumulated more dissolved ions from Cu and ZnO NPs (0.05 and 0.5 mg L\(^{-1}\), respectively), while the particles were accumulated more at high concentrations (0.1 and 1 mg L\(^{-1}\)). Scanlan et al. (2013) observed similar or higher concentrations of Ag levels in the haemolymph of *Daphnia magna* in comparison with the initial concentration of Ag NWs in the medium indicating effective bioaccumulation during filter feeding. Lovern et al. (2008) used electron microscopy to observe accumulation and to investigate the presence and distribution of Au NPs in gut tissues of *Daphnia magna* exposed for 24 hours. They observed movement of NPs to the posterior region of the gut, and there were no large blockages and minimal deaths were observed. Therefore, they concluded that the particles are cleared with time in waste pellets.

Correlation between accumulation of NPs in daphnids and their eggs is also reported. Sá-Pereira et al. (2018) found NPs in the digestive tract, mainly in the gut and in the eggs of the brood pouch of *Daphnia magna* when exposed to TiO\(_2\) NPs. Also, the penetration of Ti into epithelial region was higher at higher concentration levels. When *Daphnia magna* was exposed to polystyrene NPs, Brun et al. (2017) noted accumulation of NPs in or on the lipophilic cells in the early stages of embryonic development, while the embryo is still surrounded by a chorion. However, they did not observe any NPs accumulated neither in the gut epithelium nor in lipid droplets in the adults. Sakka et al. (2016) observed higher mortality and reproductive effects in *Daphnia magna* correlated with the uptake of Ag NPs.

**2.7.2 Trophic transfer of NPs**

Studies show that NPs are taken up and accumulated inside organisms which are transferred to higher trophic levels. Transfer of NPs up through the food chain is a primary concern since it affects the balance of the ecosystem putting ecosystem health at risk (Bhuvaneshwari et al., 2018a, Wu et al., 2017b). Since organisms may feed on NP-contaminated food, it is important to understand the role of the trophic route (Bour et al., 2015). Dietary intake of NPs may cause significant effects on growth, survival and reproduction (Bhuvaneshwari et al., 2018a). Certain metals and NPs are accumulated more through dietary intake than waterborne exposure (Ribeiro et al., 2017), while certain other NPs are accumulated more through the waterborne exposure (Bhuvaneshwari et al., 2018b). The effects from NPs ingested via dietary intake may have different mechanism of toxicity compared to direct exposure (Bour et al., 2015). Werlin et al. (2011) showed that the CdSe quantum dots can be
transferred from the bacteria *Pseudomonas aeruginosa* to the protozoa *Tetrahymena thermophila* with the Cd concentration in the protozoa five times higher than that found in the bacteria. Chae and An (2016) observed that the Ag NWs were transferred from the alga *Chlamydomonas reinhardtii* to *Danio rerio* through *Daphnia magna*. Renault et al. (2008) showed that Au NPs were transferred from the freshwater alga *Scenedesmus subspicatus* to *Corbicula fluminea*. Bouldin et al. (2008) observed the transfer of carboxyl quantum dots from *Raphidocelis subcapitata* to *Ceriodaphnia dubia* through dietary intake. Chen et al. (2016) found that the trophic transfer of fullerene NPs from *Scenedesmus obliquus* to *Daphnia magna* was dependent on sub-cellular distribution of NPs in alga cell. They observed that the highest NP transfer occurs via the cell wall followed by cell organelle and cell membrane. McTeer et al. (2014) reported the transfer of Ag to *Daphnia magna* from AgNP and AgNO₃ treated alga, *Chlamydomonas reinhardtii*. Bhuvaneshwari et al. (2018a) observed the transfer of ZnO NPs from the alga *Scenedesmus obliquus* to *Ceriodaphnia dubia* with the biomagnification factor (BMF) found to be nearly one, causing ultra-structural damage and degradation of internal organs in daphnia. Larguinho et al. (2014a) reported that Au NPs transferred from the alga *Dunaliella salina* to the bivalve *Mytilus galloprovincialis*. However, they did not observe any significant morphological alterations in mussel digestive glands or activation of any antioxidant enzymes tested. Zhu et al. (2010b) observed trophic transfer of TiO₂ NPs from *Daphnia magna* to *Danio rerio* by dietary exposure. Although they observed lower biomagnification from dietary intake than from aqueous exposure, the higher body burden in the dietary exposure group led them to conclude that trophic transfer is a major route of potential NP exposure. Skjolding et al. (2014b) observed trophic transfer of ZnO NPs and ZnO-octyle NPs from *Daphnia magna* to *Danio rerio*. However, daphnids did not uptake ZnO-OH NPs, and therefore, these NPs were not available for trophic transfer. This demonstrates that surface functionalization influences the trophic transfer of NPs. Cano et al. (2018) observed the trophic transfer of MWCNTs from *Daphnia magna* to *Pimephales promelas* which was found to be dependent on the size of the particles. However, Bhuvaneshwari et al. (2017) did not observe any transfer of nZVIs from the treated alga *Scenedesmus sp.* to *Ceriodaphnia dubia* though the algae had taken up NPs. Similarly, Bhuvaneshwari et al. (2018b) did not observe any trophic transfer of TiO₂ NPs from the treated alga *Dunaliella salina* to *Artemia salina*. However, Hu et al. (2017) observed the transfer of Ag from AgNP-treated *Chlorella pyrenoidosa* to *Daphnia magna*. In this case, the BMF was 0.5, and therefore, they concluded that there was no biological magnification of NPs from algae to daphnids.
2.8 Effects of NPs on behaviour of aquatic organisms

In addition to the direct effects of the contaminants to organisms, behavioural effects are also critically important. Behaviour is a sensitive measure of an organism’s response to stress, and noticeable changes can be observed at concentrations of contaminants which are orders of magnitude less than that which cause mortality (Weis and Candelmo, 2012). Behavioural ecotoxicity tests are becoming increasingly popular because of their high sensitivity at low concentrations and early response (Yeardley et al., 1996). Though the importance of behavioural tests is appreciated in ecotoxicology tests, far less attention has been received (Melvin and Wilson, 2013). Most currently available standard tests mention the obligation to document abnormal behaviour, but this is not quantitatively sufficient for any risk assessments (Postma and Keijzers, 2014). Most of the behavioural activities which are used in experiments are related to feeding (feeding rate, filtration rate, predator response) or movement (swimming, avoidance, burrowing).

There are a few studies on the effects of the NPs on the behaviour of aquatic species with most relating to daphnids. The adhesion of aggregates of NPs to the exoskeleton of *Daphnia sp.* may lead to different probability of survival, loss of mobility and physical damage (Baun et al., 2008). Stanley et al. (2016) exposed *Daphnia magna* to MWCNTs for 48 h and found LC$_{50}$ as 29.3 mg L$^{-1}$ and EC$_{50}$ (swimming velocity) as 6.7 mg L$^{-1}$. They concluded that behavioural tests are more sensitive than traditional acute toxicity tests for materials which are toxic physically rather than chemically. Also, they suggested that use of only survival endpoints to set environmental guidelines could underestimate potential hazards and risks of NPs to the environment. Lovern and Klaper (2006) observed *Daphnia magna* showing abnormal behaviour such as sporadic swimming in small circles, persistent ramming to vessel walls and inability to swim down from the surface when exposed to fullerene NPs. Artells et al. (2013) studied the effects of CeO$_2$ NPs on the survival and swimming behaviour of *Daphnia similis* and *Daphnia pulex*. Swimming velocities decreased in the range of 30% to 40% in both species when treated with 1 mg L$^{-1}$ NPs. At higher concentrations (10 and 100 mg L$^{-1}$), the swimming velocity of *Daphnia similis* was more impacted than *Daphnia pulex*. Noss et al. (2013) studied the swimming behaviour of *Daphnia magna* after treating with TiO$_2$ NPs. They observed a treatment-dependent swarming in the centre of the test vessels during the initial period. The swimming velocities increased with increased body length but significantly reduced after 96 hours of exposure. Vijayakumar et al. (2016) observed abnormal behaviour in *Ceriodaphnia*
cornuta and Moina micrura upon exposure to ZnO NPs. The restricted and reduced movements were attributed to the adhesion and agglomeration of NPs on the carapace and the filter apparatus. When Strigul et al. (2009) exposed Daphnia magna to 2.5 mg L⁻¹ B NPs, they were actively swimming compared with the control group. However, when the concentration increased to 8 mg L⁻¹, they were less active, while they were very slow at 25 mg L⁻¹.

O'Keefe et al. (1998) suggested that the predation risk of daphnids depends on their swimming behaviour. Pokhrel and Dubey (2012) investigated the potential impacts of citrate-coated Ag NPs on the behaviour of Daphnia magna in the presence of the predatory dragonfly Anax junius. In the absence of Ag NPs, daphnids avoided predators with both horizontal and vertical movements which are different to the control. However, they did not show any difference in vertical movement when treated with Ag NPs suggesting that Ag NPs may have potential implications on daphnid populations with increased vulnerability to predation. Lovern et al. (2007) quantified the behavioural responses of Daphnia magna at sub-lethal concentrations of TiO₂ and fullerene NPs. Both treatments caused significant increase in hopping frequency and appendage movement suggesting increased risk of predation and reproductive decline. Lu et al. (2017) observed a decrease in the ingestion and filtration rate of algae by Daphnia magna upon exposure to increased concentrations of TiO₂ NPs, and the researchers attributed this to the observed chronic toxicity. Similarly, Lv et al. (2017) saw a reduction in ingestion and filtration rate of Daphnia magna upon exposure to C₆₀ and Si NPs. Heinlaan et al. (2017) suggested that the reduced algal feeding rate of Co₃O₄ and Mn₂O₃ NPs-exposed Daphnia magna was not particle specific since similar results were obtained for daphnids exposed to relevant metals. Gaiser et al. (2011) observed reduced feeding in Daphnia magna when they were exposed to CeO₂ NPs which was ascribed to potential replacement or coating of algae by NPs and filling the intestine with particles. Zhu et al. (2010a) observed drastic reductions in food intake when Daphnia magna was exposed to TiO₂ NPs. The chronic toxicity of NPs was ascribed to poor food intake and malnutrition. McTeer et al. (2014) observed a significant reduction of feeding when Daphnia magna were fed with Ag NP- and AgNO₃-contaminated algae compared to the control. They concluded that this reduction was due to the presence of Ag in algae.

In general, behavioural tests are fast and more sensitive than conventional acute and chronic ecotoxicity tests. These characteristics are particularly useful in assessing NP toxicity. NPs tend to transform and aggregate in the medium exerting huge challenges in assessing
toxicity by conventional tests. Also, due to numerous types of NPs entering into the market, it is a huge challenge to assess the toxicity due to time consuming nature and the cost of conventional tests. These issues can be overcome by choosing comparatively faster and cheaper behavioural tests. There is a growing interest to develop lab-on-a-chip behavioural tests (Wang et al., 2017, Cartlidge et al., 2017) which are fast and sensitive with added advantages.

2.9 Summary

Both the use and the number of applications of nanoparticles (NPs) have expanded rapidly in recent years. This has led to increasing concern regarding the impact of NPs on ecosystem health. Toxicological research in this area is therefore of utmost importance in order to determine the risks of NPs to organisms in the environment. The goal of this literature review was to analyse recent literature in this interdisciplinary research field, with special focus on the freshwater environment. The review begins with summarizing knowledge of current production and use of NPs, production and exposure concerns in the environment. The major physicochemical characteristics of NPs are examined and their subsequent fate, behaviour and toxicity to aquatic organisms. We review literature regarding the toxicity of NPs to freshwater organisms at different trophic levels involving studies on hydra, algae, daphnia and shrimps. Finally, the review examines the less understood behavioural and ageing effects of NPs on freshwater organisms. This aspect necessarily focuses on inorganic NPs including AgNPs due to their industrial use and production although the effects of organic nanoparticle should not be overlooked. It is a huge challenge to accurately predict the environmental concentrations of NPs, their fate and behaviour in the environment and to assess the risks posed to aquatic organisms. Through analysis, this review contributed to improved understanding on the effects of NPs while also identifying current research gaps which helped me to set up the aims for the current project.
Chapter 3. NP synthesis, characterisation and culturing organisms

3.1. Synthesis of silver nanoparticles (AgNPs)

Several methods are used for the synthesis of AgNPs (Brahamdutt et al., 2018); however these can be categorised into two categories; top-down and bottom-up approaches (Fig. 3.1) (Fabrega et al., 2011, Tolaymat et al., 2010). Top-down approach involves mechanically reducing bulk materials by techniques such as lithography or laser ablation. In contrast the bottom-up approach involves the reduction of the metal salts to NPs followed by the addition of a reducing agent and possibly supplemented by a stabilizing agent (Fabrega et al., 2011, Reidy et al., 2013). The stabilizing agent has a profound effect on the surface characteristics, particle size and the aggregation potential of AgNPs (El Badawy et al., 2010b) while the solvents and reducing agents used also affect the physicochemical characteristics of AgNPs (Tolaymat et al., 2010). Several types of reducing agents are used and biological reduction using plant extracts and microorganisms is gaining popularity due to their reduced cost and toxicity (Vaseeharan et al., 2010, Selvakannan et al., 2004). The use of plant extracts also eliminates the use of harsh chemicals since they act as simultaneous reducing and stabilising agents (Ahmed et al., 2016).

Fig. 3.1: Schematic representation of the two approaches to nanoparticle synthesis - source: (Abraham, 2016).
3.1.1. Chemicals and materials

L-Tyrosine, Curcumin, Epigallocatechin gallate (EGCG) (Fig. 3.2A, 3.2B and 3.2C), KOH and Ag$_2$SO$_4$ were obtained from Sigma-Aldrich. Dialysis tubing cellulose membrane (3 kDa cut-off) was purchased from Sigma-Aldrich.

![Molecular formulae of (A) Tyrosine, (B) Curcurmin, and (C) EGCG molecules.](image)

3.1.2. Synthesis of AgNPs

The procedure followed for the in-house NP synthesis was as described previously by Selvakannan et al. (2004). Briefly, 10 mL of 10$^{-3}$ M Ag$_2$SO$_4$ solution was mixed with 10 mL of 10$^{-3}$ M aqueous solution of coating material (Tyrosine, EGCG or Curcumin) and diluted to 100 mL with MilliQ water. To this, 1 mL of 10$^{-1}$ M KOH solution was added, and the mixture was allowed to boil until the colour of the solution turned yellow which indicates NP formation. The AgNP solutions were allowed to age for 1 day and then concentrated by rotary evaporation. The solutions were then dialyzed for 48 h in a dialysis tube (MWCO: 3 kDa) submerged in copious amounts of MilliQ water with stirring to remove any uncoordinated silver ions (Ag$^+$),
excess KOH and unbound coating materials. Water was replaced twice with fresh MilliQ water after 6 and 24 hours. The dialyzed AgNP solutions were stored in the dark. Each type of NP (0.1 mL) was acid digested with ultra-pure grade 70% HNO₃ (Thermo Fisher Scientific, NSW, Australia) on a heating block at 105 °C for 12 h. The digested samples were diluted with MilliQ water and the Ag⁺ ion concentration was measured by ICP-MS (7700x, Agilent Technologies).

3.2. Characterisation of AgNPs

For risk assessment, physical and chemical characterisation is necessary for nanomaterials. The NPs were characterised in the MilliQ water as follows;

3.2.1. Ultraviolet–visible (UV-vis) absorbance spectroscopy

The surface plasmon resonance (SPR) is formed by the optically excited collective electronic oscillations through the interaction between metals and incident light (Fig. 3.3) (Li et al., 2012). Factors such as solvent dielectric constant, NP size, NP shape, surface functionalization can influence the SPR curve (Smitha et al., 2008). Therefore, any changes to the SPR curve can signify the changes of the fate and behaviour of NPs such as change in size and aggregation. Thus, the SPR can be used to monitor the stability of NPs in media. Other than that, SPR can be used to determine the formation of NPs and to detect the presence of compounds on the surface of the NPs since the AgNPs used in this study were coated with phenolic compounds. Change of SPR can be used to quantify sedimentation rate of NPs in aqueous media (Pettibonei et al., 2008). UV–vis absorption spectroscopy is the most widely used technique for characterizing the optical properties of NPs (Smitha et al., 2008) and the SPR analysis of AgNPs in the current study was performed using a Varian Cary 50 UV-visible spectrophotometer in a quartz cuvette with a path length of 1 cm. Strong, well defined absorptions peaks at 406 (T-AgNP), 400 (E-AgNP) and 414 nm (C-AgNP) in the SPR curves (Fig. 3.4) indicate the presence of AgNPs (Selvakannan et al., 2004, Rodríguez-León et al., 2013, Varaprasad et al., 2011, Ahmad et al., 2010, Cruz et al., 2010); these peaks were absent in controls which included media, pure coating material and coating material plus KOH. The secondary peaks of SPR spectra at 272 (T-AgNP), 270 (E-AgNP) and 256 nm (C-AgNP) are due to the Ag-bound tyrosine (Kierdaszuk et al., 1995, Selvakannan et al., 2004), EGCG (Snitsarev et al., 2013) and Curcumin molecules (Rodrigues et al., 2012) respectively.
3.2.2. Dynamic light scattering (DLS)

Size and size distribution are the main important factors in defining a nanomaterial which is important for legal and regulatory purposes since their value define whether a given material is regarded as a nanomaterial or not (Rasmussen et al., 2018). As per the EU directive, 2011/696/EU (EU, 2011), the term nanomaterial is defined as “material with any external dimension in the nanoscale (1 – 100 nm) or having internal structure or surface structure in the nanoscale” (Bhattacharjee, 2016). DLS is a non-invasive, optical technique used to measure the size distribution of particles. Light from a laser light source illuminates the sample in the cell and the small particles in suspension undergo random thermal motion known as Brownian motion. The Brownian motion of particles or molecules in suspension causes laser light to be scattered at different intensities. The scattered light signal is collected with detectors, either at
a 90 degree (right angle) or 173 degree (back angle) scattering angle. Analysis of these intensity fluctuations yields the velocity of the Brownian motion and hence the particle size using the Stokes-Einstein relationship. Data are usually evaluated based on the assumption that the particles are spherical, and the result is the equivalent hydrodynamic particle diameter (Rasmussen et al., 2018). Therefore, the shape of NPs plays a major role in the interpretation of the results. Particles can be dispersed in a variety of liquids of which the liquid refractive index and viscosity needs to be known for interpreting the measurement results. The sample should be clean, homogeneous and transparent without any precipitation. DLS cannot distinguish between individual NPs and aggregates/agglomerates (Calzolai et al., 2011). In this study, the hydrodynamic diameter (HDD) of the NPs was measured using a folded capillary cell with inbuilt electrodes, on a Zetasizer (Dynamic light scattering; Malvern Zetasizer Nano series, NanoZS). HDD values of AgNPs in MilliQ water are shown in the Table 3.1.

Table 3.1: Summary of AgNP sizes and zeta potential in MilliQ water.

<table>
<thead>
<tr>
<th></th>
<th>T-AgNP</th>
<th>E-AgNP</th>
<th>C-AgNP</th>
</tr>
</thead>
<tbody>
<tr>
<td>HDD</td>
<td>51.58 ± 0.55</td>
<td>40.06 ± 1.50</td>
<td>36.37 ± 0.58</td>
</tr>
<tr>
<td>TEM average size (r.nm)</td>
<td>10.56 ± 2.27</td>
<td>9.27 ± 1.29</td>
<td>13.68 ± 0.76</td>
</tr>
<tr>
<td>Zeta potential (mV)</td>
<td>-42.13 ± 0.33</td>
<td>-38.93 ± 1.37</td>
<td>-44.65 ± 1.65</td>
</tr>
</tbody>
</table>

3.2.3. Zeta potential

All particles in an aqueous media carry a positive, negative, or neutral electric charge. The zeta potential is the measurement of the electric double layer (Figure 3.5) produced by the surrounding ions in solution (Berg et al., 2009). It gives important information on NP mobility, aggregation rates and interactions with surfaces (Cattaneo et al., 2009a). Also, surface charge of NPs influence the interactions with biological systems (Caputo, 2015). The zeta potential of the NPs was measured using a folded capillary cell and glass cuvette, respectively, on a Zetasizer (Dynamic light scattering; Malvern Zetasizer Nano series, NanoZS). Zeta potential values of AgNPs in MilliQ water are shown in the Table 3.1. Zeta potential values below -30 mV suggest that organic molecules formed strong coatings on the AgNPs (Daima et al., 2014).
Figure 3.5: Schematic representation of the electric double layer and definition of zeta potential - source: Caputo (2015).

3.2.4. Transmission electron microscopy (TEM)

TEM is a technique that uses an electron beam to image a sample, providing much higher resolution than is possible with light-based imaging techniques. NP samples are prepared for imaging by drying them on a copper grid that is coated with a thin layer of carbon. Materials with electron densities that are significantly higher than amorphous carbon, such as most metals, most oxides and polymers can be easily imaged with TEM. The electrons emitted from a gun containing a LaB6 electrode travel through a vacuum and are focused on the specimen using electromagnets. The electron beam that travels through the specimen is focused and magnified by an objective lens and is collected on a fluorescent screen below. Electrons that have been scattered by the specimen are removed through an aperture, allowing the generation of a ‘shadow image’ of the specimen with brighter areas where there was no electron scattering. The image is then captured using a charge-coupled device camera. To determine the size of AgNPs in this study, the NP solutions were drop caste onto carbon-coated copper grids. The films on the TEM grids were allowed to stand for 2 min, following which the extra solution was removed using a blotting paper, and the grid was allowed to dry prior to measurement. The images were obtained by a TEM operated at 100 kV (JEOL 1010) equipped with a Gatan
imaging system. The images (Fig. 3.6A, 3.6B and 3.6C) revealed spherical shaped NPs which were uniform in size. The mean core size and size distribution of NPs was determined from TEM images using ImageJ software. The histograms show that majority of particle sizes within the range of 0 to 40 nm (Fig. 3.6D, 3.6E and 3.6F). The average sizes of AgNPs (Table 3.1) indicate that the core size may be considered approximately of the same diameter for the investigations performed in this project. The coatings on the NPs were further confirmed by the fact that hydrodynamic diameter of all NPs were greater than the core sizes determined by TEM images (Daima et al., 2014).

![TEM images and histograms](image)

3.2.5. Inductively coupled plasma mass spectrometry (ICP-MS)

ICP-MS is a type of mass spectrometry used for the elemental determination of metals and several non-metals. It combines a high-temperature ICP (Inductively Coupled Plasma) source with a mass spectrometer (MS). The ICP source converts the atoms of the elements in the sample to ions and the MS separate and quantify those ions. Nominal concentrations of test solutions for toxicity tests in this study were prepared based on the ICP-MS results. Required
volumes of AgNP stock solutions and AgNO₃ were pipetted into relevant test media to obtain the desired exposure concentrations.

3.3. Culturing and maintenance of test species

3.3.1. Hydra vulgaris

3.3.1.1. Source of organism

The original animal stock of H. vulgaris was obtained from Southern Biological Pty Ltd, Australia.

3.3.1.2. Culturing and maintaining

Animals were cultured in 1 L glass bowls. Hydra culture medium (CaCl₂.2H₂O: 147 mg L⁻¹, TES buffer: 110 mg L⁻¹, EDTA: 4 mg L⁻¹) was prepared based on the protocol described by Trottier et al. (1997). The pH was adjusted to 7.0 ± 0.1 with 1 M NaOH. The culture was kept at 25 °C with a photoperiod of 16:8 h light: dark cycle. All glassware used were washed with 5% nitric acid and rinsed with de-ionised water prior to use.

3.3.1.3. Feeding

Animals were fed daily with live Artemia salina nauplii (brine shrimp) for 1 hour. After feeding, the bowls were washed, and the medium was replaced.

3.3.2. Raphidocelis subcapitata

3.3.2.1 Source of organism

R. subcapitata cells were obtained from the pure cultures maintained in the algal lab of the Bundoora West Campus, RMIT University.

3.3.2.2. Culturing and maintaining

Algal cells were inoculated in MLA medium to prepare algal stock cultures. MLA medium was prepared as described by Bolch and Blackburn (1996) with slight modifications. Briefly, concentrated nutrient stock solutions were prepared by filter sterilization and the medium was prepared in MilliQ water by autoclaving for 15 min at 121 °C. The algal stock cultures were maintained axenically as per the organisation for economic co-operation and
development (OECD) guidelines (OECD, 2011). Cultures were aerated and incubated in a light-temperature controlled chamber at 23 ± 1 °C under continuous illumination (6000 lux). White fluorescent tubes were used as the light source while the light intensity in the test setup was measured using a LI-COR light meter (model LI-189). The pH was maintained at 7.5 ± 0.5.

3.3.3. Daphnia carinata

3.3.3.1 Source of organism

D. carinata were obtained from the ponds at the Bundoora West campus, Royal Melbourne Institute of Technology (RMIT) University and the animals were housed in the laboratory.

3.3.3.2. Culturing and maintaining

Daphnids were maintained at 20 ±1 °C with 16:8 h light : dark cycle to obtain neonatal cladocerans. ASTM standard medium which consists of 192 mg L⁻¹ NaHCO₃, 120 mg L⁻¹ CaSO₄.2H₂O, 120 mg L⁻¹ MgSO₄ and 8 mg L⁻¹ KCl (Barry and Meehan, 2000) was used as the culture media. The medium was aerated to saturation with oxygen before addition of daphnids and renewed three times a week. pH was maintained at 7.5 ± 0.2 throughout and cultures were maintained in glass beakers, which were loosely covered to prevent any contamination and evaporation.

3.3.3.3. Feeding

Daphnids were fed with the alga R. subcapitata (5 × 10⁵ cells mL⁻¹), a highly suitable nutrient source for daphnids.

3.3.4. Paratya australiensis

3.3.4.1 Source of organism

P. australiensis were collected using a sieve (4 mm mesh size) from the Yarra River at Warrandyte, Victoria, Australia (Latitude: 37° 43' 31.9454" S Longitude 145° 14' 8.1744" E). This is a pristine site in the natural reserve area (pH: 7.12; t°: 12 °C and DO: 80%). They were then transported to the laboratory in plastic containers with aeration provided.
3.3.4.2. Culturing and maintaining

The shrimp were maintained in an aerated aquarium containing a mixture of river water and dechlorinated carbon filtered tap water (referred as “shrimp medium” from now onwards) which had a mean conductivity of 0.126 mS cm⁻¹ and an ionic composition of Ca: 6.8 mg L⁻¹, Mg: 2.1 mg L⁻¹, K: 1.0 mg L⁻¹, Na: 8.9 mg L⁻¹, Cl: 15.0 mg L⁻¹, SO₄: 9.6 mg L⁻¹, CO₃ < 5 mg L⁻¹ and HCO₃: 23.0 mg L⁻¹. The temperature was maintained at 15 ± 1 °C and the light intensity was 800 lux at the surface of the water with a 16 : 8 h light : dark cycle. The river water was replaced with one fifth of shrimp medium daily and at the end of the first week, they were kept in 100% shrimp medium. The animals were acclimated for 2 weeks before the start of toxicity tests.

3.3.4.3. Feeding

Shrimps were fed with Seramin® tropical flake food twice a day but were not fed for 24 h prior to and during toxicity testing.
Chapter 4. The toxicity of AgNPs to freshwater organisms with different life strategies

This chapter has been published in the peer-reviewed literature and is presented here with only minor modifications to adjust formatting to the requirements of the thesis.


Abstract

The toxicity of manufactured nanoparticles varies greatly depending on the test species in consideration and estimates of toxicity may also be confounded by test media in which the organisms are cultured. For a more comprehensive toxicity evaluation, species at different trophic levels or with life strategies, tested in different media should be included. In this study, we examined the toxicity of tyrosine-coated silver nanonparticles (T-AgNP) to three Australian freshwater invertebrates: Hydra vulgaris, Daphnia carinata and Paratya australiensis. T-AgNPs were synthesized, characterized and their behaviour was examined in different media used for acute toxicity tests. Additionally, the sensitivity of tested organisms to T-AgNPs was compared to ionic silver (Ag⁺ ions). Based on the LC₅₀ values of both T-AgNPs and Ag⁺ ions at different time points, D. carinata was found to be the most sensitive species followed by P. australiensis and H. vulgaris. NP stability studies revealed that T-AgNPs were least stable in hydra medium followed by daphnid and shrimp media. This study demonstrates that significant differences in NP toxicity to aquatic organisms exist and the test media and the life strategy of the species play a key role in these differences. Therefore, it is recommended that a multispecies approach is used in predictive risk assessment of NPs and to ensure protection of native species from possible toxic effects from NPs released into aquatic systems. Also recommended is to carefully investigate the fate and behaviour of NPs in different media in assessing NP toxicity and emphasize the need to use native species in developing relevant regulatory frameworks.
4.1. Introduction

Over the last 30 years, the number of products containing engineered nanomaterials increased across diverse fields mainly due to our growing capacity to synthesize and manipulate such materials (Nowack and Bucheli, 2007a, Sun et al., 2017). Among the nanomaterials, silver nanoparticles (AgNPs) are considered as one of the most important due to its exceptional broad spectrum bactericidal properties (Tian et al., 2018, Kumar et al., 2018, Durán et al., 2016), relatively low cost of manufacturing AgNPs (Capek, 2004), unique properties and ability to form diverse nanostructures (Sohn et al., 2015). AgNPs are used in a growing number of applications across a diverse range of commercial consumer products such as food storage containers, coating materials, liquid fabric softeners and detergents, fabrics and clothing, sporting goods, cosmetics, wound dressings, toothbrushes, and antimicrobial coatings (Wei et al., 2015, Alarcon et al., 2015, McGillicuddy et al., 2017).

There are however concerns regarding the extensive application of NPs and rapid proliferation of NP-enabled products which may lead to a high release into the environment and cause deleterious effects on the organisms that are constantly exposed to these materials (Navarro et al., 2008a, Keller et al., 2013b, McGillicuddy et al., 2017, León-Silva et al., 2016). Tons of nanosilver are added into the aquatic environment annually from wastewater plants (Sohn et al., 2015), septic tanks (Benn and Westerhoff, 2008) and agricultural lands (Kaegi et al., 2011) with concentrations expected to range from 0.03 to 0.32 micrograms per litre (Keller and Lazareva, 2014, Keller et al., 2013b, Batley et al., 2013). Despite the fact that most AgNPs tend to accumulate in the sludge solids in waste water treatment plants (WWTPs) (Wang et al., 2012), it is estimated that a significant proportion of AgNPs will be released in the effluent (Gottschalk et al., 2009, Limbach et al., 2008a). The presence of AgNPs has already been detected in the treated effluent at concentrations of 12 ng L\(^{-1}\) (Siripattanakul-Ratpukdi and Fürhacker, 2014) and 0.1 mg L\(^{-1}\) (Mitrano et al., 2012a).

The environmental impacts of AgNPs including their effects on different organisms within the aquatic environment are still largely unknown (Hu et al., 2016). The toxicity of NPs to organisms depend on the physicochemical characteristics of NPs (Fekete-Kertész et al., 2017). Organic coatings are widely investigated for NPs especially in biomedical research due to their green and ecofriendly characteristics (Daima et al., 2014, Park, 2014). Also, in aquatic systems, NPs undergo physical and chemical transformation (e.g. agglomeration, settling) and therefore, the organisms are not exposed solely to dissolved chemicals, for which test protocols
in ecotoxicology were originally devised (Petersen et al., 2015). Therefore, it is necessary to characterise and assess the fate and behaviour of NPs in the test medium (Steinhäuser and Sayre, 2017, Hund-Rinke et al., 2016, Rasmussen et al., 2018). The toxicity of Ag\textsuperscript{+} ions which are possibly released from AgNPs is a cause for concern since Ag\textsuperscript{+} ions have been traditionally considered as the most toxic form of Ag in water prior to the interest in NPs (Ratte, 1999). Uncertainty on what causes their toxicity is one of the major issues when assessing effects of AgNPs (Yue et al., 2017). While the toxicity of AgNPs is partly explained by the release of Ag\textsuperscript{+} ions, the contribution of AgNPs remains unclear. Some studies presented evidence that toxicity is mainly due to the release of Ag\textsuperscript{+} ions (Shen et al., 2015, Sakamoto et al., 2015, Li et al., 2015a), but others reported a specific nanoparticle effect that could not be simply explained by the dissolution of Ag\textsuperscript{+} ions (Fabrega et al., 2011, Sørensen et al., 2015). Interestingly, some recent studies have begun to point out that in certain cases metal NPs may in fact be more toxic than the corresponding metal ions (Pakrashi et al., 2017, Abramenko et al., 2018, Li et al., 2017b).

Complex and largely unknown properties of nanomaterials (Grieger et al., 2012) accompanied with lack of toxicological and exposure data (USEPA, 2015, Skjolding et al., 2016) are currently among the major barriers for robust risk assessment, which is otherwise critical to formulate sound policies around environmental regulation of nanomaterials (Hjorth et al., 2017c). Knowledge gaps remain for many factors including dose-response relationships and differences across species to characterize the risk of NPs to aquatic organisms (Adam et al., 2015b). Besides, there is no commensurate investment in environmental safety research while we invest heavily in the development of new nanotechnologies (Miller and Wickson, 2015). There are several known and unknown hurdles in evaluating the toxicity of NPs (Kumar et al., 2017, Fabrega et al., 2011) and the reported recommendation values for the protection of aquatic species in the literature vary greatly (Kwak et al., 2016, USEPA, 2012, Gubbins et al., 2011, Fabrega et al., 2011, USEPA, 1985, CCME, 2015). Multispecies toxicity studies which include different trophic levels or species with different life strategies are scarce. Previous studies using more than one species showed that the toxicity of both Ag\textsuperscript{+} ions and AgNPs significantly differed based on the test species (Ribeiro et al., 2014b, Kwak et al., 2016). Therefore, it is important to develop a species sensitivity distribution (SSD) which can be used to estimate the potentially affected fraction of species and to set up threshold concentrations (Garner et al., 2015a, Belanger et al., 2017). Several studies have focused on deriving
hazardous concentration values based on SSD (Nam et al., 2015, Gottschalk et al., 2013a, Adam et al., 2015b, Garner et al., 2015a).

Kwak et al. (2016) and Chen et al. (2018) have studied SSDs of AgNPs and suggested hazardous concentration values (HC5) in the range of 1.1 – 31.73 µg L⁻¹ at which 95% of species are not harmed in the aquatic environments. The objectives of the present study were to examine the toxicity of organic (tyrosine)-coated AgNPs (T-AgNP) and Ag⁺ ions to three freshwater invertebrates at different trophic levels: the cnidarian *Hydra vulgaris*, the daphnid *Daphnia carinata* and the freshwater shrimp *Paratya australiensis* and to compare species sensitivity and the influence of culture media on the toxicity of AgNPs. *Hydra* (Medusozoa: Anthomedusae: Hydridae) is a freshwater coelenterate native to tropical and temperate regions. Their diploblastic body nature, recognizable morphological changes, ubiquitous presence in freshwater environments, easy maintenance in the laboratory, cost effectiveness and the ability to reproduce asexually resulting in large number of genetically similar individuals make them an ideal organism for acute toxicity tests (Beach and Pascoe, 1998, Trottier et al., 1997). *Daphnia sp.* (Crustacea: Cladocera: Daphniidae) is widely used as a model organism in aquatic toxicology tests since they are one of the most sensitive organisms and their ecology, phylogeny, toxicology, and physiology are relatively well understood and a great number of toxicants have been evaluated using daphnids (Von der Ohe and Liess, 2004, Altshuler et al., 2011, Edwards and Pascoe, 2018). The freshwater atyid shrimp, *P. australiensis* (Crustacea: Decapoda: Atyidae) inhabits streams and other freshwater habitats along the east coast of Australia (Hughes et al., 1995). There are no studies conducted to date on toxicity of NPs to *P. australiensis* though it has been used in toxicity studies as a bioindicator for other contaminants in the Australian environments (Kumar et al., 2010b, Lanctôt et al., 2016, Kumar et al., 2010a, Phyu et al., 2005).

4.2. Materials and methods

4.2.1. Test materials

Silver nitrate (AgNO₃), L-tyrosine and KOH were obtained from Sigma-Aldrich. T-AgNPs were produced in-house as described previously by Daima et al. (2014). Briefly, 10 mL of 10⁻³ M of aqueous AgNO₃ solution, 10 mL of 10⁻³ M aqueous tyrosine solution and 1mL of 10⁻¹ M solution of KOH were mixed, diluted to 100 mL with deionized water and allowed to boil until the colourless solution turned yellow, indicating the formation of AgNPs. The NPs
were prepared in the required concentrations through boiling the aqueous dispersions, followed by dialysis using a dialysis membrane (MWCO: 12.4 kDa) to remove potentially unreduced Ag\(^+\) ions and other reactants. For dialysis of NPs, the membrane containing NPs was submerged in 3 L of MilliQ water with stirring over 48 h. The water was replaced twice with fresh MilliQ water after 6 and 24 h. The dialysed NP stock solution was kept in the dark. 0.1 mL of NP was acid digested in 0.2 mL of ultra-pure grade 70% HNO\(_3\) on a heating block at 105 °C for 12 hours. The digested sample was diluted with MilliQ water and the Ag\(^+\) ion concentration in solution was measured with ICP-MS (7700X, Agilent Technologies).

4.2.2. Characterization of AgNPs

Please see Chapter 3. NP Synthesis, characterisation and culturing organisms, Section 3.2. Characterisation of AgNPs.

4.2.3. Test organisms and culture conditions

Please see Chapter 3. NP Synthesis, characterisation and culturing organisms, Section 3.3. Culturing and maintenance of test species for information on test organisms used in this study and the culture conditions used for culturing H. vulgaris, D. carinata and P. australiensis.

4.2.4. T-AgNP temporal stability and dissolution in the test media

The stability of T-AgNPs in different media and MilliQ water was investigated as described by Tejamaya et al. (2012a) with some modifications. NP solutions of 5 mg L\(^{-1}\) were incubated in glass vials for 96 h under similar conditions to the acute toxicity tests. The surface plasmon resonance (SPR), hydrodynamic diameter (HDD), zeta potential and pH of the suspensions were monitored at 0, 24, 48 and 96 h. Release of Ag\(^+\) ions from T-AgNPs in each medium was investigated as described by Xia et al. (2008) with some modifications. Briefly, NP suspensions were sampled by extracting 1 mL into Eppendorf tubes and centrifuging for 15 minutes at 21000 g in a Sigma 3-KL centrifuge. The tubes were carefully removed, and 0.75 mL of the supernatant was transferred to 15 mL tubes. Samples were diluted with MilliQ water and acidified (2% HNO\(_3\)) with HNO\(_3\) before ICP-MS (7700X, Agilent Technologies) analysis to measure Ag\(^+\) ion concentrations. The percentages of soluble Ag to the nominal concentrations of AgNP suspensions were calculated (Zhao and Wang, 2012a).
4.2.5. *Hydra acute toxicity test*

Acute tests were conducted as per the methodology described by Trottier et al. (1997) in the hydra medium described above. The definitive test concentrations for all acute tests were selected based on the data obtained from range finding tests (data not shown). The toxicity of Ag\(^+\) ions was significantly higher than AgNPs and therefore, two different range of concentrations were used for toxicity tests. The exposure tests were conducted in 12 well plates in triplicate as each well contains 4 mL test solution. The T-AgNP concentrations tested were 10.8, 27, 54, 108, 216, 539.3, 1078 and 2157 µg L\(^{-1}\) plus a blank control (0 µg L\(^{-1}\)). For each concentration, one Petri dish with the same test solution concentration were used as intermediate “transfer wells” to minimize dilution of test concentrations (Trottier et al., 1997). Ethylenediaminetetraacetic acid (EDTA) is a chelating agent involved in metal complexation which may affect the toxicity (O’Brien et al., 1990, Nugegoda and Rainbow, 1988, Smékalová et al., 2018). Therefore, the experiment was repeated for hydra for the same AgNP concentration range in the absence of EDTA in the medium. The tests were conducted in the dark with gentle agitation for a period of 96 h. Similarly, the concentrations tested for toxicity of Ag\(^+\) ions in the hydra medium in the presence and absence of EDTA were 0.94, 1.9, 3.8, 7.5, 15, 30, 50 and 100 µg L\(^{-1}\). At time 0, all the organisms were observed under the microscope at 6 to 10 times magnification to make sure all organisms were healthy. Any hydra with clubbed or shortened tentacles were replaced with healthy individuals with extended tentacles which were approximately as same length as the body column. The morphological changes in hydra were observed at 24, 48, 72 and 96 h and any hydra at a tulip stage were considered dead (Trottier et al., 1997).

4.2.6. *Daphnid acute toxicity test*

Semi-static acute toxicity testing of T-AgNP to *D. carinata* was conducted based on organisation for economic co-operation and development (OECD) guidelines (OECD, 2004b). ASTM standard medium was used and six concentrations (10, 20, 40, 60, 80 and 100 µg L\(^{-1}\)) of T-AgNP plus a blank control (0 µg L\(^{-1}\)) were used in quadruplicate. Toxicity of Ag\(^+\) ions to daphnids was assessed with the Ag\(^+\) ion concentrations of 0.2, 0.4, 0.6, 0.8, 1.0 and 1.2 µg L\(^{-1}\). Less than 24 h old daphnid neonates from the third brood progeny were used for experiments and each replicate had 6 neonates in a 40 mL glass vial containing 15 mL of test solution. The daphnids were not fed during the 48 h testing period. They were considered immobile if they could not move even after a gentle agitation and immobilization was recorded after 24 and 48
h. The toxicity tests were considered valid if the mortality was not greater than 10% in the control and the DO concentration in all the test solutions exceeded 3 mg L\(^{-1}\).

4.2.7. Shrimp acute toxicity test

Acute toxicity of T-AgNPs to \(P. australiensis\) was assessed by exposing them to 8 concentrations of T-AgNPs (5.4, 10.8, 21.6, 43.2, 75.5, 107.9, 539.3 and 1078.7 µg L\(^{-1}\)) plus a blank control (0 µg L\(^{-1}\)). The shrimps (10 – 15 mm long) were exposed for 96 h in triplicate under static conditions with no replacement of the test solution. Each replicate had 10 animals in an aquarium containing 2 L of test solution and each aquarium was covered with a glass lid to reduce volatilization. The temperature was maintained at 15 ±1 °C and a light intensity of 800 lux at the surface of the water with a 16 : 8 light : dark cycle. Mortality was defined as lack of movement or response to gentle prodding (ASTM, 1998). Dead animals were removed every 12 h and the individual survival was assessed by counting the total number of dead animals at 24, 48, 72 and 96 h. pH, conductivity and dissolved oxygen (DO) were measured daily. The toxicity tests were considered valid if the mortality was not greater than 10% in the control and the DO level in all the test solutions exceeded 60% saturation. Toxicity of Ag\(^{+}\) ions to shrimps was assessed with the Ag\(^{+}\) ion concentrations of 0.94, 1.9, 3.8, 7.5, 15 and 30 µg L\(^{-1}\).

4.2.8. Deriving the species sensitivity distribution

For deriving the SSD, experimental toxicity data (LC\(_{50}\)) of the present study and additional toxicity data retrieved from other published studies (amphibian, algae, bacteria, fish, nematodes, protozoa, and yeast) were used. Multiple toxicity data for the same species were summarised as geometric means. The SSD plots that show the proportion of species affected at different exposure levels in laboratory toxicity tests were developed by using the CADDIS SSD generator (Barron et al., 2013, Garner et al., 2015a) that was downloaded from https://www.epa.gov/caddis-vol4/caddis-volume-4-data-analysis-download-software. The SSD toolbox that fits distributions to acute toxicity data was downloaded from https://www.epa.gov/endangered-species/provisional-models-endangered-species-pesticide-assessments.

4.2.9. Data analysis

The median lethal concentrations (LC\(_{50}\)) were obtained using the Trimmed Spearman-Karber method (Hamilton et al., 1977). This method is not subject to the problems of Probit
and Logit models and has good statistical properties, is easy to use and recommended for
accurate and precise calculations of LC_{50} values and their 95% confidence interval end points
(Hamilton et al., 1977). All statistical analyses were based on a 0.05 significance level and are
reported as the value with the range calculated as the standard deviation (± SD). Burr
distribution and the maximum likelihood method were chosen for calculating the HC_{5} value
based on the highest probability value generated from Chi square goodness of fit test. The HC_{5}
was determined based on SSDs at a 95% protection level.

4.3. Results and discussion

4.3.1. Characteristics of NPs

The UV-visible absorbance spectra of the tyr-AgNPs (Fig 4.1A) shows an SPR band at
406 nm, which is characteristic of AgNPs, while the peak at approximately 272 nm is due to
the tyrosine coating (Daima et al., 2014, Selvakannan et al., 2004). The presence of NPs was
further confirmed by TEM images. Also, TEM images revealed that the NPs were spherical in
shape and were reasonably uniform in size. The HDD was 86.8 nm (Fig. 4.1B) while core size
determined by TEM was ~30 nm in diameter (Fig. 4.1C). The negative charge of the AgNPs, -
42.4 mV (Fig. 4.1D), as determined by zeta potential suggest that tyrosine molecules formed
strong coatings on the AgNPs (Daima et al., 2014).

4.3.2. T-AgNP stability and dissolution

T-AgNP suspensions were yellowish in colour initially with absorption peaks at 404, 402 and 406 nm in hydra, daphnid and shrimp media, respectively. Visual observation revealed
that the colour of NP suspension in hydra medium changed to grey within a few minutes (< 10
min) and in daphnid medium changed to grey within a few hours (< 3 h). The colour in MilliQ
water was stable even after 96 h. SPR (Fig. 4.2A, 4.2B, 4.2C and 4.2D) revealed that the initial
(< 2 min) peak absorbance values in hydra (0.33) and daphnid media (0.50) were considerably
lower than that in MilliQ water (0.77) and led to emergence of a second absorbance peak
(broader) at higher wavelengths. The absorption peak in hydra medium completely disappeared
within the first 24 h. There was a significant reduction of peak height in daphnid (53%) and
shrimp (32%) media while in the case of MilliQ water only a marginal (~1%) decrease was
observed. A further 12% decrease in the SPR peak was observed in all media and MilliQ water
at 72 h. The HDD of T-AgNPs increased significantly in the hydra and daphnid media
approximately by 3.5 and 6.5 times respectively in less than 5 min and daphnid media
approximately by 3.5 and 6.5 times respectively in less than 5 min and approximately by 15 and 18 times respectively after 24 h (Table 4.1). The percentage intensity of different sizes (data not shown) in both media (weighted according to the scattering intensity of each particle fraction) revealed that the majority (90%) of aggregates remained in sub-micron level (100 – 1000 nm) after 96 h. Less than 1.5 times size increase was observed in shrimp medium and MilliQ water at any time point. Percentage of aggregates at micrometre size in both shrimp medium and MilliQ water did not exceed 2.5% at any given time. Immersion of AgNPs in hydra, daphnid and shrimp media led to a considerable immediate decrease (34 – 67%) in zeta potential of NPs compared with the zeta potential of NPs in MilliQ water (Table 4.2). However, the decrease was 1 to 8% only in the next three days. In contrast, zeta potential changed only by 13% in MilliQ water at 4 days. Change of HDD and zeta potential of NPs in hydra and daphnid media and MilliQ water were in good agreement with changes observed in SPR spectra and colour. Change of HDD of NPs in shrimp medium was in good agreement, but the significant decrease in zeta potential does not correlate with the other changes observed. Percentage dissolution of Ag ions after 96 h was 0.14, 0.26 and 0.13%, respectively in hydra, daphnid and shrimp media while it was 0.97% in the MilliQ water (Table 4.3).

Fig. 4.1: (A) UV-vis spectra of the T-AgNPs with a peak at 406 nm, (B) SPR spectra of the T-AgNPs indicating hydrodynamic diameter of 86.82 nm, (C) TEM image of T-AgNPs indicating core size of ~30 nm (Scale bar represents 0.2 µm), and (D) Zeta potential of the T-AgNPs was -42.4 mV.
Fig. 4.2: The effect of exposure time on the SPR of T-AgNPs in (A) MilliQ water, (B) hydra, (C) daphnid, and (D) shrimp media.

Table 4.1: Effect of exposure time on HDD of T-AgNPs in different media and MilliQ water. Values are mean of triplicate determinations ± standard deviation.

<table>
<thead>
<tr>
<th>Media</th>
<th>5 min (nm)</th>
<th>24 h (nm)</th>
<th>48 h (nm)</th>
<th>96 h (nm)</th>
<th>&lt;5 min (pdi)</th>
<th>96 h (pdi)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MilliQ</td>
<td>89.9 ± 4.6</td>
<td>93.8 ± 2.7</td>
<td>96.9 ± 1.8</td>
<td>116.7 ± 12.2</td>
<td>0.30 ± 0.01</td>
<td>0.45 ± 0.01</td>
</tr>
<tr>
<td>Hydra</td>
<td>170.6 ± 1.2</td>
<td>772.5 ± 6.0</td>
<td>841.9 ± 19.3</td>
<td>843.8 ± 24.9</td>
<td>0.32 ± 0.01</td>
<td>0.71 ± 0.01</td>
</tr>
<tr>
<td>Daphnid</td>
<td>326.1 ± 8.2</td>
<td>934.0 ± 45.4</td>
<td>955.7 ± 14.0</td>
<td>1054.6 ± 67.1</td>
<td>0.53 ± 0.08</td>
<td>0.65 ± 0.02</td>
</tr>
<tr>
<td>Shrimp</td>
<td>91.2 ± 8.5</td>
<td>105.5 ± 6.5</td>
<td>107.6 ± 6.0</td>
<td>121.2 ± 7.4</td>
<td>0.44 ± 0.05</td>
<td>0.42 ± 0.02</td>
</tr>
</tbody>
</table>

Table 4.2: Effect of exposure time on zeta potential of T-AgNPs in different media and MilliQ water. Values are mean of triplicate determinations ± standard deviation.

<table>
<thead>
<tr>
<th>Media</th>
<th>5 min (mV)</th>
<th>24 h (mV)</th>
<th>48 h (mV)</th>
<th>96 h (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MilliQ</td>
<td>-42.1 ± 0.3</td>
<td>-38.9 ± 2.5</td>
<td>-37.7 ± 1.5</td>
<td>-36.5 ± 2.5</td>
</tr>
<tr>
<td>Hydra</td>
<td>-13.8 ± 0.1</td>
<td>-11.6 ± 0.3</td>
<td>-11.8 ± 0.3</td>
<td>-10.6 ± 0.3</td>
</tr>
<tr>
<td>Daphnid</td>
<td>-27.5 ± 0.2</td>
<td>-26.8 ± 0.4</td>
<td>-27.2 ± 0.8</td>
<td>-26.8 ± 0.6</td>
</tr>
<tr>
<td>Shrimp</td>
<td>-24.7 ± 1.1</td>
<td>-24.4 ± 0.4</td>
<td>-22.8 ± 1.1</td>
<td>-22.1 ± 0.6</td>
</tr>
</tbody>
</table>
Table 4.3: Influence of exposure time on percentage of soluble Ag⁺ released from T-AgNPs in different media and MilliQ water. Values are mean of triplicate determinations ± standard deviation.

<table>
<thead>
<tr>
<th>Media</th>
<th>24 h</th>
<th>48 h</th>
<th>96 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>MilliQ</td>
<td>0.04 ± 0.07</td>
<td>0.47 ± 0.22</td>
<td>0.97 ± 0.04</td>
</tr>
<tr>
<td>Hydra</td>
<td>0.08 ± 0.08</td>
<td>0.05 ± 0.07</td>
<td>0.14 ± 0.13</td>
</tr>
<tr>
<td>Daphnid</td>
<td>0.06 ± 0.23</td>
<td>0.01 ± 0.07</td>
<td>0.26 ± 0.11</td>
</tr>
<tr>
<td>Shrimp</td>
<td>0.06 ± 0.20</td>
<td>0.02 ± 0.09</td>
<td>0.13 ± 0.08</td>
</tr>
</tbody>
</table>

Fate and behaviour of NPs in the media affect bioactivity and bioavailability. Achieving a constant concentration of AgNPs in the media or actual measurement is challenging. However, monitoring certain attributes such as particle agglomeration size, SPR and dissolution can favour understanding of observed toxicity. Appearance of additional and broader absorbance peaks, rapid absorbance loss, increased particle size and colour change are indicative of formation of aggregates (Stebounova et al., 2011, Römer et al., 2011, Tejamaya et al., 2012a). Results indicate that NPs highly aggregated in the hydra and daphnid media while it was marginal in shrimp medium. No aggregation was observed in MilliQ water. Decreased zeta potential values in the media with high ionic strength compared to MilliQ water with relatively low ionic strength are in accordance with the classical colloid theory (Hunter, 1981, Elimelech et al., 1995). A reduction of the thickness of the diffuse double layer with increased ionic strength allows for the attractive van der Waals interactions to dominate and increases the particle-particle interaction resulting in increased aggregation (Badawy et al., 2010, Brant et al., 2005a, Sharma, 2009). Aggregation of NPs affect the bioavailable concentration of NPs (Tejamaya et al., 2012a, Römer et al., 2011) and hence, inter alia plays an important role in potential toxicity (Park et al., 2015, Reidy et al., 2013, Völker et al., 2013, Carnovale et al., 2016). Smaller particles can penetrate in to the body more easily (Smékalová et al., 2018) and release more Ag⁺ ions due to higher surface area than larger particles (Zhao and Wang, 2012a, Allen et al., 2010). The observed insignificant ion release from AgNPs in the media in this study may also be due to the aggregation. SPR and HDD data from our study show that T-AgNPs aggregate fast in the hydra medium, forming a bigger fraction (85%) of aggregates with HDD being larger than 650 nm. This leads to deposition of particles making them less bioavailable in a short period of time. Daphnids and shrimps can eat precipitated NPs
on the bottom, increasing their respective exposure rates, but hydras are not able to do that. Another reason for lower toxicity from T-AgNPs and Ag+ ions to hydra is the less bioavailability of NPs in the medium due to aggregation and precipitation.

4.3.3. Comparison of T-AgNPs and Ag+ ion toxicity to hydra, daphnids and shrimps

Our data suggest that the acute toxicity of T-AgNPs and Ag+ ions to the three species tested is considerably different (Table 4.4). The results of our study indicated that the toxicity of Ag+ ions to all three species was higher than the toxicity of the T-AgNPs. In the presence of EDTA, Ag+ ions were 25 times more toxic to hydra at all time points. This was 27 times in the absence of EDTA at 24h, but the ratio decreased to 10.5 at 48 h since the toxicity of AgNPs increased with time. Due to similar reason, the Ag+ ions were 90 times more toxic to daphnids at 24 h but decreased to 57 times at 48 h. Interestingly, the toxicity of Ag+ ions to shrimps increased from 17 times at 24 h to 553 times at 48 h compared with AgNPs due to the toxicity of Ag+ ions increased with time. T-AgNPs were 30 and 55 times more toxic to D. carinata than to hydra when 24 h and 48 h LC50 values are compared. However, this gap reduced to 11 and 7 times respectively when compared with the LC50 values for hydra in the medium without EDTA. In a similar pattern, Ag+ ions were 150 and 130 times toxic to D. carinata than to hydra when 24 h and 48 h LC50 values are compared, but the gap reduced to 37 and 41 times when EDTA was absent in hydra medium. The toxicity of T-AgNPs to P. australiensis (LC50: 62.2 µg L⁻¹) was similar to that of D. carinata (LC50: 62.0 µg L⁻¹) in the first 24 h though they were more toxic to daphnids at 48 h (LC50: 35.4 µg L⁻¹) compared to shrimps (LC50: 55.3 µg L⁻¹). Interestingly, though Ag+ ions were 5 times more toxic to daphnids than shrimps at 24 h, they were 6 times more toxic to shrimps (LC50: 0.1 µg L⁻¹) than to daphnids (LC50: 0.62 µg L⁻¹) at 48 h. Overall, the toxicity of T-AgNPs and Ag+ ions to the three species was respectively in the order of daphnids > shrimps > hydra and shrimps > daphnids > hydra.

Bondarenko et al. (2013) summarized LC50 values of AgNPs (48 h LC50: 1.0 – 40.0 µg L⁻¹) from 17 previous studies with a median value of 10 µg L⁻¹ and LC50 values of Ag+ ions (48 h LC50: 1.0 – 9.40 µg L⁻¹) from 8 studies with a median value of 0.85 µg L⁻¹. The authors could find only one study where 24 h LC50 (3.5 – 10.8 µg L⁻¹) and 48 h LC50 (1.75 – 4.61 µg L⁻¹) values of AgNPs using D. carinata were reported (Qin et al., 2015). These researchers exposed daphnids in two different media to AgNPs synthesized with the fungus Gibberella sp. and the HDD of the NPs were between 20 to 50 nm. The 48 h LC50 values obtained for D. carinata in our study (35.4 µg L⁻¹) is in general agreement with LC50 values reported by others for Daphnia.
though it is considerably less sensitive to T-AgNPs in comparison with the values obtained by Qin et al. (2015) for *D. carinata*. This may be due to different physicochemical characteristics of AgNPs tested and different chemical characteristics of the media used. This is the first study which used *P. australiensis* to assess NP toxicity. Similar toxicity of AgNPs to both shrimps and daphnids at 24 h, but higher toxicity to daphnids at 48 h compared with shrimps may be due to their different food habits. Daphnids being filter feeders take up larger volumes of the aqueous medium by habit as opposed to shrimps which are detritus feeders and utilise water only for respiration. Higher toxicity of Ag$^+$ ions to daphnids at 24 h, but less toxicity at 48 h compared with shrimps may be a result of a change in the interaction of Ag$^+$ ions with other trace elements in the daphnid medium over time changing the bioavailability of Ag$^+$ ions (Wasmuth et al., 2016). Also, higher toxicity of AgNPs to daphnids at 48 h in contrast to shrimps which were more sensitive to Ag$^+$ ions at 48 h is attributed to different mechanisms of toxicity on two organisms. Though hydra is a simple diploblastic organism with two simple cell layers in their body, the entry of the tested toxicants across their ectodermal cells may be less than across the gills of daphnids and shrimps, resulting in the lower toxicity of T-AgNP and Ag$^+$ ions to hydra. Hydra is a predator and hence does not uptake the aquatic medium as much as daphnids that are filter feeders resulting in less exposure to the toxicants as a result.

It is well understood that soluble Ag compounds are highly toxic to aquatic organisms and best explained by the biotic ligand model (Bianchini and Wood, 2003). However, the mechanisms of how AgNPs are toxic to organisms are still in debate and there are divergent opinions on the effects (Völker et al., 2013). Several researchers have shown that the dissolved ions from the NPs is the dominant cause for acute toxicity (Li et al., 2015a, Sakamoto et al., 2015, Shen et al., 2015) while some have claimed that the toxicity is attributed not only to the liberated ions from AgNPs, but also to the NPs themselves (Lapresta-Fernández et al., 2012, Davoudi et al., 2014, Navarro et al., 2008b). Corresponding Ag$^+$ ion concentrations at LC$_{50}$ values of T-AgNPs, calculated based on percentage dissolution are lower than the LC$_{50}$ values of Ag$^+$ ions. This indicates that there is a synergistic effect from both T-AgNPs and Ag$^+$ ions in causing toxicity. Certain aquatic organisms such as algae alter the toxicity of NPs by releasing exopolymeric substances (Zhou et al., 2016) which is not expected with the tested organisms in the current study. However, reduced Ag concentrations in the media due to sorption of NPs to the organisms or excreta cannot be excluded (Bone et al., 2012).
Table 4.4: *H. vulgaris*, *D. carinata* and *P. australiensis* lethality (LC$_{50}$) for T-AgNPs and Ag$^+$ ($\pm$ 95% confidence interval, n = 3) (CI: Confidence interval, ND: Not defined, NT: Not tested).

<table>
<thead>
<tr>
<th>Organism</th>
<th>Substance</th>
<th>24 h LC$_{50}$</th>
<th>48 h LC$_{50}$</th>
<th>96 h LC$_{50}$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>µg L$^{-1}$ (95% CI)</td>
<td>µg L$^{-1}$ (95% CI)</td>
<td>µg L$^{-1}$ (95% CI)</td>
<td></td>
</tr>
<tr>
<td>Hydra</td>
<td>T-AgNPs (EDTA)</td>
<td>1941.66 (ND)</td>
<td>1941.66 (ND)</td>
<td>1941.66 (ND)</td>
</tr>
<tr>
<td></td>
<td>Ag$^+$ (EDTA)</td>
<td>80.0 (75 - 95)</td>
<td>80.0 (75 - 95)</td>
<td>80.0 (75 - 95)</td>
</tr>
<tr>
<td></td>
<td>T-AgNPs (no EDTA)</td>
<td>690.37 (388.33 - 1078.7)</td>
<td>269.67 (ND)</td>
<td>269.67 (ND)</td>
</tr>
<tr>
<td></td>
<td>Ag$^+$ (no EDTA)</td>
<td>25.6 (14.4 - 48.4)</td>
<td>25.6 (14.4 - 48.4)</td>
<td>25.6 (14.4 - 48.4)</td>
</tr>
<tr>
<td>Daphnid</td>
<td>T-AgNPs</td>
<td>62.04 (54.53 - 69.76)</td>
<td>35.48 (29.02 - 42.19)</td>
<td>NT</td>
</tr>
<tr>
<td></td>
<td>Ag$^+$</td>
<td>0.69 (0.64 - 0.73)</td>
<td>0.62 (0.56 - 0.67)</td>
<td>NT</td>
</tr>
<tr>
<td>Shrimp</td>
<td>T-AgNPs</td>
<td>62.24 (44.34 - 49.51)</td>
<td>55.34 (39.48 - 76.7)</td>
<td>55.34 (39.48 - 76.7)</td>
</tr>
<tr>
<td></td>
<td>Ag$^+$</td>
<td>3.6 (1.6 - 10)</td>
<td>0.1 (0.1 - 0.9)</td>
<td>0.1 (0.1 - 0.9)</td>
</tr>
</tbody>
</table>

4.3.4. Effect of media and exposure time on toxicity of T-AgNPs

The 24, 48 and 96 h LC$_{50}$ values of T-AgNPs to *H. vulgaris* were similar (1941.66 µg L$^{-1}$ in the presence of EDTA and 690.37, 269.67 and 269.67 µg L$^{-1}$ in the absence of EDTA. The 96 h LC$_{50}$ values of Ag$^+$ ions at each time point were similar in the presence (80.0 µg L$^{-1}$) and absence (25.6 µg L$^{-1}$) of EDTA. However, NP toxicity to *D. carinata* approximately doubled from 24 h (LC$_{50}$: 62.0 µg L$^{-1}$) to 48 h (LC$_{50}$: 35.4 µg L$^{-1}$) while there was no significant change observed for Ag$^+$ ions toxicity from 24 h (LC$_{50}$: 0.69 µg L$^{-1}$) to 48 h (LC$_{50}$: 0.62 µg L$^{-1}$). Toxicity of NPs to *P. australiensis* only slightly increased from 24 h (LC$_{50}$: 62.2 µg L$^{-1}$) to 96 h (LC$_{50}$: 55.3 µg L$^{-1}$). Conversely, the toxicity of Ag$^+$ ion increased by 36 times from 24 h (LC$_{50}$: 3.6 µg L$^{-1}$) to 96 h (LC$_{50}$: 0.1 µg L$^{-1}$). To summarize, a significant increase in toxicity of T-AgNPs and Ag$^+$ ions with time was observed for daphnids and shrimps respectively. T-AgNPs were effectively toxic to hydra and shrimps within 24 h only and thereafter, no toxic responses were observed.

The composition and the pH of the media affect the ecotoxicological potential of NPs (Metreveli et al., 2016, Seitz et al., 2015b). The hydra medium Trottier et al. (1997) used contains EDTA. The hydra toxicity test was extended to see the effect of EDTA on toxicity by
exposing hydra to both T-AgNPs and Ag\(^+\) ions in the absence of EDTA. The LC\(_{50}\) values at each time point for both AgNPs and Ag\(^+\) ions in the absence of EDTA are less than the corresponding LC\(_{50}\) values in the media with EDTA. EDTA chelates metals which may cause reduced toxicity due to decreased rate of metal uptake (O’Brien et al., 1990). Therefore, lower toxicity of both T-AgNPs and Ag\(^+\) ions in the EDTA containing hydra medium is mainly attributed to the complexation of Ag\(^+\) ions by EDTA. Also, hydra in the medium with EDTA are more robust than in the medium without EDTA since the former is the recommended recipe for optimum growth of hydra (Trottier et al., 1997). Some of the ions released are trapped by the halides (e.g. Cl\(^-\)) present in the media making insoluble precipitations (Römer et al., 2011) resulting in less detectable free ions. Precipitation of Ag\(^+\) ions as AgCl by high concentration of Cl\(^-\) in the hydra medium is also a possible factor for observed less toxicity of AgNPs and Ag\(^+\) ions to hydra. AgNPs oxidize more easily with increasing pH resulting in higher dissolution (Zhao and Wang, 2012a, Liu and Hurt, 2010a). Higher pH of the media compared with MilliQ (pH: 6.44) water is a possible factor for observed lower dissolution of Ag\(^+\) ions in media.

4.3.5. Hazardous concentration (HC) of coated AgNPs and Ag\(^+\) ions

There are several methodologies which are employed by regulatory bodies in different countries to derive HC\(_5\) values and we chose the species sensitivity distribution (SSD) approach. Since we only had acute toxicity test results, the USEPA protocol was adopted since it supports using acute toxicity values (Kwak et al., 2016). To predict more accurate HC\(_5\) values, toxicity values of sufficient species should be available representing a comprehensive ecosystem. USEPA (1985) recommends having results of acceptable acute tests with 8 different families to derive the SSD for freshwater aquatic organisms. Since we had acute toxicity data of only three families, more data were sourced from published journal papers and the ETOX database (ETOX, 2018). SSDs are models of variance and the model fits of the probability distributions were excellent with \(R^2\) values of 0.98 and 0.93 for AgNO\(_3\) and AgNPs respectively (Fig. 4.3 and 4.4). The HC\(_5\) value obtained for Ag\(^+\) ions and AgNPs in this study were 0.51 and 18.41 \(\mu\text{g L}^{-1}\).

The HC\(_5\) value we found (0.51 \(\mu\text{g L}^{-1}\)) for Ag\(^+\) ions based on the acute toxicity values in this study and 12 other freshwaters species is about 6 times lower than the USEPA Criterion Maximum Concentration (CMC) value (3.2 \(\mu\text{g L}^{-1}\)) (USEPA, 1985). However, it is 2 and 10 times higher than the recommended long-term freshwater Canadian water quality guideline.
(CWQG) for ionic silver (0.25 µg L\(^{-1}\)) (CCME, 2015) and the Australia and New Zealand’s water quality guideline (ANZWQG) for ionic silver (0.05 µg L\(^{-1}\)) (ANZECC, 2000) respectively. Relatively higher ionic silver guideline values by USEPA is likely because CMC values reflect only acute effects in contrast to lower values by CWQG and ANZWQG which use chronic toxicity data (Kwak et al., 2016). The HC\(_5\) value (0.51 µg L\(^{-1}\)) for ionic silver from this study is lower than the reported values by Garner et al. (2015b) (~1.0 µg L\(^{-1}\)) and Chen et al. (2018) (3.6 and 5.7 µg L\(^{-1}\)). Ionic silver is significantly toxic compared to the HC\(_5\) values reported for other metallic ions (10 – 4000 µg L\(^{-1}\)) (Chen et al., 2018, Garner et al., 2015a). The HC\(_5\) value derived in this study for ionic silver is 36 times lower than the HC\(_5\) value obtained for AgNPs (18.41 µg L\(^{-1}\)). The HC\(_5\) value derived in this study for AgNPs is less than the HC\(_5\) value reported by Kwak et al. (2016) for polyvinylpyrrolidone (PVP) coated AgNPs (31.73 µg L\(^{-1}\)). However, Chen et al. (2018) reported comparatively lower HC\(_5\) values for PVP coated (1.1 µg L\(^{-1}\)), sodium citrate coated (3.0 µg L\(^{-1}\)) and uncoated (6.3 µg L\(^{-1}\)) AgNPs. The reported HC\(_5\) values for other metallic NPs fall in the range of 22 to 4000 µg L\(^{-1}\).

Fig. 4.3: Species sensitivity distribution (SSD) of AgNO\(_3\) acute toxicity derived by the CADDIS SSD generator and SSD toolbox using the acute toxicity values of fifteen species.

There is a growing interest in using SSDs to derive hazard levels of contaminants. SSD approach has significant influence on assessment and decision making of chemical exposure to ecosystems (Belanger et al., 2017). The risk assessment approaches of NPs have not been tested and validated yet (Mattsson and Simkó, 2017). There are many reasons for the
uncertainty in measuring the hazard of AgNPs such as broad range of parameters of NPs, indirect effects, lack of information, modifications of NPs in the environment, questionable validity and methods of test systems (Mattsson and Simkó, 2017). Several researchers highlighted the importance of SSDs in deriving HCs to protect native fauna from NPs (Adam et al., 2015b, Garner et al., 2015b, Kwak et al., 2016). Traditionally, SSDs were derived using the toxicity values based on the effects of the contaminant on organisms at different hierarchies. Experiments with several species with the same batch of each nanoparticle is challenging and therefore, it is not possible to safeguard the biota by concentrating on specific NPs. In line with this, several researchers (Adam et al., 2015b, Garner et al., 2015b) have sourced toxicity values from literature to derive SSDs. Further, the accuracy of HCs predicted will increase when more data are available (Garner et al., 2015b). Since this is the only study with tyrosine coated AgNPs, we sourced the toxicity values of AgNPs from published literature. However, this will bring some uncertainty due to several variabilities such as different exposure conditions, physicochemical characteristics of NPs, which has caused to question the applicability of using SSDs for deriving HCs for NPs (Adam et al., 2015b, Garner et al., 2015b). Better estimates can be obtained by developing SSDs based on grouping of NPs (Garner et al., 2015b, Gottschalk and Nowack, 2013). However, Chen et al. (2018) did not observe a significant difference in the derived HC5 values for AgNPs based on the size, shape and exposure duration. This similarity was attributed to the transformation of particles in the medium and the biological effects are likely to result from the release of ions. Also, chronic sublethal effects have enormous sublethal effects on organisms (Garner et al., 2015b) and thus, chronic test results should be used in developing SSDs. However, studies on chronic end points are scarce in comparison to the acute test results which leads to a bias towards SSDs based on acute test data. Therefore, more useful chronic tests are recommended to generate SSDs. Besides, as observed in this study, it is acknowledged the difficulty in maintaining constant NP concentrations in the test media for such experiments.

This study shows the different sensitivity to tyrosine coated AgNPs across species using Australian native species and highlights the importance of considering species with different life habits in assessing toxicity of NPs. There is a considerable difference in fate and behaviour of T-AgNPs in different media and quantification of such changes is important when performing and interpreting toxicity assessments. Also, it appreciates the importance of SSD in evaluating HC levels of differently coated NPs and adds new knowledge which will be important in AgNP risk assessment (Posthuma et al., 2001) and setting up water quality
guidelines (Smetanová et al., 2014). Based on the results, the authors recommend further NP exposure studies for differently coated NPs using a broad range of freshwater aquatic species with different life habits to assess the toxicity of the range of novel manufactured NPs.

![Species sensitivity distribution (SSD) of AgNP acute toxicity derived by the CADDIS SSD generator and SSD toolbox using the acute toxicity values of eight species.](image)

**Fig. 4.4**: Species sensitivity distribution (SSD) of AgNP acute toxicity derived by the CADDIS SSD generator and SSD toolbox using the acute toxicity values of eight species.

### 4.4. Conclusions

Freshwater aquatic organisms are potentially exposed to AgNPs and Ag$^+$ ions. The objectives of the present study were to examine the toxicity of tyrosine-coated AgNPs (T-AgNPs) and Ag$^+$ ions to three freshwater invertebrates at different trophic levels, to compare species sensitivity and the influence of culture media on the stability of AgNPs. The toxicity of T-AgNPs and Ag$^+$ ions to the three species was respectively in the order of daphnids > shrimps > hydra and shrimps > daphnids > hydra. Fate and behaviour of AgNPs and Ag$^+$ ions were dependent on the test media and should be considered a key factor in assessing NP toxicity. To protect aquatic organisms from AgNP exposures, it is necessary to set water quality guidelines based on data gathered from toxicity studies relevant to different species in such environments. Assessing the sensitivity of species with different life habits and native species to AgNPs and Ag$^+$ ions are highlighted.
Chapter 5. The toxicity of coated silver nanoparticles to the alga Raphidocelis subcapitata

This chapter has been submitted to a journal and is presented here with only minor modifications to adjust formatting to the requirements of the thesis.


Abstract

The use of silver nanoparticles (AgNPs) is growing exponentially, especially in consumer products due to their excellent antimicrobial properties. However, concerns are growing on their possible negative effects on environmental and human health. AgNPs from consumer products enter aquatic ecosystems where their physicochemical properties including surface functionalization are critical to their impact on aquatic organisms. The effects of AgNPs coated with three different ligands; tyrosine (T-AgNP), epigallocatechin gallate (E-AgNP) and curcumin (C-AgNP) and Ag+ ions on the freshwater green alga Raphidocelis subcapitata was investigated. Stability tests of AgNPs revealed that the coating significantly affects the fate and behaviour of AgNPs. All types of AgNPs and ionic silver (Ag+ ions) were found to be toxic to the alga and differential growth inhibition of algae were observed from differently coated AgNPs, with the 48 h EC50 of T-AgNPs, E-AgNPs and C-AgNPs being 0.163, 0.243 and 0.155 mg L⁻¹ respectively in comparison to 0.051 mg L⁻¹ for Ag+ ions. Associated Ag in the algae increased with increased concentrations of all AgNPs and Ag+ ions and the toxicity positively correlated to the associated Ag content in algae. The antioxidant enzymes glutathione S-transferase and catalase were activated in algal cells by the AgNPs and Ag+ ions, but a consistent difference in response was not identified with different concentrations of NPs. This study confirms the ecotoxicity of AgNPs to algae and the importance of surface functionalization on their effects, highlighting the importance of considering the type of coating on AgNPs in environmental risk assessment.

5.1. Introduction

Nano-silver is already highly commercialized, and its applications are growing, mainly due to its excellent antimicrobial qualities. It is one of the most widely used nanoparticles (NPs) (Zhang et al., 2015) occurring in 25% of all nano-based products (Bundschuh et al., 2018,
Vance et al., 2015). The Nanodatabase (Nanodatabase, 2018) currently lists more than 500 commercial products that contain silver nanoparticles (AgNPs) which are available to the European market. Healthcare, electronics, textile and food and beverage industries are the largest segments of AgNPs (Vance et al., 2015, Yu et al., 2013, McGillicuddy et al., 2017, Zhang et al., 2016a, Grandviewresearch, 2015). Their global consumption is estimated to rise to 450 metric tonnes per year (Lazareva and Keller, 2014) and the global market for Ag NPs is expected to reach USD 2.45 billion by 2022 (Grandviewresearch, 2015). AgNPs in most consumer products will end up in the environment including natural aquatic systems (Keller et al., 2013a). Despite many advantages, extensive usage of Ag NPs may cause environmental pollution and concerns are rising about their possible impacts on ecosystem and human health. New uses of AgNPs are continuously being discovered, yet the environmental implications are not fully understood (Keller et al., 2013a).

Algae play an important role in the aquatic ecosystem as the primary producers in the food web (Kusk et al., 2018). They are also known to function as vectors for self-purification of polluted waters (Ji et al., 2011). Primary producers in the aquatic system are vulnerable and are targeted first by contaminants. Any adverse effects to the autotrophs will have an effect on the heterotrophs (Renault et al., 2008, Baker et al., 2014). Several studies have proven that AgNPs are toxic to algae (Bondarenko et al., 2013, Oukarroum et al., 2012, Miao et al., 2009, Ivask et al., 2014b, Sendra et al., 2017b). Also, algae serve as a model organism to assess effects of NPs in aquatic systems (Wang et al., 2011, Bondarenko et al., 2016, OECD, 2011a). Studies on NP toxicity so far concluded that either or both NPs and liberated ions from NPs could cause toxicity (Schultz et al., 2014). Navarro et al. (2008b) studied the effects of short-term toxicity of AgNPs and ionic silver (Ag+ ions) on Chlamydomonas reinhardtii. They concluded that both AgNPs and Ag+ ions cause toxicity. Li et al. (2015b) observed the cell morphology of Euglena gracilis was significantly affected when exposed to AgNPs, but the effects were not visible in the presence of cysteine which is a strong Ag+ ion ligand. From this, the researchers concluded that the effects were mediated by dissolved Ag+ ions.

Physicochemical characteristics of both NPs and the surrounding environment influence the toxicity of NPs (Park et al., 2015, Hartmann et al., 2012, Köser et al., 2017). Size, shape, surface chemistry, chemical composition, surface area, crystal structure are all intrinsic NP properties (Barbero and Yslas, 2016, Shang et al., 2014, Clément et al., 2013a, Qu et al., 2017); ionic strength, pH, the presence of natural organic matter, and hardness are media-
specific properties that affect toxicity of NPs (Li et al., 2013a, Van Hoecke et al., 2008, Römer et al., 2011). Biofunctionalized AgNPs are used in many applications (Ravindran et al., 2013, Shukla et al., 2012, Sindhu et al., 2014, Daima et al., 2014). The effects of this surface functionalization of NPs on their physicochemical characteristics, fate and toxicity to organisms are critical (Baumann et al., 2014, Bozich et al., 2014, Sharma et al., 2014), but not well understood yet (Saei et al., 2017). (Navarro et al., 2015) studied the toxicity of nine differently coated AgNPs to alga Chlamydomonas reinhardtii and found the toxicity was not related to the coating. However, Kalman et al. (2015) observed differential toxicity and bioaccumulation dynamics when the alga Chlorella vulgaris was exposed to differently coated AgNPs. From a similar study, Perreault et al. (2012) found polymer-coated CuONPs were more toxic to Chlamydomonas reinhardtii than bare CuONPs and the increased toxicity of coated NPs was attributed to the higher penetration capacity into the cells than bare NPs.

Uptake of NPs is a crucial factor in assessing the effects of NPs (Ivask et al., 2014a). When Ag NPs are associated with algae, it is possible for them to transfer up the food web (Zhao and Wang, 2010, Lee et al., 2015). Piccapietra et al. (2012) reported intracellular silver accumulation in Chlamydomonas reinhardtii upon exposure to carbonate coated AgNPs and Ag\(^+\) ions. Miao et al. (2010a) reported AgNP internalization in Ochromonas danica with the observation of AgNPs in the vacuole of cells. Kalman et al. (2015) also demonstrated AgNP internalization in alga Chlorella vulgaris while Li et al. (2015b) observed higher amounts of associated silver in Euglena gracilis upon exposure to AgNPs than with exposure to Ag\(^+\) ions. Merdzan et al. (2014) found the surface coatings influenced the bioaccumulation of ZnNPs in the alga Chlamydomonas reinhardtii. It is widely accepted that the AgNPs cause oxidative damage (Oukarroum et al., 2012, Qian et al., 2016, Taylor et al., 2016a). AgNPs cause the generation of reactive oxygen species (ROS) which lead to activation of the antioxidant system resulting in increased production of antioxidants (Melegari et al., 2013, Ivask et al., 2014a). The antioxidant status of an organism can be used to assess oxidative stress and therefore represents a good indicator to evaluate the effects of NPs (von Moos and Slaveykova, 2014).

This study was aimed at understanding the effects of Ag NPs coated with three different organic ligands; tyrosine-coated (T-AgNP), curcumin-coated (C-AgNP) and epigallocatechin gallate-coated (E-AgNP) and Ag\(^+\) ions on the freshwater green alga Raphidocelis subcapitata. These organic ligands act as reducing and stabilizing agents (Hebbalalu et al., 2013) and are widely studied for different applications (Khaskel et al., 2015,
Contino et al., 2016, Selvakannan et al., 2004). The production and characterization of the NPs used were performed in-house and the stability of NPs in the algae medium, their toxicity, bioaccumulation and effects on antioxidant enzyme activity were all assessed upon acute (72 h) exposure of algae to AgNPs and Ag$^+$ ionic suspensions.

5.2. Materials and methods

5.2.1. Test materials

Please see Chapter 3. NP Synthesis, characterisation and culturing organisms, Section 3.1. Synthesis of AgNPs.

5.2.2. Characterization of AgNPs

Please see Chapter 3. NP Synthesis, characterisation and culturing organisms, Section 3.2. Characterisation of AgNPs.

5.2.3. Test organisms and culture conditions

Please see Chapter 3. NP Synthesis, characterisation and culturing organisms, Section 3.3. Culturing and maintenance of test species for information on culturing R. subcapitata which was used in this study.

5.2.4. AgNP temporal stability and dissolution in the test media

The stability of AgNPs in the algae medium and MilliQ water was investigated as previously described (Tejamaya et al., 2012b) with some modifications. Nanoparticle solutions of 5 mg L$^{-1}$ were incubated in glass vials for 72 h at similar environmental conditions to the algae growth inhibition test (please see Section 5.2.5.). The surface plasmon resonance (SPR), hydrodynamic diameter (HDD) and zeta potential of the suspensions were investigated using three independent samples and pH was monitored at 0, 24, 48 and 72 h. The release of Ag$^+$ ions from NPs in the algae medium and MilliQ water was investigated at 0, 24, 48 and 72 as described previously by Xia et al. (2008) with some modifications. Briefly, 1 mL from each NP suspension was extracted into Eppendorf tubes in triplicate and centrifuged at 21,000 rpm for 15 min (Sigma 3-KL centrifuge). Supernatant (0.75 mL) from carefully removed tubes was transferred to 15 mL tubes, diluted with MilliQ water and acidified (2%) with HNO$_3$. The Ag$^+$ ion concentrations were measured by ICP-MS (7700x, Agilent Technologies). The samples
need to be in ionic form prior to entering the mass analyser in order to be detected. The coated NPs were not detectable as validated by independent experiments by using undigested NPs. Therefore, the influence of NPs which would have been present in the supernatant to the readings was assumed negligible.

5.2.5. Algae growth inhibition test

Algae growth inhibition tests with alga *R. subcapitata* were conducted as per the organisation for economic co-operation and development (OECD) guidelines with some adaptations (OECD, 2011b). Algae cells from a pure culture were inoculated into MLA medium (Bolch and Blackburn, 1996) and algal cells were counted periodically. The test was started when the algae growth rate was at the exponential stage. Algal biomass was adjusted to $5 \times 10^4$ cells mL$^{-1}$ in each flask which contains 40 mL of test solution. AgNP test solutions of 0.020, 0.050, 0.080, 0.110, 0.140, 0.170, 0.200 and 0.230 mg L$^{-1}$ were prepared by dispersing relevant volumes of NP stock solutions in algae culture. Ionic silver (Ag$^+$ ions) stock solution was prepared by dissolving Ag$_2$SO$_4$ in MilliQ water and test solutions of 0.010, 0.020, 0.040, 0.060, 0.080, 0.100 and 0.120 mg L$^{-1}$ Ag$^+$ ion concentrations were prepared by dissolving relevant volumes from the stock solution. All treatments were tested in triplicate while six controls with only M4 medium and algae were incubated using the same algal cell concentration.

Flasks were kept on shakers (100 rpm) (OM6, RATEK, Aus) to allow mixing and CO$_2$ diffusion and incubated in a light-temperature controlled chamber under continuous illumination (6000 lux) at 23 ± 1 °C. White fluorescent tubes were used as the light source while the light intensity in the test setup was measured using a LI-COR light meter (model LI-189). Flasks were manually shaken every 24 h to resuspend any settled cells and pH was monitored throughout the testing period. Samples (50 µg L$^{-1}$) were taken from each vessel at 24, 48 and 72 h and the number of cells were quantified by measuring fluorescence intensity as described by Aruoja et al. (2009). Algae samples (50 µL) were added to 200 µL of ethanol in triplicate in a 96 well plate and the plate was shaken for 3 h in the dark. Fluorescence intensity was measured with a fluorescence spectrophotometer (POLARstar omega, BMG Labtech) using an excitation wavelength of 400 ± 80 nm and emission wavelength of 600 ± 80 nm. The nanoparticle suspensions neither fluoresced nor absorbed any light under these conditions. Chlorophyll fluorescence correlated with the cell density which was determined and calibrated by cell counting with a TC20™ Automated Cell Counter, Bio-Rad Laboratories, Hercules, CA.
Microscopic observations were performed to verify the healthy appearance of the inoculum culture and to verify readings inferred with data obtained with fluorescence microscopy. All experiments were conducted under aseptic conditions. The specific growth rate was calculated as the logarithmic increase in the biomass of each single vessel using the following equations:

\[ \mu_{i-j} = \frac{(\ln X_j - \ln X_i)}{(t_j - t_i)} \]

Where, \( \mu_{i-j} \) is the average specific growth rate from time i to time j, \( X_j \) is the biomass at time j and \( X_i \) is the biomass at time i.

The percent inhibition of growth rate for each treatment was calculated as:

\[ \% I_r = \frac{(\mu_c - \mu_T)}{\mu_c} \times 100 \]

Where, \( \% I_r \) is the percent inhibition in average specific growth rate, \( \mu_c \) is the mean value for average specific growth rate in the control group and \( \mu_T \) is the average specific growth rate for the treatment replicate.

The biomass in the control cultures increased exponentially in the range of 17 to 22 within the 72-h test period and the coefficient of variation of average specific growth rates in replicate control cultures were less than 7%, thus fulfilling the OECD validity criteria (OECD, 2011a).

5.2.6 Algae digestion and metal analysis

Algae digestion and metal analysis were carried out as described by Li et al. (2015b) and Miller et al. (2017). Briefly, 30 mL of AgNP exposed algae cultures from each flask were concentrated by centrifugation in 50 mL polypropylene tubes at 3500 g for 15 min (Heraeus Multifuge 1S-R, Thermoscientific) and resuspended in MilliQ water. After 2 wash cycles, algae were washed with 0.5 mM cysteine-MOPS for 5 min to remove metals loosely bound to the surface as described and validated previously (Li et al., 2015b, Piccapietra et al., 2012). Algae were then filtered (SM16510, Sartorius) and acid digested with ultra-pure grade 70% HNO₃ and 30% H₂O₂ at 105 °C for 6 hours. The digested samples were diluted with MilliQ water and the Ag⁺ ion concentrations were measured with ICP-MS. The measured Ag content after wash
cycles was operationally defined as the cell-associated Ag (Ag_{cell}) and expressed as nanograms per cell (ng Cell^{-1}).

5.2.7. **GST and CAT enzyme activity assays**

Enzyme activity assays were conducted as described by Melegari et al. (2013) with some modifications. Aliquots of 200 mL algae culture (1 × 10^6 cells mL^{-1}) at the exponential growth phase were exposed to AgNPs and Ag^+ ions (0.1, 0.2, 0.4 and 0.8 mg L^{-1}) for 72 h at conditions similar to the above described toxicity test. Controls did not include contaminants and all samples were exposed in triplicate. After exposure, algal cultures were collected, centrifuged at 3,500 g for 15 min (Heraeus Multifuge 1S-R, Thermoscientific) and the pellets were resuspended in 400 µL of 0.1 M phosphate buffer (pH 6.5) for glutathione S-transferase (GST) analysis and 800 µL of 0.05 M phosphate buffer (pH 7) for catalase (CAT) analysis. The suspensions were homogenized in 2 mL lysing tubes (FastPrep™, MP bio) using a FastPrep-24™ 5G Homogenizer, MP bio and centrifuged at 9,000 g for 30 min at 4 °C (Sigma 3-16KL centrifuge) for GST analysis and 15,000 g for 15 min at 4 °C for CAT analysis. The supernatants were stored at -80 °C. GST activity was evaluated as described by Habig et al. (1974) adapted to a microplate (Frasco and Guilhermino, 2002). CAT activity was evaluated as described by Aebi (1984). The optical absorbance was measured at 340 nm for GST and at 240 nm for CAT by UV-visible spectrophotometer (POLARstar Omega, BMG Labtech). GST and CAT activity was expressed as micro moles (µM) or milli moles (mM) of substrate (H_2O_2) hydrolysed per min per mg of protein, respectively.

5.2.8. **Data analysis**

All Effective concentration (EC_x) calculations were conducted using the software ToxRat Professional v 3.0.0 (ToxRat Solutions, Aachen, Germany). Data from bioassays were analysed by a two-way ANOVA followed by Tukey’s or Bonferroni t-test for pairwise multiples comparison of means. Shapiro Wilk’s test was performed to check the normal distribution followed by Levene’s test for variance homogeneity analysis. Data were natural log (ln)/log/ arcsin transformed to meet the ANOVA assumptions of normality prior to all analyses. Statistical calculations were done using the software SigmaPlot v.13. All tests were performed at the 5% level of significance.
5.3. Results and discussion

5.3.1. Characteristics of AgNPs

Please see Chapter 3. NP Synthesis, characterisation and culturing organisms, Section 3.2. Characterisation of the AgNPs.

5.3.2. AgNP stability and dissolution

Suspensions of AgNPs in algae medium were initially yellowish in colour with absorption peaks at 406, 400 and 414 nm for T-AgNPs, E-AgNPs and C-AgNPs respectively. Visual observation revealed that the E-AgNP suspension turned to yellowish red within minutes, but the yellowish colour of T-AgNP and C-AgNP was stable even after 72 h. SPR analysis revealed a larger decrease in the absorption peak (~32%) of T-AgNPs and E-AgNPs compared with C-AgNPs (~10%) within 72 h (Fig. 5.1). Broadening of the plasmon bands were more visible in T-AgNPs followed by E-AgNPs, but not for C-AgNPs. The mean HDD of T-AgNPs, E-AgNPs and C-AgNPs increased approximately by 1.5, 1.2 and 1.3-fold within 72 h (Table. 5.1). A reduction of the thickness of the diffuse double layer with increased ionic strength increases the particle-particle interaction resulting in increased aggregation (Badawy et al., 2010). An instant (< 5 min) increase in the zeta potential values for all the particles was observed in the algae medium. However, the remaining zeta potential values were close to −30 mV, indicative of quite higher stability of NPs (Berg et al., 2009) during the 72 h exposure duration (Table. 5.1). The initial percentage dissolution of Ag⁺ ions from T-AgNPs and E-AgNPs remained below 0.5% in M4 medium within the test duration while it was approximately 1.5% for C-AgNPs by 72 h (Table 5.2).

A broader absorbance peak, large background signal and increased HDD indicate aggregation of NPs (Stebounova et al., 2011, Tejamaya et al., 2012b). As per the SPR and HDD results, aggregation in the M4 medium was highest for T-AgNPs followed by E-AgNPs while C-AgNPs showed no signs of aggregation. Aggregation of NPs depends on particle concentration, pH, ionic strength, ionic composition, concentration and composition of natural organic matter, and other characteristics of the aqueous media (Zhou and Keller, 2010, Keller et al., 2010a). Since these parameters are not considerably different, it can be suggested that the observed differences in destabilization was due to different coating materials (Behra et al., 2013, Huynh and Chen, 2011). The colour, SPR peak (Fig. 5.2A, 5.2B and 5.2C), HDD (Table 5.3) and Zeta potential (Table 5.3) of all three types of NP suspensions in MilliQ water
remained largely unchanged. Less aggregation of NPs in MilliQ water, but not in the M4 medium, could be attributed to the higher ionic strength of the medium. However, the dissolution of all types of AgNPs was slightly higher in MilliQ water (Table 5.4) than in the algae medium. This may be due to the precipitation of Ag ions by halides (Cl\textsuperscript{-}) in the medium (Römer et al., 2011) leaving less detectable ions in the solution. Halides may reduce the exposure of organisms to any free ions released from NPs. Also, MilliQ water was slightly acidic (pH: 6.44) compared to media which may have caused higher oxidation of AgNPs releasing more Ag\textsuperscript{+} ions into the solution (Zhao and Wang, 2012a). The dissolution experiments were conducted using AgNP concentrations which were 1 to 3 orders of magnitude higher than the concentrations used in the experiment. Ag\textsuperscript{+} ion release kinetics tend to level off at concentrations, but this possibility was excluded since the observed dissolved ion concentrations were marginal. However, studies have shown that far higher percentages of Ag\textsuperscript{+} ion release are commonly observed at low concentrations as a function of time. Therefore, the percentage Ag\textsuperscript{+} ions released from the NPs in tests vials may be higher than the values presented.

Fig. 5.1: The SPR curves of (A) T-AgNPs, (B) E-AgNPs, and (C) C-AgNPs in M4 medium.
Table 5.1: HDD, Zeta potential and Polydispersity index (Pdi) of AgNPs with different coatings in M4 medium measured at different times within the test duration. NPs were dispersed in the media at Ag concentration of 5,000 µg L⁻¹. Standard deviations (± SD) are from triplicates.

<table>
<thead>
<tr>
<th>Substance</th>
<th>HDD (nm)</th>
<th>Zeta Potential (mV)</th>
<th>PdI</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>&lt; 5 min</td>
<td>72 h</td>
<td>&lt; 5 min</td>
</tr>
<tr>
<td>T-AgNP</td>
<td>50.4 ± 7.1</td>
<td>75.1 ± 4.8</td>
<td>-29.7 ± 1.7</td>
</tr>
<tr>
<td>E-AgNP</td>
<td>40.1 ± 10.3</td>
<td>51.1 ± 2.6</td>
<td>-30.8 ± 1.1</td>
</tr>
<tr>
<td>C-AgNP</td>
<td>45.0 ± 3.8</td>
<td>48.9 ± 2.2</td>
<td>-29.0 ± 2.1</td>
</tr>
</tbody>
</table>

Table 5.2: Dissolution of AgNPs with different coatings in M4 medium measured at different times within the test duration. NPs were dispersed in the media at Ag concentration of 5,000 µg L⁻¹. Standard deviations (± SD) are from triplicates.

<table>
<thead>
<tr>
<th>Substance</th>
<th>% Dissolution</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>24 h</td>
</tr>
<tr>
<td>T-AgNP</td>
<td>0.06 ± 0.14</td>
</tr>
<tr>
<td>E-AgNP</td>
<td>0.16 ± 0.26</td>
</tr>
<tr>
<td>C-AgNP</td>
<td>0.66 ± 0.81</td>
</tr>
</tbody>
</table>

The behaviour of NPs in test systems is not predictable using the traditional methods of partitioning and bioavailability (Petersen et al., 2015). The stability of organic-coated AgNPs in biological media has received significant attention in the last few years in nanotechnology and toxicology studies (Sharma et al., 2014). Toxicological studies have shown that aggregation, dissolution and change in NP characteristics all influence the bioavailability of NPs and hence, play an important role in determining toxicity (Jiang et al., 2009, Sager et al., 2007, Miao et al., 2010b, Tejamaya et al., 2012b). Culture media may impact the behaviour and properties of AgNPs, ultimately leading to various toxicological responses (Ji et al., 2011). Therefore, it is required to consider those when performing toxicity assessments (Van Hoecke et al., 2008). This study shows that the type of NP coating and the physicochemical characteristics of the medium influence the degree of aggregation and the behaviour of NPs.
Fig. 5.2: The SPR curves of (A) T-AgNPs, (B) E-AgNPs, and (C) C-AgNPs in MilliQ water.

Table 5.3: HDD, Zeta potential and Polydispersity index (Pdi) of AgNPs with different coatings in MilliQ water measured at different times within the test duration. NPs were dispersed in the media at Ag concentration of 5,000 µg L⁻¹. Standard deviations (± SD) are from triplicates.

<table>
<thead>
<tr>
<th>Substance</th>
<th>HDD (nm)</th>
<th>Zeta Potential (mV)</th>
<th>Pdi</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>&lt; 5 min</td>
<td>72 h</td>
<td>&lt; 5 min</td>
</tr>
<tr>
<td>T-AgNP</td>
<td>42.7 ± 4.7</td>
<td>44.6 ± 3.0</td>
<td>-39.7 ± 5.8</td>
</tr>
<tr>
<td>E-AgNP</td>
<td>36.0 ± 9.4</td>
<td>37.1 ± 0.4</td>
<td>-46.6 ± 2.0</td>
</tr>
<tr>
<td>C-AgNP</td>
<td>41.2 ± 8.0</td>
<td>42.7 ± 4.7</td>
<td>-48.5 ± 1.9</td>
</tr>
</tbody>
</table>
Table 5.4: Dissolution of AgNPs with different coatings in MilliQ water measured at different times within the test duration. NPs were dispersed in the media at Ag concentration of 5,000 µg L\(^{-1}\). Standard deviations (± SD) are from triplicates.

<table>
<thead>
<tr>
<th>Substance</th>
<th>% Dissolution</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>24 h</td>
</tr>
<tr>
<td>T-AgNP</td>
<td>0.04 ± 0.07</td>
</tr>
<tr>
<td>E-AgNP</td>
<td>0.36 ± 0.10</td>
</tr>
<tr>
<td>C-AgNP</td>
<td>4.39 ± 0.52</td>
</tr>
</tbody>
</table>

5.3.3. Acute toxicity to *R. subcapitata*

Concentration-effect curves (Fig. 5.3) show that the average specific growth rates decreased with increasing concentration of all three types of NPs and Ag\(^+\) ions indicating that the toxicity positively correlated to the concentration. Also, a time-dependent effect was observed for all three type of AgNPs and Ag\(^+\) ions at all concentrations since the differences between growth rates of algae in exposed groups decreased compared with control groups during 72 h of exposure period. The 72 h EC\(_{10}\), EC\(_{20}\) and EC\(_{50}\) values which were calculated based on the percentage inhibition of growth of algae upon exposure to AgNPs and Ag\(^+\) ions are shown in the table 3. Among the three coated AgNPs, toxicity of C-AgNPs and T-AgNPs (72 h EC\(_{50}\): 0.155 and 0.163 mg L\(^{-1}\)) were not significantly different and caused the highest toxicity whilst E-AgNPs were the least toxic (72 h EC\(_{50}\): 0.243 mg L\(^{-1}\)). However, EC\(_{20}\) and EC\(_{10}\) values of T-AgNPs (133 and 119 µg L\(^{-1}\)) were considerably higher than both E-AgNPs (0.072 and 0.038 mg L\(^{-1}\)) and C-AgNPs (0.033 and 0.029 mg L\(^{-1}\)). Although there was no difference between the toxicity of T-AgNPs and C-AgNPs based on EC\(_{50}\) values, this demonstrates that the toxicity of T-AgNPs increased rapidly with increased concentrations; the percentage of viable cells compared to the control at the highest concentration tested (0.2 mg L\(^{-1}\)) was only 6% for T-AgNPs in comparison to 15.3 and 26.3% for C-AgNPs and E-AgNPs respectively confirming that the toxicity of AgNP is in the order of T-AgNP > C-AgNP > E-AgNP. The toxicity of AgNPs to freshwater algae has been examined in several studies and the values obtained in the present study are consistent with the reported EC\(_{50}\) values which are ranging from 0.008 to 1.2 mg L\(^{-1}\) (Ribeiro et al., 2014a, Li et al., 2015b, Sørensen and Baun, 2015, Ivask et al., 2014a). However, the toxicity of Ag\(^+\) ions observed (72 h EC\(_{50}\): 0.051 mg L\(^{-1}\)) was less than the reported values, ranging from 0.005 to 0.034 mg L\(^{-1}\) (Ribeiro et al., 2014a, Li et
al., 2015b, Sørensen and Baun, 2015, Ivask et al., 2014a). This may be due to the source of Ag$^+$ ions in the current study was Ag$_2$SO$_4$ instead of AgNO$_3$, which was the preferred source of Ag$^+$ ions in other studies and AgNO$_3$ may be more toxic to algae than Ag$_2$SO$_4$.

Fig 5.3: Concentration-effect curves showing the influence of AgNPs with different coatings and Ag$^+$ ions on the growth rate of Raphidocelis subcapitata as observed from 0 to 72 h.

Most previous studies reported that the nano form of Ag was less toxic in comparison to their ionic counterparts (Ag$^+$ ions) (Notter et al., 2014); it is likely that the liberated ions from the NPs cause toxicity (Yin et al., 2011, Potera, 2012, Xiu et al., 2012, Franklin et al., 2007). In the present study, the EC$_{50}$ value (72 h) of Ag$^+$ ions (0.051 mg L$^{-1}$) observed is significantly lower than the EC$_{50}$ values of the AgNPs which suggests that the tested coated AgNPs are less toxic than Ag$^+$ ions. However, interestingly, the EC$_{10}$ of Ag$^+$ ions (0.029 mg L$^{-1}$) and C-AgNPs (0.029 mg L$^{-1}$) were similar along with the EC$_{50}$ values of E-AgNPs (0.038 mg L$^{-1}$). Ribeiro et al. (2014a) exposed R. subcapitata to alkane-coated AgNPs (size < 10 nm) and Ag$^+$ ions and observed that AgNPs were twice as toxic as Ag$^+$ ions at concentrations of 0.025 mg L$^{-1}$. However, Navarro et al. (2008b) reported that the toxicity of AgNPs were much
higher than that of the Ag\(^+\) ions to Chlamydomonas reinhardtii when compared as a function of Ag\(^+\) ions, since the free Ag\(^+\) ions from AgNPs in the medium could not fully explain the toxicity. The EC\(_{50}\) values calculated (data not shown) as a function of Ag\(^+\) ion concentrations (by using the percentage dissolution values) for all three types of NP concentrations used are far below the EC\(_{50}\) value obtained for Ag\(^+\) ions (Ag\(_2\)SO\(_4\)). Therefore, the observed toxicity of NPs to alga is attributed to the effects of both particle effects plus Ag\(^+\) ions released.

The unicellular algae R. subcapitata was selected for the experimental analysis since it is a popular alga species in ecotoxicology due to its wide availability, ease of culture, ecological relevance and high sensitivity to contaminants. It is used as a model organism in the OECD algal growth inhibition test (OECD, 2011b) and in other regulatory agencies worldwide. Algal growth inhibition at the exponential growth stage is the most frequent criterion used to evaluate toxicity. Growth inhibition is inferred in terms of change of biomass which is determined by cell count, chlorophyll \(a\) measurement or dry weight (Janssen and Heijerick, 2003). MLA medium (Wahid et al., 2013, Bolch and Blackburn, 1996) was selected for the study since it was highly effective for culturing the particular alga in our lab in comparison to the Hoagland and standard OECD algae growth medium. Aruoja et al. (2009) assessed the effects of shading by NPs on the performance of algae and concluded that the shading effect from NPs was negligible. The NP concentrations they used were three orders of magnitude higher than the concentrations used in this study and therefore, it was assumed that shading from NPs had no effect on the results obtained. Quantification of algae cells in tests was done by measuring the in vivo chlorophyll fluorescence (POLARstar omega, BMG LABTECH) which proved highly effective. Automated cell counting (TC20\textsuperscript{TM} Automated Cell Counter, Bio-Rad Laboratories, Hercules, CA) was effective with viable cells, but gave unrealistic readings in the presence of dead cells, especially when samples treated with higher concentrations of highly toxic Ag\(^+\) ions were measured. The suitability of the fluorometric method for quantification of algae cells was demonstrated in a previous study conducted by Eisentraeger et al. (2003) and used by several researchers (Aruoja et al., 2009, Hartmann et al., 2010).

5.3.4. Associated AgNPs and Ag\(^+\) ions

The associated Ag content in algae against the concentration of AgNPs and Ag\(^+\) ions are shown in Fig. 5.4A and 5.4B respectively. The results demonstrate that the associated Ag quantity increases with increased concentrations for both Ag NPs and Ag\(^+\) ions. However, the Ag accumulation in algal cells exposed to Ag\(^+\) ions is comparatively higher than the Ag NPs
while the $Ag_{\text{cell}}$ was approximately 6.5 times higher in the algal cells exposed to $Ag^+$ ions (0.1 mg L$^{-1}$) compared to C-AgNPs (0.11 mg L$^{-1}$). A linear increase of $Ag_{\text{cell}}$ was observed in the algal cells exposed to $Ag^+$ ions and AgNPs up to 0.14 mg L$^{-1}$ ($r^2$ between 0.84 and 0.98 respectively) while an exponential increase in $Ag_{\text{cell}}$ was observed at higher (0.17 and 0.2 mg L$^{-1}$) concentrations. Piccapietra et al. (2012) found higher internalization of Ag in Chlamydomonas reinhardtii exposed to $Ag^+$ ions ($AgNO_3$: 0.002 – 0.053 mg L$^{-1}$) compared to carbonate coated AgNPs (AgNPs: 0.053 – 1.078 mg L$^{-1}$, size: 29 nm) and the accumulation correlated with the concentration. They also observed a linear increase in $Ag_{\text{cell}}$ in algae exposed to $Ag^+$ ions but not in the algae exposed to AgNPs where a constant accumulation level was observed at NP concentrations above 0.215 mg L$^{-1}$. When the alga Euglena gracilis was exposed to AgNPs and $Ag^+$ ions, Li et al. (2015b) also observed a linear increase in $Ag_{\text{cell}}$ in algae exposed to $Ag^+$ ions, but not for AgNPs where $Ag_{\text{cell}}$ remained constant above the AgNP concentration of 0.269 mg L$^{-1}$. Merdzan et al. (2014) investigated the bioaccumulation of differently coated ZnNPs and $Zn^{2+}$ ions in the alga C. reinhardtii and observed lower bioaccumulation of Zn from ZnNPs compared to Zn salt or bare ZnONPs which liberate more $Zn^{2+}$ ions. Size of the pores and the change of permeability of cell wall limit the passage of Ag through the cell wall (Navarro et al., 2008a), but not for ionic forms which can be explained by commonly accepted models including the free ion activity and biotic ligand models (Di Toro et al., 2001, Slaveykova and Wilkinson, 2005). When compared with other studies, $Ag_{\text{cell}}$ in AgNP-exposed algae cells in the present study did not reach a constant or steady state with increased concentration of AgNPs. This may be due to the maximum AgNP concentration tested in this study was 0.2 mg L$^{-1}$ whereas others observed $Ag_{\text{cell}}$ level became constant when the algae were exposed to AgNP concentrations above 0.2 mg L$^{-1}$. We previously showed that $Ag^+$ ions were most toxic while E-AgNPs were least toxic. The highest associated Ag content was found in algae exposed to $Ag^+$ ions followed by T-AgNP, C-AgNP and E-AgNPs. Therefore, it can be inferred that the toxicity correlates to the associated Ag content.

Difference in associated Ag content can be attributed to difference in bioavailability of NPs as suggested by Piccapietra et al. (2012). Further, it can be assumed that the type of coating of AgNPs used in this study influenced the bioavailability of AgNPs resulting in different uptake of AgNPs. However, data obtained from this study are insufficient to identify the reasons for the observed difference in uptake. Metallic NPs may cause toxicity to algae by ions released from NPs while they are in the suspension, attached to the surface of cells or after internalization (Miao et al., 2010a). However, toxicity of NPs other than from released ions
Fig 5.4: Cell associated silver measured in *Raphidocelis subcapitata* after 72 h upon exposure to (A) AgNPs with different coatings, and (B) Ag⁺ ions at different concentrations. The error bars indicate the SD (*p* < 0.05, *n* = 3). The *p*-values for multiple pairwise comparisons were obtained from two-way ANOVA followed by Bonferroni t-test using Sigmaplot. Letter sign denotes comparison of *p*-values for particles within each concentration and control while the number sign denotes comparison of *p*-values for each particle within different concentrations.
was reported (Miao et al., 2010a). The coatings of AgNPs used in this study considerably reduced the dissolution of ions from AgNPs in suspension. The concentrations of Ag⁺ ions released which were calculated based on percentage dissolution were well below the EC₅₀ of Ag⁺ ions observed. Therefore, the causes of toxicity could be attributed to the ions released from the associated AgNPs (surface attached or internalized) plus other adverse effects from AgNPs including physical damage. Associated Ag quantification was performed based on ICP-MS analysis after acid digestion. Any AgNPs associated with the algae would have been converted to Ag⁺ ions with acid digestion. Therefore, from the results obtained, it is not possible to differentiate correct quantities of associated AgNPs from any Ag⁺ ions released from NPs.

5.3.5. Enzyme activity

Increased activities of GST and CAT were found in alga upon exposure to AgNPs and Ag⁺ ions at higher concentrations (Fig. 5.5A and 5.5B). NPs induce ROS production (Xia et al., 2015, Oukarroum et al., 2012, Angel et al., 2015, Melegari et al., 2013) and the upregulation of enzyme activities during exposure could be associated with increased production of ROS in the algae (Dauda et al., 2017). Generation of ROS inhibits algal growth and photosynthesis by damaging the cell membrane, nucleus and chloroplasts (Xia et al., 2015, Dauda et al., 2017); the activity of some antioxidative enzymes are triggered in defence to protect from those negative effects (Pinto et al., 2003, Dewez et al., 2005, Mittler, 2002, Bhattacharjee, 2005, Fu et al., 2014). CAT catalyzes the dismutation of H₂O₂ to H₂O and O₂ while GST inactivates secondary metabolites such as lipid hydroperoxides in response to ROS (Lu et al., 2016b) and is responsible for the repair of ROS oxidized macromolecules of damaged cellular components (Angelucci et al., 2005). None of the AgNPs or Ag⁺ ions caused increased activation of GST below 0.2 mg L⁻¹ compared with the control while the activity was strongly triggered in algal cells exposed to 0.4 mg L⁻¹ of C-AgNPs and 0.4 and 0.8 mg L⁻¹ of Ag⁺ ions. Also, the GST activation in algal cells exposed to 0.4 mg L⁻¹ of C-AgNPs was significantly different to the cells exposed to same concentrations of T-AgNP and E-AgNP. CAT activity also was not significantly increased by AgNPs at concentrations below 0.2 mg L⁻¹. However, an increased activation of CAT was observed in algae exposed to increased concentrations of T-AgNPs, E-AgNPs and Ag⁺ ions (0.4 and 0.8 mg L⁻¹) compared with the control. C-AgNPs increased CAT activity only at 0.8 mg L⁻¹ concentration though it caused significantly less activation of CAT levels compared with other types of NPs and Ag⁺ ions within the same concentration group.
Fig 5.5: Effects of AgNPs with different coatings and Ag\(^+\) ions on antioxidant enzymes (A) GST, and (B) CAT activities in *Raphidocelis subcapitata* after 72 h upon exposure to different AgNP and Ag\(^+\) ion concentrations. GST: Glutathione S-transferase CAT: Catalase. The error bars indicate the SD (\(p < 0.05, n = 3\)). The *p*-values for multiple pairwise comparisons within concentration and particles were obtained from two-way ANOVA followed by Bonferroni t test using Sigmaplot. Letter sign denotes comparison of *p*-values for particles within each concentration and control while the * sign denotes comparison of *p*-values between concentrations for each particle.
To date, the data regarding the effects of AgNPs on antioxidant activities in algae are scarce. However, increased GST and CAT activities were previously recorded after exposure to other NPs such as TiO$_2$ NPs (Dauda et al., 2017) and CuO NPs (Melegari et al., 2013). The activation of enzymes is different for AgNPs with different coatings and the toxicity may depend on the ability of the antioxidant system to activate the antioxidant enzymes at different concentrations of AgNPs with different surface functionalization. However, results from this study do not provide any strong basis for explaining the differential enzyme activation by differently coated AgNPs. Therefore, correlations between differently coated AgNPs and activation of antioxidant enzymes need further investigation. Also, the activation of antioxidant system is complex (Dewez et al., 2005, Mittler, 2002) and the mechanisms of toxicity of each type of differently coated AgNPs on algae may be equally complex (Chang et al., 2012, Miao et al., 2010a). Therefore, it is difficult to explain the cause of the differential toxicity of differently coated AgNPs by the response of tested enzymes activities and thus, it is required to evaluate the responses of suit of antioxidant enzymes to fully explain the different toxic effects.

5.4. Conclusions

This study shows that the physicochemical characteristics of the surface coatings of AgNPs play a major role in determining their behaviour in the algae growth medium, the toxicity, bioaccumulation and antioxidant enzyme responses of algae. T-AgNPs seemed less stable compared with E-AgNPs and C-AgNPs though all three types of NPs remained quite stable in the 72 h test duration. The highest toxicity to algae was caused by Ag$^+$ ions followed by C-AgNPs, T-AgNPs, and E-AgNPs respectively. The results clearly showed that toxicity increased with the concentration of Ag$^+$ ions and AgNPs; the associated Ag content in algae followed the same pattern. The toxicity of AgNPs was several magnitudes less than that of Ag$^+$ ions. Though AgNPs proved to be less toxic compared to their ionic counterparts, they are highly toxic in comparison to other types reported toxicity values of NPs. Increased GST and CAT levels indicate increased antioxidant activity in response to possible ROS production caused by AgNPs and Ag$^+$ ions. Taken together, these findings indicate that AgNPs pose ecological risk to aquatic organisms depending on the inherent characteristics of the NP and surrounding environment. Therefore, these factors should be considered in environmental risk assessment of AgNPs. In addition, the sensitivity of algae varies with differently functionalized
AgNPs; further studies are recommended to understand the influence of different coatings on the toxicity of AgNPs to primary producers in aquatic ecosystems.
Chapter 6. The toxicity of coated silver nanoparticles to Daphnia carinata and trophic transfer from alga Raphidocelis subcapitata

This chapter has been published in the peer-reviewed literature and is presented here with only minor modifications to adjust formatting to the requirements of the thesis.


Abstract

Nanoparticles (NPs) are causing threats to the environment. Silver NPs (AgNPs) are increasingly used in commercial products and may end up in freshwater ecosystems. The freshwater organisms are vulnerable due to water-borne and dietary exposure to AgNPs. Surface properties play an important role in the fate and behavior of AgNPs in the aquatic environment and their effects on organisms. However, effects of surface properties of AgNPs on organisms are poorly understood. In this study, we explored the effects of AgNPs coated with three different ligands; Tyrosine (T-AgNP), Epigallocatechin gallate (E-AgNP) and Curcumin (C-AgNP) in relation to the toxicity to a key aquatic organism; Daphnia carinata. The study focused on how coatings determine fate of NPs in the medium, mortality, feeding behaviour, bioaccumulation and trophic transfer from the freshwater alga, Raphidocelis subcapitata to daphnids. NP stability tests indicated that T-AgNPs were least stable in the ASTM daphnid medium while C-AgNPs were most stable. 48 h EC50 values of AgNPs to D. carinata were in the order of E-AgNP (19.37 µg L−1) > C-AgNP (21.37 µg L−1) > T-AgNP (49.74 µg L−1) while the 48 h EC50 value of Ag+ ions was 1.21 µg L−1. AgNP contaminated algae significantly decreased the feeding rates of daphnids. However, no significant differences were observed in feeding rates between algae contaminated with differently coated AgNPs. Trophic transfer studies showed that AgNPs were transferred from algae to daphnids. The bioaccumulation of AgNPs in algae and the diet-borne bioaccumulation of AgNPs in daphnids varied for differently coated AgNPs. Bioaccumulation of C-AgNPs in algae was 1.5 time higher than T-AgNPs. However, the accumulation of T-AgNPs in daphnids via trophic transfer was 2.6 times higher than C-AgNPs. The knowledge generated from this study enhances the understanding of surface property dependent toxicity, bioaccumulation and trophic transfer of AgNPs in aquatic environments.
6.1. Introduction

Engineered nanoparticles (ENPs) are man-made materials with a size range of 1 to 100 nm (Klaine et al., 2012). Global production of ENPs are increasing exponentially as they are widely being used in many applications such as healthcare, personal care, construction, energy, electronics, catalysts, packaging, textiles, environmental remediation and agriculture (Hamers, 2017, Keller et al., 2013a, Cecchin et al., 2016). Despite their useful applications, the physicochemical characteristics that make NPs unique are causing possible threats to the health of the environment including humans. Unfortunately, understanding the implications of NPs have not kept pace with the advancements of nanotechnology and therefore, concerns are growing about their possible environmental health and safety risks among the scientific community, regulatory agencies and general public (Klaine et al., 2012, Maurer-Jones et al., 2013a, Beer et al., 2012, Erbis et al., 2016, Karimi et al., 2018). There are 1800 plus nano-enabled consumer products in the market while silver nanoparticles (AgNPs) is the most frequently used nano-material (435 products) as reported by Vance et al. (2015). Certain physicochemical, chemical and structural features of AgNPs are useful as an excellent antimicrobial agent (Nowack et al., 2011, Prabhu and Poulose, 2012, Rai et al., 2012, Tran and Le, 2013, Schluesener and Schluesener, 2013, Silva et al., 2017) and in the fields of material science, chemistry and physics (Asghari et al., 2012b). With increased usage in consumer products, large quantities of AgNPs end up in aquatic ecosystems posing a huge threat to aquatic organisms. Primary producers are vulnerable to water-borne exposure while higher organisms are affected by both water-borne and diet-borne exposure (Bhuvaneshwari et al., 2017, Wu et al., 2017a). Complete assessment of health and environmental impacts of engineered nanomaterials is not possible due to lack of nanotoxicity and ecotoxicity data and it will take many more years to produce actionable information leaving all concerned parties with little guidance (Erbis et al., 2016). Therefore, further studies are required to minimize the harm caused by NPs considering the diversity of NP characteristics (Seitz et al., 2015b, El Badawy et al., 2010a, Liu and Hurt, 2010a, Lowry et al., 2012, Asghari et al., 2012a).

AgNPs may cause mechanical (Dabrunz et al., 2011, Mendonça et al., 2011, Artells et al., 2013) and physiological (Domínguez et al., 2015, Li et al., 2015a) damage. Some researchers claim that liberated ions from AgNPs are the only cause of toxicity to aquatic organisms (Shen et al., 2015, Sakamoto et al., 2015) while other studies indicate that particles are the major cause of toxicity (Li et al., 2017b, Abramenko et al., 2018). The toxicity of NPs
are compared to the toxicity of the counterpart bulk material, usually metal salts to test this hypothesis (Djurišić et al., 2015). Surface coatings of NPs have a strong influence on their physicochemical properties and can also influence the toxicity to organisms (Silva et al., 2014a). Though there are studies on effects of coated NPs, further studies are required due to addition of new coating materials, different views on effects of coatings and to protect native freshwater species. Zhao and Wang (2012a) found AgNPs coated with sodium dodecylbenzene sulfonate caused highest toxicity (48 h LC\(_{50}\): 1.1 µg L\(^{-1}\)) to *Daphnia magna* followed by polyvinylpyrrolidone (PVP) (48 h LC\(_{50}\): 2.0 µg L\(^{-1}\)) and lactate-coated AgNPs (48 h LC\(_{50}\): 28.7 µg L\(^{-1}\)). Silva et al. (2014a) studied the toxicity of three types of organo-coated AgNPs to *D. magna*. They found that the branched polyethyleneimine-coated AgNPs (48 h LC\(_{50}\): 0.41µg L\(^{-1}\)) were most toxic followed by citrate (48 h LC\(_{50}\): 2.88 µg L\(^{-1}\)) and PVP-coated AgNPs (48 h LC\(_{50}\): 4.79 µg L\(^{-1}\)). Newton et al. (2013) exposed *D. magna* to three types of coated AgNPs for 48 h in two different media. They found gum Arabic-coated AgNPs (48 h LC\(_{50}\): 2.14 – 3.48 µg L\(^{-1}\)) were most toxic followed by polyethylene glycol (48 h LC\(_{50}\): 2.27 – 13.08 µg L\(^{-1}\)) and PVP-coated AgNPs (48 h LC\(_{50}\): 14.04 – 14.81 µg L\(^{-1}\)).

NPs can be bioaccumulated and transferred from one trophic level to another through the food chain (Bundschuh et al., 2016a, Ribeiro et al., 2017, Gardea-Torresdey et al., 2014). Trophic transfer studies allow us to differentiate the importance of different exposure routes which is useful in risk analysis. Algae are primary producers of energy as a food source and any impacts at this level may affect the health of organisms at higher trophic levels (Kalman et al., 2015, Bhuvaneshwari et al., 2018a). Several studies have shown that food is the major source of AgNP accumulation in *D. magna* (Kalman et al., 2015, Zhao and Wang, 2010). Zhao and Wang (2010) found that AgNPs were more efficiently assimilated in daphnids and was more difficult to depurate when NPs were ingested through the dietary intake than water-borne exposure. McTeer et al. (2014) observed trophic transfer of NPs to *D. magna* from AgNP treated alga *Chlamydomonas reinhardtii*. Chae and An (2016) saw trophic transfer of Ag nanowires (AgNWs) from *C. reinhardtii* to *D. magna* and then to the fish *Danio rerio*. Lee et al. (2015) observed trophic transfer of gold NPs (AuNPs) to *D. magna* from *C. reinhardtii* and *Euglena gracilis*. Bouldin et al. (2008) observed transfer of Carboxyl quantum dots (QD) from QD-exposed alga *Raphidocelis subcapitata* to *Ceriodaphnia dubia*. Elsewhere, trophic transfer of ENPs were demonstrated from algae to mussels (Larguinho et al., 2014b), daphnids to zebrafish (Zhu et al., 2010b) and biofilms to snails (Yeo and Nam, 2013). Exposure to AgNPs and Ag\(^{+}\) ions contaminated algae also cause changes in feeding behaviour in daphnids. McTeer
et al. (2014) also reported a significant reduction in feeding when daphnids were fed with AgNP and Ag⁺ ion contaminated algae compared to untreated algae. Zhu et al. (2010a) observed dose dependent reductions in ingestion and filtration rate when *D. magna* was exposed to TiO₂ NPs.

In this study, we studied the toxicity of AgNPs coated with different organic ligands; Tyrosine-coated (T-AgNP), Curcurmin-coated (C-AgNP) and Epigallocatechin gallate-coated (E-AgNP) AgNPs to the freshwater filter-feeding cladoceran, *Daphnia carinata*. In addition, the effects of associated Ag with alga on daphnid feeding behaviour and trophic transfer from the alga diet to daphnids were investigated. Tyrosine, Curcurmin and Epigallocatechin gallate have different number of phenol structures and classified as mono-phenol, bi-phenol and poly-phenols respectively. Since they are organic compounds, their usage is considered as a green and ecofriendly approach to produce NPs while they are biocompatible which is a useful characteristic in therapeutic applications (Daima et al., 2014). These coatings are used to produce NPs for different applications, mainly being found in medical applications (El Khoury et al., 2015, Shukla et al., 2012, Daima et al., 2014, Selvakannan et al., 2004, Dubey et al., 2015). Though there are studies on the effects of AgNPs with most commonly used coatings, studies on organic coatings used in this study are lacking. *Daphnia sp.* is one of the most sensitive species used in aquatic toxicological studies and is recommended as a model test organism by international agencies. The most common species used for studies is *D. magna* which is considered as an invasive species in some parts of the world. Therefore, it is required to improve test species and protocols to better reflect species sensitivity in different ecosystems (Freitas and Rocha, 2011), for environmental risk assessment and to protect native species. Also, daphnids represent the bottom level of the freshwater food chain and any qualitative or quantitative effect to the population will affect higher organisms (Asghari et al., 2012a). To the best of our knowledge, this is the first study where the effects of differently coated NPs were studied against *D. carinata* while other studies so far have used *D. magna*. Also, the coating materials are quite novel and have not being used in similar kind of studies.

6.2. Materials and methods

6.2.1. Test materials

Please see Chapter 3. NP Synthesis, characterisation and culturing organisms, Section 3.1. Synthesis of AgNPs.
6.2.2. Characterization of AgNPs

Please see *Chapter 3. NP Synthesis, characterisation and culturing organisms*, section 3.2. Characterisation of AgNPs.

6.2.3. Test organisms and culture conditions

Please see *Chapter 3. NP Synthesis, characterisation and culturing organisms*, Section 3.3. Culturing and maintenance of test species for information on culturing *D. carinata* and *R. subcapitata* which was used in this study. Algal cells were counted periodically with an automated cell counter (TC20TM, Bio-Rad Laboratories, Hercules, CA) to make sure the culture was at exponential growth stage when it was treated with NPs for the daphnid feeding and bioaccumulation tests.

6.2.4. AgNP temporal stability and dissolution in the test medium

The stability of AgNPs in the ASTM medium and MilliQ water was investigated for 24 h under similar environmental conditions used for the daphnid acute toxicity test (please see Section 6.2.5.), as previously described in the *Chapter 5. The toxicity of coated silver nanoparticles to the alga Raphidocelis subcapitata* Section 5.2.4. AgNP Temporal Stability and Dissolution in the Test Media.

6.2.5. Acute toxicity bioassay

AgNP test solutions of relevant nominal concentrations for the acute test were prepared based on ICP-MS results just before the test began by sonicating and dispersing relevant volumes of stock solutions in daphnid culture medium. Ionic silver (Ag⁺ ions) stock solution was prepared by dissolving Ag₂SO₄ in culture medium followed by ICP-MS analysis and required concentrations for acute tests were prepared by dissolving relevant volumes from the stock solution in daphnid culture medium. Controls contained only the culture medium. Semi-static renewal acute toxicity tests were performed according to the Organisation for economic co-operation and development (OECD) standard procedure (OECD, 2004a). Less than 24 h old third brood progeny was used for experiments. Daphnid neonates were exposed to seven different concentrations of each test substance for 48 h. Test concentrations were chosen based on results obtained from range finding tests. Concentrations employed for the tests were in the range of 10.0 – 40.0 µg L⁻¹ for E-AgNP, 30.0 – 90.0 µg L⁻¹ for T-AgNP, 10.0 – 35.0 µg L⁻¹ for
C-AgNP and 0.6 – 1.8 μg L\(^{-1}\) for Ag\(^{+}\) ions. For each concentration, 5 neonates (age: < 24 h) were placed in a 35 mL glass vial containing 15 mL of test solution. The test solution was renewed after 24 h and all experiments were conducted in quadruplicate (n = 4). Daphnids were not fed during the 48 h time period. The tests were conducted at 20 ± 1°C with 16:8 h light and dark photoperiod, pH was maintained at 7.5 ± 0.2 while the dissolved oxygen concentration in all the test solutions exceeded 3 mg L\(^{-1}\). Immobilization was recorded after 24 and 48 h while they were considered immobile if they couldn’t move within 15 s of gentle agitation of the test container. The toxicity tests were considered valid if the mortality was not >10% in the control.

6.2.6. Feeding analysis and AgNP trophic transfer

Algae feeding experiment was conducted as described by McTeer et al. (2014) and Grintzalis et al. (2017) with some modifications. Cell density of the algae culture which was in exponential growth stage was adjusted to 5 × 10\(^{4}\) cells mL\(^{-1}\). Algae cells were propagated in a 1 L Erlenmeyer flask containing 500 mL algae culture in the presence of particles (50 μg L\(^{-1}\)) or absence (blank control) in triplicate. The flasks were incubated as per the OECD guideline (OECD, 2011a) on orbital shakers (OM6, RATEK, Aus) at 100 rpm under the same environmental conditions used for algae culturing. Algal cell counts of each flask were taken with the automated cell counter and the specific algal growth rate of all treatments were calculated for each day. Algae cells were fed to daphnids after 6 days when the cells had reached stationary growth phase. Since the cells were no longer dividing, number of cells consumed could accurately be measured. A volume of 350 mL from each culture was centrifuged (3000 g, 5 min, 20 °C) and washed three times with MilliQ water to remove any loosely adsorbed contaminants from algae. The algae were then resuspended in ASTM medium in a 50 mL Falcon tube as it contained approximately 5 × 10\(^{6}\) cells mL\(^{-1}\) and stored at 4 °C. Feeding experiments were carried out under similar environmental conditions used for the acute toxicity test in 100 mL glass beakers in triplicate with 50 mL ASTM medium in each beaker. Daphnids (15, age: < 24 h) were placed in each beaker and fed with AgNP treated and untreated algae for 5 days. Daphnids were transferred to fresh media after every 24 h and the starting and final algal cell numbers were determined with the automated cell counter. The solutions were stirred on a magnetic stirrer for 20 s before samples were taken for cell counting. The 5 day feeding phase was followed by a 3 days depuration phase in fresh medium where daphnids were fed with unexposed algae. Then, daphnids were oven dried at 60 °C for 48 h and acid digested on a block heater for 6 h at 100 °C with 70% HNO\(_3\) and 30% H\(_2\)O\(_2\). Digested
samples were diluted with MilliQ water and analysed using ICP-MS. A volume of 50 mL from each remaining culture was harvested by centrifugation (3000 g, 5 min) and washed three times with MilliQ water. The resulting algal pellets were oven dried at 60 °C for 48 h and acid digested separately as described above. The digested samples (0.5 mL) were diluted with MilliQ water and the Ag⁺ ion concentrations were measured using ICP-MS (7700X, Agilent Technologies).

6.2.7. Data analysis

The 24 and 48 h EC₅₀ values and their associated 95% confidence intervals (95% CI) were calculated using the TOX Rat software (TOX Rat solutions GmbH, version 3.0). The statistical method used was the probit analysis using linear maximum likelihood regression. The daily feeding rate was determined by dividing the number of algal cells consumed by the number of daphnids that were alive in each beaker. Trophic transfer was quantitatively determined by calculating the transfer of Ag to a daphnid from 10⁵ algal cells consumed. For each dataset, mean and SD are presented and data were considered statistically significantly different at p < 0.05. All data were normally distributed according to Shapiro-Wilk test. Differences between treatments were analysed using one-way analysis of variance (ANOVA). When significant differences were detected at a 95% level of confidence, the Tukey’s multiple comparisons were applied. ANOVA was performed using Sigmaplot statistics version 13. Differences of treatments over time for repeated measures were determined by 2-factor ANOVA followed by Holm-Sidak method.

6.3. Results and discussion

6.3.1 Characteristics of AgNPs

Please see Chapter 3. NP Synthesis, characterisation and culturing organisms, Section 3.2. Characterisation of the AgNPs.

6.3.2 AgNP stability and dissolution

Suspensions of AgNPs in MilliQ water were initially yellowish in colour with absorption peaks at 406, 400 and 414 nm for T-AgNPs, E-AgNPs and C-AgNPs respectively. Once the AgNPs were dispersed in the ASTM medium, visual observation revealed that the T-AgNP and E-AgNP suspensions turned to yellowish brown within minutes, but the yellowish
colour of C-AgNP suspension was stable even after 24 h. SPR analysis revealed a larger decrease in the absorption peaks of T-AgNPs (34 and 70%) and E-AgNPs (33 and 57%) after 5 min and after 24 h respectively in comparison to the initial relevant absorption peaks of AgNPs in the MilliQ water measured after 5 min (Fig 6.1A and 6.1B). In contrast, the decrease of the absorption peak was 4 and 5% for C-AgNPs after 5 min and 24 h (Fig 6.1C). In addition, the SPR bands of T-AgNPs and E-AgNPs significantly broadened which was not observed for C-AgNPs. However, the SPR bands of AgNPs didn’t change considerably in the MilliQ water (Fig. 6.2A, 6.2B and 6.2C). In the ASTM medium, the percentage change of dissolution after 24 h in comparison to the dissolution after first 5 min was 0.06, 0.04 and 1.51% for T-AgNPs, E-AgNPs and C-AgNPs respectively while it was 0.04, 0.36 and 4.39%, respectively in MilliQ water. The mean HDD of T-AgNPs and E-AgNPs in the ASTM medium increased approximately by 2.7 and 4.7 times respectively after 5 min and by 9 and 8 times after 24 h. However, the mean HDD of C-AgNPs did not change considerably (Table 6.1). In comparison, none of the AgNPs showed considerable change in HDD in the MilliQ water (Table 6.2). The percentage intensity distribution of particle size (weighted according to the scattering intensity of each particle fraction) in media revealed that approximately 63, 79 and 87% of aggregates of T-AgNPs, E-AgNPs and C-AgNPs remained at the sub-micron level after 24 h. In comparison to the zeta potential of NPs in the MilliQ water (Table 6.2), it sharply increased by 13.4, 23.9 and 24.3 mV of T-AgNPs, E-AgNPs and C-AgNPs respectively after 5 min but did not change considerably thereafter (Table 6.1). The polydispersity index (pdI) of T-AgNPs and E-AgNPs slightly increased in both ASTM medium (Table 6.1) and MilliQ water (Table 6.2).

Table 6.1: HDD, Zeta potential and PdI of AgNPs in ASTM daphnid medium measured after 5 min and 24 h. AgNPs were dispersed in ASTM daphnid medium at Ag concentration of 5,000 µg L⁻¹. Standard deviations (± SD) are from triplicates.

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<th>Substance</th>
<th>HDD (nm)</th>
<th>Zeta Potential (mV)</th>
<th>PdI</th>
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<td></td>
<td>&lt; 5 min</td>
<td>24 h</td>
<td>&lt; 5 min</td>
</tr>
<tr>
<td>T-AgNP</td>
<td>116.3 ± 6.4</td>
<td>394.1 ± 90.0</td>
<td>- 26.3 ± 1.6</td>
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<tr>
<td>E-AgNP</td>
<td>170.8 ± 33.8</td>
<td>290.4 ± 46.6</td>
<td>- 22.7 ± 3.1</td>
</tr>
<tr>
<td>C-AgNP</td>
<td>44.2 ± 8.6</td>
<td>49.7 ± 12.1</td>
<td>- 24.2 ± 1.4</td>
</tr>
</tbody>
</table>
Fig 6.1: The SPR curves of (A) T-AgNPs, (B) E-AgNPs, and (C) C-AgNPs in ASTM medium.

Table 6.2: HDD, Zeta potential and pdI of AgNPs in MilliQ water measured after 5 min and 24 h. AgNPs were dispersed in MilliQ water at the Ag concentration of 5,000 µg L⁻¹. Standard deviations (± SD) are from triplicates.

<table>
<thead>
<tr>
<th>Substance</th>
<th>HDD (nm)</th>
<th>Zeta Potential (mV)</th>
<th>pdI</th>
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<tr>
<td></td>
<td>&lt; 5 min</td>
<td>24 h</td>
<td>&lt; 5 min</td>
</tr>
<tr>
<td>T-AgNP</td>
<td>42.7 ± 4.7</td>
<td>41.1 ± 2.9</td>
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<tr>
<td>E-AgNP</td>
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<tr>
<td>C-AgNP</td>
<td>41.2 ± 8.0</td>
<td>42.7 ± 5.6</td>
<td>-48.5 ± 1.9</td>
</tr>
</tbody>
</table>

A broader absorbance peak, a large background signal and increased HDD indicate aggregation of NPs (Stebounova et al., 2011, Tejamaya et al., 2012b). As per the results, T-AgNPs and E-AgNPs aggregated rapidly in ASTM medium in comparison to C-AgNPs. Aggregation of NPs depends on particle concentration, pH, ionic strength, ionic composition,
Concentration and composition of natural organic matter, and other characteristics of the aqueous media (Zhou and Keller, 2010, Keller et al., 2010a). The observed differences in aggregation among coated AgNPs may be due to different coating materials since other characteristics of AgNPs such as initial HDD, shape and zeta potential are not significantly different ($p > 0.05$) (Behra et al., 2013, Huynh and Chen, 2011). Also, all NPs showed no signs of aggregation in MilliQ water within the same time duration. The observed higher aggregation of AgNPs in ASTM medium in comparison to the MilliQ water could be attributed to the higher ionic strength of the medium. Decreased zeta potential values in ASTM medium with high ionic strength compared to MilliQ water with relatively low ionic strength are in accordance with the classical colloid theory (Hunter, 1981, Elimelech et al., 1995). A reduction of the thickness of the diffuse double layer with increased ionic strength allows for the attractive van der Waals interactions to dominate and increases the particle-particle interaction resulting in increased aggregation (Badawy et al., 2010, Brant et al., 2005a). Interestingly, the dissolution of all types of AgNPs was higher in MilliQ water than in the ASTM medium. Köser et al. (2017) and Levard et al. (2013) reported that the percentage dissolution of AgNPs correlates to the Cl$^-$ ion content (Cl/Ag ratio) in the medium which was contradictory to our results. This may be due to the precipitation of Ag ions by halides (Cl$^-$) in the medium (Römer et al., 2011) which is not expected in the MilliQ water. Halides may reduce the exposure of organisms to any free ions released from NPs. Also, the size of the AgNPs is one of deciding factors for the release of Ag$^+$ ions (Zhang et al., 2011). Small size particles have high surface to volume ratios and therefore, more atoms on the surface come into contact with oxidants in comparison to larger particles (Zhao and Wang, 2012b). Aggregation of NPs results in larger particles with increased HDD reducing the surface area of particles available to release free Ag ions (Allen et al., 2010, Zhao and Wang, 2012a). Higher dissolution of AgNPs in MilliQ water than in ASTM medium may also be due to the less aggregation of AgNPs. The observed higher dissolution of C-AgNPs in both ASTM medium and MilliQ water than T-AgNPs and E-AgNPs is attributed to the less aggregation of C-AgNPs, while dissolution depends on several other factors (Lopes et al., 2014).

Aggregation, dissolution and change in NP characteristics such as HDD as observed in this study may influence the bioavailability of NPs and hence, play an important role in determining toxicity (Jiang et al., 2009, Sager et al., 2007, Miao et al., 2010b, Tejamaya et al., 2012b). Also, culture medium impacted the behaviour and properties of AgNPs which may ultimately lead to various toxicological responses (Ji et al., 2011). This study shows that the
type of NP coating and medium significantly influence the degree of aggregation and the behaviour of AgNPs, which are required to consider in environmental risk assessment (Van Hoecke et al., 2008).

6.3.3 Acute toxicity to *D. carinata*

Evaluation of acute toxicity is crucial in environmental risk assessment of NPs in protecting the organisms and setting up water quality guidelines. According to the results in this study, the toxicity of AgNPs and Ag$^+$ ions correlate with the concentration while the toxicity of Ag$^+$ ions is significantly higher than AgNPs as per the 48 h EC$_{50}$ values (Table 6.3). Among differently coated AgNPs, E-AgNPs showed the highest toxicity, but it was approximately 16 times less toxic compared to Ag$^+$ ions. There was no much difference in toxicity between E-AgNPs and C-AgNPs (48 h EC$_{50}$: 19.3 and 21.3 µg L$^{-1}$), but T-AgNP was almost 2.5 times less toxic (48 h EC$_{50}$: 49.7 µg L$^{-1}$).
Table 6.3: 48 h EC_{50, 20, 10} values of *Daphnia carinata* exposed to T-AgNP, E-AgNP, C-AgNP and Ag^{+} ions in ASTM medium.

<table>
<thead>
<tr>
<th>Substance</th>
<th>EC_{50} µg L^{-1}</th>
<th>95 % CI</th>
<th>EC_{20} µg L^{-1}</th>
<th>95 % CI</th>
<th>EC_{10} µg L^{-1}</th>
<th>95 % CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ag^{+} ions</td>
<td>1.21</td>
<td>1.12</td>
<td>1.3</td>
<td>0.96</td>
<td>0.84</td>
<td>1.05</td>
</tr>
<tr>
<td>T-AgNP</td>
<td>49.74</td>
<td>45.4</td>
<td>53.86</td>
<td>38.7</td>
<td>33.31</td>
<td>42.76</td>
</tr>
<tr>
<td>E-AgNP</td>
<td>19.37</td>
<td>16.86</td>
<td>21.79</td>
<td>12.91</td>
<td>10.08</td>
<td>15.11</td>
</tr>
</tbody>
</table>

NP size, type of coating, shape, charge are some major factors that influence toxicity (Allen et al., 2010, Dominguez et al., 2015, Zhao and Wang, 2012a, Silva et al., 2014a, Baumann et al., 2014, Newton et al., 2013). The shape of all NPs is spherical, and the initial size and charge do not significantly differ (p > 0.05) which may not explain the observed differences in acute toxicity. Therefore, the difference in toxicity is presumed to be the effects of different coating materials which reaffirm that different types of coating of NPs do have different effects on toxicity of NPs. T-AgNPs were least stable in the ASTM medium exhibiting the highest aggregation and settling of particles. Thus, the reduced toxicity of T-AgNPs compared to the other two types could be due to the lower bioavailability of NPs (Zhou and Keller, 2010, Wong et al., 2010). However, the toxicity of E-AgNPs was similar to that of C-AgNPs which showed less aggregation and high dissolution compared with E-AgNPs. Therefore, the observed difference in toxicity could be a result of several factors which can’t be exclusively explained from the results of this study. Several previous studies have assessed the acute toxicity of AgNPs to *Daphnia sp.* and the reported EC_{50} values fall in the range of 0.26 to 236.3 µg L^{-1} for AgNPs and 0.16 to 12.9 µg L^{-1} for Ag^{+} ions (Zhao and Wang, 2011, Seo et al., 2014, Seitz et al., 2015b, Sakamoto et al., 2015, Becaro et al., 2015, Blinova et al., 2013). The toxicity of coated AgNPs in this study is comparatively less than the values reported by majority of studies for other coated AgNPs. The broad range of toxicity values among published studies can be explained by different test scenarios and particle characteristics (Allen et al., 2010, Hoheisel et al., 2012). *D. magna* has been the preferred species in many previous studies while this study used *D. carinata*. 

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Many research findings support the idea that the toxicity comes exclusively from Ag\(^+\) ions as the main source of toxicity (Liu et al., 2010, Newton et al., 2013, Kittler et al., 2010). Ag\(^+\) ions could prevent the absorption of Na across the membranes of gills by inhibiting the Na\(^+\), K\(^+\)-ATPase activity and this could lead to ionoregulatory failure causing death (Bianchini and Wood, 2003). The significantly lower EC\(_{50}\) value for Ag\(^+\) ions in comparison to NPs shows that Ag\(^+\) ions are much more toxic than AgNPs. Coatings control the release of ions from AgNPs to the surrounding medium (Liu et al., 2010, Zhao and Wang, 2012a). As per the percentage dissolution of AgNPs, the computed concentrations of dissolved fractions of Ag\(^+\) ions from T-AgNPs, E-AgNPs and C-AgNPs at EC\(_{50}\) concentrations in the ASTM medium were 0.21, 0.13 and 1.06 µg L\(^-1\) respectively which were below the 48 h EC\(_{50}\) value of Ag\(^+\) ions. The toxicity may therefore not exclusively come from released Ag\(^+\) ions from AgNPs; NPs may have other toxic effects such as generation of reactive oxygen species (ROS) causing oxidative stress (Asharani et al., 2008, Dominguez et al., 2015, Zhao and Wang, 2011, Choi and Hu, 2008), attachment to daphnid’s body surface or appendages leading to physical impairment and behavioral changes (Lovern et al., 2007).

6.3.4 Feeding behaviour of D. carinata

Daphnid feeding rate significantly increased from day 1 to 5 for all treated algae and in controls due to higher food consumption with ageing (Fig 6.3). The mean 5 day feeding rate was highest in the control followed by C-AgNP, E-AgNP and T-AgNP treated algae fed daphnids. Compared with the control, there was no significant deviation (\(p > 0.05\)) in feeding rate in the day 1 while only the feeding rate of E-AgNP treated algae was significantly different in the day 2. In contrast, the feeding rates of AgNP treated algae were significantly different to the relevant control from day 3 to 5 (\(p < 0.05\)). When compared with each treatment group, Daphnid feeding rates of algae treated with E-AgNPs in day 2 and T-AgNPs in day 5 were significantly different from other two types (\(p < 0.05\)). However, a consistent difference was not observed and therefore, the data obtained is not sufficient to prove any significant variations of feeding rates among daphnids based on algae treated with AgNPs with different coatings. Besides, less favoritism for untreated algae compared to treated algae shows that AgNPs have some effect on feeding behaviour. Previous studies also have shown that the feeding rate of daphnids became depressed when contaminants were associated with algae (Taylor et al., 1998, Allen et al., 1995, JONES et al., 1991). McTeer et al. (2014) observed a reduction in feeding rates when daphnids were fed with algae treated with polymer-coated AgNPs. Nutritional
characteristics of all algae samples were similar and therefore, they emphasized that the feeding reduction was due to Ag toxicity but not due to the nutritional quality of the algae diet. Zhao and Wang (2011) speculated that the reduced feeding rate was due to accumulation of NPs in the gut or due to higher sedimentation of contaminated algae to the bottom of the vessel causing less availability for filter feeding.

Fig 6.3: Algal feeding rates of *Daphnia carinata* over a 5 d feeding period exposed to *Raphidocelis subcapitata* cells that were treated with 50 µg L⁻¹ concentrations of AgNPs. Data are mean ± SD from three independent experiments, each with 15 daphnids. The error bars indicate the SD ($p < 0.05$, $n = 3$). The $p$-values for multiple pairwise comparisons were obtained from two-way ANOVA followed by Holm-Sidak method using Sigmaplot. Letter sign denotes comparison of $p$-values of feeding rates for each day separately while the * sign denotes comparison of $p$-values of feeding rates over 5 d. Treatments that do not share lowercase letters or number of * signs are significantly different.

Inputs of energy from food is critically important for population growth and survival and therefore, the feeding rate in primary consumers like *Daphnia sp.* could have profound implications at the population level (Taylor et al., 1998). Feeding inhibition may cause reduction of growth, targeted inhibition of internal organs and reproduction of daphnids (Hanazato, 2001, Ribeiro et al., 2014a, Zhao and Wang, 2011, Lu et al., 2016b). It may also have effects on water clarity, altered nutrient regeneration rates, population size of predators (Moore and Folt, 1993) and elevated phytoplankton biomass due to reduced grazing (Jak et al.,
Therefore, the observed feeding inhibition of algae associated with NPs by daphnids in this study is a cause of concern and should be considered in assessing aquatic NP pollution.

6.3.5 Trophic transfer of AgNPs from R. subcapitata to D. carinata

The mean AgNP accumulation in algae treated with 50 µg L\(^{-1}\) C-AgNP (0.68 ± 0.09 ng 10\(^5\) cells\(^{-1}\)), E-AgNP (0.61 ± 0.08 ng 10\(^5\) cells\(^{-1}\)) and T-AgNP (0.44 ± 0.06 ng 10\(^5\) cells\(^{-1}\)) concentration was significantly different to the control (0.09 ± 0.04 ng 10\(^5\) cells\(^{-1}\)) (Fig 6.4A). C-AgNP accumulation in algae was significantly higher than T-AgNPs. However, the Ag accumulation profile in algae does not mirror the Ag accumulation profiles in daphnids. The mean Ag content in daphnids treated with T-AgNPs (0.016 ± 0.006 ng daph\(^{-1}\) 10\(^5\) cells\(^{-1}\)) was significantly higher than the Ag content in daphnids treated with C-AgNPs (0.006 ± 0.0004 ng daph\(^{-1}\) 10\(^5\) cells\(^{-1}\)) (Fig 6.4B). Also, Ag content in daphnids treated with T-AgNPs and E-AgNPs was significantly higher than the control. Though the T-AgNP treated algae accumulated lowest amount of NPs, it led to the highest mean percentage of Ag retention (3.6%) in daphnids followed by E-AgNP (2.1%) and C-AgNP treated algae (0.95%) (Fig 6.4C). In contrast, McTeer et al. (2014) found metal accumulation profiles in *D. magna* from trophic transfer correlated with the metal accumulation profiles in algae treated with ZnNPs. The Ag bioaccumulation in daphnids did not correlate with the daphnid survival percentage (Fig 6.4D) where no significant difference was observed between each treatment group.

Any remaining treated algae in the digestive tract may lead to overestimation of bioaccumulation. Gillis et al. (2005) determined the length of time required to completely depurate metal contaminated sediments from the digestive track of *D. magna* in the presence of algae and recommend a minimum of 8 h. Petersen et al. (2009) observed a limited depuration of carbon nanotubes from *D. magna* after 48 h whereas Zhu et al. (2010a) observed complete depuration of food associated TiO\(_2\) NPs from the gut of *D. magna* after 26 h. Following exposure to the treated algae, daphnids were allowed to feed on fresh algae for 72 h in fresh medium and it was assumed all NPs in the digestive track were removed. The algae treatment concentration of AgNP (50 µg L\(^{-1}\)) was chosen to ensure sufficient Ag concentrations were available for detection in daphnid tissues without causing mortality to algae, based on EC\(_{50}\) values obtained through an algae acute test for all types of NPs in a different study (please see Chapter 5, Section 5.3.3.). However, since the toxicity of Ag\(^+\) ions from Ag salt (Ag\(_2\)SO\(_4\)) to the alga was very high (72 h EC\(_{50}\): 51 µg L\(^{-1}\)), the feeding and trophic transfer experiments were not conducted for algae contaminated with Ag salt at this concentration.
Fig 6.4: Bioaccumulation and trophic transfer of AgNPs. (A) Elemental Ag content per $10^5$ *Raphidocelis subcapitata* cells measured after 5 d of growth in 50 µg L$^{-1}$ concentrations of AgNPs or with no Ag treatment (control), (B) Elemental Ag content (per daphnid), (C) Percentage Ag retained (per daphnid), and (D) Percentage survival of algae-fed *Daphnia carinata* after 5 d of exposure to *Raphidocelis subcapitata* cells that were grown in 50 µg L$^{-1}$ or no Ag (control). The error bars indicate the SD ($p < 0.05$, $n = 3$). The $p$-values were obtained from one-way ANOVA followed by Tukey’s test using Sigmaplot. Treatments that do not share lowercase letters are significantly different.

However, it is not possible to conclude whether Ag was transferred from algae to daphnids in the form of NPs or Ag$^+$ ions. McTeer et al. (2014) hypothesized that Ag$^+$ ions liberated from AgNPs by dissolution were accumulated by algae and then transferred in to the daphnids. Van Hoecke et al. (2008) demonstrated that NPs could not cross the double cell layer of *R. subcapitata* when exposed to silica NPs as confirmed by transmission electron microscopy (TEM) images. Piccapietra et al. (2012) found AgNP internalization was limited when *C. reinhardtii* was exposed to carbonate coated AgNPs. However, NPs less than 20 nm may pass through the algal cell walls since the cell walls are porous (5 – 20 nm in size) and their permeability changes during mitosis. Also, high concentrations of AgNPs may increase
the permeability of algae cell wall resulting in more internalization (Kalman et al., 2015). Miao et al. (2010a) confirmed internalization of NPs in the cell after exposing Ochromonas danica to AgNPs and Kalman et al. (2015) found AgNP localized in starch granules within the chloroplast of Chlorella vulgaris as determined by TEM images. Therefore, the trophic transfer of NPs from algae to daphnids may occur in the forms of NPs or Ag\(^+\) ions which depend on several factors such as the type of algae, life stage of algae and size of NPs. Data generated from this study clearly show that the type of coating affects the NP accumulation in algae and trophic transfer from algae to daphnids. However, it is not possible to predict the toxicity to daphnids based on bioaccumulation through trophic transfer from this study, and hence, further studies are recommended. The transfer of AgNPs along the aquatic food chain could have adverse implications and therefore there is a need to take this into consideration in protecting aquatic organisms. In doing so, great caution must be taken when assessing the risk of differently coated NPs.

6.4. Conclusions

The type of AgNP coating and medium significantly influenced the degree of aggregation and the behaviour of AgNPs. Based on the 48 h EC\textsubscript{50} values of D. carinata, we found that the Ag\(^+\) ions are significantly more toxic than AgNPs. The toxicity of E-AgNP and C-AgNP were not significantly different, but T-AgNPs were comparatively about 2.5 times less toxic. Since other characteristics such as shape, size and charge are quite similar, the difference in toxicity could be attributed to the effect of different coatings. Feeding experiments revealed that the ingestion rates of NP treated algae were significantly lower than untreated algae revealing associated AgNPs with algae change daphnid feeding behaviour which could have longer term negative effects on D. carinata population. However, findings from this study are not sufficient to conclude the cause of changed behaviour. Ingestion rates of algae treated with differently coated NPs were not markedly different showing that different types of coatings had little effect on D. carinata feeding. Our findings also demonstrated the diet-borne transfer of AgNPs from AgNP contaminated R. subcapitata to D. carinata. In the algae exposed to AgNPs, T-AgNP bioaccumulation was the highest while C-AgNPs were the lowest. However, bioaccumulation of Ag in daphnids through trophic transfer did not correlate with the accumulation profiles of Ag in algal cells. The percentage Ag retained in daphnids was highest for T-AgNP treated algae while it is lowest for C-AgNPs. These results demonstrate that type of coating may have effects on AgNP accumulation profiles at different trophic levels.
The behaviour of differently coated NPs in medium, their toxicity profile and trophic transfer data generated in this study demonstrate the importance of considering type of coating in environmental risk assessment.
Chapter 7. The toxicity of non-aged and aged coated silver nanoparticles to the freshwater alga Raphidocelis subcapitata

This chapter has been submitted to a journal and is presented here with only minor modifications to adjust formatting to the requirements of the thesis.


Abstract

The fate and transformation of coated AgNPs together with their impacts on aquatic organisms remain a research area requiring further study, given the increasing release of AgNPs into the environment. In this study, the role of three organic surface coatings (Tyrosine, Epigallocatechin gallate and Curcumin) on the stability of AgNPs in aquatic environment was studied. This study also aimed to examine and compare the toxic effects of these AgNPs of different ages (i.e., newly manufactured and aged for 29 days) using the sub-lethal indices in the freshwater alga, Raphidocelis subcapitata. The stability of the AgNPs was evaluated by monitoring surface plasmonon resonance, zeta average hydrodynamic diameter, zeta potential and dissolution of the NPs over 32 days. Results indicated that transformation of all three types of AgNPs occurred during incubation; however, coating-specific effects were observed. The presence of the three types of AgNPs resulted in increased reactive oxygen species (ROS) formation compared with the control ($p < 0.05$) while aged T-AgNPs and C-AgNPs induced excessive ROS generation (2 to 4-fold increase) compared with the fresh counterparts. Increased ROS levels caused increased lipid peroxidation in treatment groups exposed to both fresh and aged NPs while the thiobarbituric acid reactive substances (TBARS) levels were higher in algae exposed to aged AgNPs. The observed increase in catalase (CAT) activity of algal cells was attributed to early stress responses induced by excessive intracellular ROS generation while the CAT levels were higher in the aged NP treatment groups in line with the increased ROS levels. It was concluded that AgNPs have a negative effect on aquatic algae, as manifested by the increased ROS levels and lipid peroxidation while antioxidant enzymes such as CAT are activated to neutralize the oxidative stress. Overall, the results suggest that the coating and ageing of AgNPs have major impacts on AgNP transformation in media and their effects on algae.
7.1. Introduction

Nanoparticles (NPs) are being increasingly used in a wide variety of fields and potential release of NPs into the environment has raised concerns on their impacts on ecosystem. Silver NPs (AgNPs) are the most widely used NP in the nanotechnology industry due to their excellent antimicrobial properties, which has resulted in more than 50% of inventoried nanotechnology-based products containing NPs (Köser et al., 2017, Huynh and Chen, 2011, Lu et al., 2016a). AgNPs are used in a wide variety of products including medical and personal care products, food containers, textiles, paints and disinfectants (Reidy et al., 2013, Mackevica et al., 2016, Keller et al., 2014, McClements and Xiao, 2017, Caballero-Guzman and Nowack, 2016). Migration of AgNPs from these products into the aquatic environment after use may pose toxicity risks to organisms (Matzke et al., 2014, Kwak et al., 2016). However, their impact on ecosystems and human health are not fully evaluated (Kwok et al., 2016, Selck et al., 2016b).

The views on effects of NP ageing are variable since increasing, decreasing or similar toxicity with ageing have been reported. Su et al. (2018) observed more toxicity from 15 d aged Mo-nZVI NPs to the cyanobacteria, Microcystis aeruginosa than those aged for a shorter time (< 5 d). The researchers attributed this to the transformation of NPs due to formation of different particle composition and morphology over time. However, Pereira et al. (2011) observed increased toxicity of gold nano-rods, but decreased toxicity of organic NPs with ageing of NPs, to the bacterium Vibrio fischeri. Interestingly, Sørensen and Baun (2015) reported increased toxicity of AgNPs after 48 h ageing to R. subcapitata, but the toxicity of particles decreased with ageing beyond 48 h. Changes in toxicity correlated well with the aggregation of NPs around 3 to 4 days of ageing. Lei et al. (2016b) reported decreased toxicity of 90 d aged ion-based NPs to the alga Chlorella pyrenoidosa, which was attributed to the oxidation of NPs leading to reduced redox activity of NPs and decreased adhesion of NPs to the cells due to formation of iron oxide on the particle surfaces. Manier et al. (2013) exposed freshwater alga Raphidocelis subcapitata to freshly prepared and artificially altered 3 and 30 days old CeO$_2$ NPs. The particles highly agglomerated up to 10 μm in size within the first 3 days and reached steady state thereafter. However, 72 h EC$_{50}$ values revealed that the toxicity of aged NPs was not significantly different to the non-aged NPs. Similarly, Cupi et al. (2015) observed no difference in toxicity of 48 h aged AgNPs in comparison to freshly prepared AgNPs to Daphnia magna.
Physicochemical characteristics of the aqueous medium have also been shown to alter the behaviour of NPs and thus their toxicity levels (Li et al., 2011b, von Moos et al., 2015, Aravantinou et al., 2015, Seo et al., 2014, Muna et al., 2017, Hu et al., 2017). Aggregation, agglomeration, dissolution and degradation of NPs are important parameters which will determine their toxicity. pH has a significant effect on zeta potential and agglomerate size (Berg et al., 2009, Omar et al., 2014, Prathna et al., 2011). The availability of dissolved Ag concentrations is controlled by the presence of Cl\(^{-}\) and proteins (Köser et al., 2017) while Mg\(^{2+}\) and Ca\(^{2+}\) may compete with toxic ions for binding sites, reducing toxicity (Li et al., 2013a). Aggregation and dissolution of AgNPs increase with increasing ionic strength (Zhang et al., 2016c).

Multiple toxicological studies have shown that the properties of the NPs themselves, including size, shape, and surface chemistry play an important role in determining toxicity (Albanese et al., 2012, Silva et al., 2014a, Fekete-Kertész et al., 2017, Dominguez et al., 2015, Baumann et al., 2014). The stabilization mechanism is determined by the type of coating; there are an extensive number of coating materials being utilized in the NP industry (El Badawy et al., 2012, Tolaymat et al., 2010, Fauss et al., 2014, Keller et al., 2010b, Zheng et al., 2015). Previously, the most prevalent capping agents used for coating AgNPs were citrate, sodium borohydride (NaBH\(_4\)), and polyvinylpyrrolidone (PVP) (Badawy et al., 2010). However, the requirement for biocompatible capping agents, especially in the pharmaceutical industry (Duse et al., 2017) has resulted in the development and use of several organic based materials (biomass or extracts) as capping agents in producing NPs (Rodríguez-León et al., 2013, Sapsford et al., 2013). The capping agents which were used to synthesize AgNPs in this study were Tyrosine (T-AgNP), Epigallocatechin gallate (E-AgNP) and Curcumin (C-AgNP) (Contino et al., 2016, El Khoury et al., 2015, Khaskel et al., 2015, Nune et al., 2009).

Given current production and use of NPs, there is a pressing need for further toxicological understanding of the fate and effects of NPs following release into the environment (Mitrano et al., 2015b). Although numerous studies have investigated the stability of AgNPs with different properties under various environmental conditions, there is little information available regarding the impact of the transformation of NPs on the toxicity to aquatic organisms. The main objective of this study was to investigate the fate and behaviour of AgNPs with three different biocompatible coatings in algae media and to evaluate their effects on toxicity. The toxicity to the freshwater alga *Raphidocelis subcapitata* was evaluated
with both pristine and 29 days old AgNPs in relevant media while monitoring the stability of AgNPs. Exposure to AgNP can cause excessive production of reactive oxygen species (ROS) and membrane damage in algae (Rogers et al., 2010, Sørensen et al., 2016a, Dorobantu et al., 2015). Algae have a sophisticated system to produce antioxidant enzymes such as catalase (CAT) to self-regulate oxidative stress caused by ROS (Liu et al., 2017, Dauda et al., 2017). The toxic response of algae to AgNP exposure in terms of antioxidative potential and lipid peroxidation was assessed respectively by measuring the CAT activity and thiobarbituric acid reactive substances (TBARS) levels, a by-product of lipid peroxidation, to compare the effects of non-aged and aged AgNPs on the algae. To the best of our knowledge, this is the first comparative study where effects of differently coated AgNPs on algae were studied.

7.2. Materials and methods

7.2.1. Test materials

Please see Chapter 3. NP Synthesis, characterisation and culturing organisms, Section 3.1. Synthesis of AgNPs.

7.2.2. Characterization of AgNPs

Please see Chapter 3. NP Synthesis, characterisation and culturing organisms, Section 3.2. Characterisation of AgNPs.

7.2.3. Test organisms and culture conditions

Please see Chapter 3. NP Synthesis, characterisation and culturing organisms, Section 3.3. Culturing and maintenance of test species for information on culturing R. subcapitata which was used in this study. Algal cells were counted periodically with an automated cell counter (TC20™, Bio-Rad Laboratories, Hercules, CA). The culture was grown to a density of \( > 1 \times 10^6 \) cells mL\(^{-1}\).

7.2.4. AgNP temporal stability and dissolution in the test medium

The stability of AgNPs in the M4 medium and MilliQ water was investigated for 32 days under similar environmental conditions used for algae exposure tests (please see Section 7.2.5.), as previously described in Chapter 5. The toxicity of coated silver nanoparticles to the
alga *Raphidocelis subcapitata* Section 5.2.4. AgNP Temporal Stability and Dissolution in the Test Media. The measurements were taken at the beginning and after 1, 2, 4, 8, 16 and 32 days.

7.2.5. Algal exposure to fresh and aged AgNPs

AgNP suspensions of 300 µg L⁻¹ were prepared in M4 medium using stock solutions and aged for 29 d in Erlenmeyer flasks which were kept on shakers (100 rpm) (OM6, RATEK, Aus) in a light-temperature controlled chamber at 23 ±1 °C under 16: 8 h dark: light photoperiod with illumination of 6000 lux to mimic environmental conditions. Each test material including the blank control (containing only M4 medium) was aged in triplicate. Fresh AgNP suspensions of 300 µg L⁻¹ were prepared just before the toxicity tests from the same NP stock suspensions in triplicate while fresh M4 medium was used as the blank control. The physicochemical characteristics of NP stock suspensions were monitored, and no changes were observed during 32 days. Algal toxicity tests were performed after 29 days as per the organisation for economic co-operation and development (OECD) guidelines (OECD, 2011a) with some modifications. Algal cells were harvested by centrifugation (3,500 g, 15 min, 4 °C) (Heraeus Multifuge 1S-R, Thermoscientific) and the pellets were resuspended in autoclaved fresh M4 medium. Aliquots (400 mL) of fresh and aged NP suspensions were inoculated in triplicate with algae as the cell density was approximately 1 × 10⁶ cells mL⁻¹ and incubated under similar environmental conditions used for algae culturing. All stock suspensions were sonicated before spiking into the test vessels. Flasks were kept on shakers (100 rpm, OM6, RATEK, Aus) to allow mixing and CO₂ diffusion while they were manually shaken every 24 h to resuspend any settled cells. pH was monitored throughout the testing period. Toxicity tests for aged NPs were performed in the same vessels used for NP ageing to avoid differences in interaction with the glass vessel.

7.2.6. Qualitative evaluation of intracellular ROS

The generation of ROS was quantified using the fluorescent dye 2, 7-dichlorodihydrofluorescein diacetate (H2DCFDA) (Oukarroum et al., 2017). The H2DCFDA stock solution (10 mM) was prepared in DMSO in the dark and stored at 4 °C. Algal samples (1 mL) were incubated with H2DCFDA (10 µM) for 30 min in the dark and the intracellular ROS level was determined by measuring the fluorescence emissions with excitation and emission wavelengths of 485 and 530 nm in a 96 well plate (Nunc, Denmark) using a microplate reader (POLARstar Omega, BMG Labtech). A negative control of untreated algal
cells was also analysed to measure the interference due to autofluorescence. The relative percentage of ROS levels were expressed compared to the control. To normalise the fluorescence emission, algal cell concentration in each sample was quantified with PrestoBlue®, a cell viability reagent that uses the reducing ability in the cytosol of the cells which turns the reagent red in colour and highly fluorescent. An aliquot (190 µL) of the algal cell samples was mixed with 10 µL of the reagent in a 96 well plate and incubated for 2 h at 37 °C before proceeding with absorbance reading at 570 nm using a PolarStar Omega spectrophotometer. A calibration curve was developed with samples of known cell density, determined by cell counting with a TC20TM Automated Cell Counter, Bio-Rad Laboratories, Hercules, CA.

7.2.7. Biochemical tests

After exposure, algal cultures were collected, and centrifuged at 3,500 g for 15 min (Heraeus Multifuge 1S-R, Thermoscientific). The resultant pellets were washed three times with phosphate buffer (0.05 M, pH 7.8) and resuspended in precooled buffer. The cells were homogenized in 2 mL lysing tubes (FastPrep™, MP bio) using a FastPrep-24™ 5G Homogenizer, MP bio and centrifuged at 3,000 g for 15 min at 4 °C (Sigma 3-16KL centrifuge) for antioxidant enzyme assays. The supernatant containing the enzymes was collected and stored at -80 °C.

CAT was assessed by measuring the decomposition of H₂O₂ as described by Hadwan (2016). When heated in the presence of H₂O₂, dichromate in acetic acid is reduced to chromic acetate. The concentration of chromic acetate measured colourimetrically at 570 nm is directly proportional to the H₂O₂ concentration. H₂O₂ was added to the supernatant and incubated in a water bath at 37 °C for 3 min. Dichromate/acetic acid solution was then added, further incubated for 10 min at 90 °C and centrifuged at 2500 g for 5 min to remove precipitated protein. The resulting supernatant was plated in a 96 well plate and the absorbance was read at 570 nm using a spectrophotometer (POLARstar Omega, BMG Labtech). CAT activity was calculated by modifying the equation presented by Hadwan (2016) and expressed as U mg⁻¹ protein, with one unit of CAT defined as the amount of CAT needed to reduce 1 µM of H₂O₂ per minute. Total protein concentration of samples was measured according to Bradford (1976) using a 96-well microplate (Nunc, Denmark) and absorbance reading at 595 nm in a microplate reader (POLARstar Omega, BMG Labtech). A calibration curve was obtained with bovine serum albumin standard. CAT activity was calculated as follows:
CAT activity (kU min\(^{-1}\) mg protein\(^{-1}\)) = \frac{2.303}{t} \times \log_{10} \frac{S_t}{T-CT} \times \frac{VT}{VS} \times \frac{1}{c}

where, \(t\) is the time (min), \(S_t\) is the absorbance of the standard, \(T\) is the absorbance of the test, \(CT\) is the absorbance of the control test, \(VT\) is the total volume of reagents in the assay tube (mL), \(VS\) is the volume of the supernatant (mL) and \(c\) is the protein concentration (mg mL\(^{-1}\)).

Oxidative damage of lipids can lead to the formation of lipid hydroperoxides which further degrade into several types of aldehydes, including malondialdehyde (MDA). Oxidative damage by ROS was quantified by measuring TBARS which are a proxy for MDA levels, as described by Parrilla-Taylor et al. (2013). Briefly, the supernatant was incubated at 37 °C for 15 min in a shaking water bath and then the reaction was stopped by adding ice cold stopping solution (1.0 M HCl in 12.5% TCA and 1% TBA). The sample was incubated again at 90 °C for 10 min, cooled to room temperature, and centrifuged at 3,000 \(g\) for 10 min at 4 °C. The supernatant was collected and its absorbance was read at 532 nm. The MDA concentration was calculated using an extinction coefficient of 155 mM\(^{-1}\) cm\(^{-1}\) and the MDA content was expressed as nM TBARS per 10\(^{10}\) algal cells.

7.2.8. Data analysis

Data from bioassays were analysed by a two-way ANOVA followed by Tukey's test for pairwise multiples comparison of means. Shapiro Wilk’s test was performed to check the normal distribution followed by Levene’s test for variance homogeneity analysis. Data transformation was included when data were not normally distributed. All tests were performed at the 5% level of significance.

7.3. Results and discussion

7.3.1 Characteristics of AgNPs

Please see Chapter 3. NP Synthesis, characterisation and culturing organisms, Section 3.2. Characterisation of the AgNPs.

7.3.2 AgNP stability and dissolution

Once the AgNPs were dispersed in MLA medium, visual observation revealed that the colour of the E-AgNP suspension changed within 5 minutes. However, the colour of T-AgNP suspension remained quite stable up to 16 days and no visually observable changes occurred
in C-AgNP suspension even after 32 days. The absolute zeta potential values of T-AgNP, E-AgNP and C-AgNPs in M4 medium sharply decreased by 10, 16 and 20 mV respectively within the first 5 min while the values were stable thereafter (Fig. 7.1). The DLVO theory suggests that the increased ionic strength of the M4 medium may have shielded the charges reducing the diffuse layer thickness which allows the AgNPs to come closer resulting in aggregation (Römer et al., 2011, Metreveli et al., 2016). Typically, an absolute zeta potential value above 30 mV is considered stable (Rogers et al., 2010, Daima et al., 2014). No more major changes were observed in zeta potential while the values remained around -30 mV, suggesting that the surface corona of all three types of NPs became stable, resulting in stable NPs. Zeta potential values of AgNPs in the MilliQ water did not change significantly during the ageing process except for E-AgNPs, where the value dropped from -40 to -31 mV within last 16 days (Fig. 7.2). The decreased zeta potential values in the M4 medium with high ionic strength compared to MilliQ water with relatively low ionic strength are in accordance with the classical colloid theory (Hunter, 1981, Elimelech et al., 1995). A reduction of the thickness of the diffuse double layer with increased ionic strength allows for the attractive van der Waals interactions to dominate and increases the particle-particle interaction resulting in increased aggregation (Badawy et al., 2010, Brant et al., 2005a).

![Fig. 7.1: Change in zeta potential of AgNPs with different coatings in M4 medium within 32 d. NPs were dispersed in M4 medium at Ag concentration of 5,000 µg L^{-1}. Error bars indicate the standard deviation from triplicates.](image-url)
Fig. 7.2: Change in zeta potential of AgNPs with different coatings in MilliQ water within 32 d. NPs were dispersed in MilliQ water at Ag concentration of 5,000 µg L$^{-1}$. Error bars indicate the standard deviation from triplicates.

The z-average hydrodynamic diameter (HDD) of T-AgNPs and E-AgNPs increased by 1.4 and 1.5-fold within day one while it was 1.1-fold for C-AgNPs. The corresponding z-average HDD values respectively increased by 1.6, 1.4 and 1.3-fold within 4 days, equivalent to the acute algae exposure duration (Fig. 7.3). The size of T-AgNPs and E-AgNPs slightly increased up to 1.8 and 1.6 times respectively within the next 28 days while this value increased 2.3 times for C-AgNPs. However, the percentage intensity distribution of NPs revealed that approximately 20% of the particles of T-AgNPs were at micrometre level after 32 days while it was less than 5% for E-AgNPs and C-AgNPs. Pdi values remained unchanged in all suspensions throughout the ageing period. No changes to HDD were observed for AgNPs in the MilliQ water during the ageing process (Fig. 7.4). Tejamaya et al. (2012b) reported a 2-fold size increase in citrate and polyethylene glycol (PEG) coated AgNPs in different media over 21 days. Ribeiro et al. (2014a) reported a 2 to 4 times increase in the HDD of alkane coated AgNPs in the Woods Hole MBL, ASTM daphnia and Zebra fish culture medium over 4 days. Sørensen and Baun (2015) observed a 2.5 times size increase of citrate coated AgNPs in ISO algal medium after 7 days. The observed differences in aggregation among coated AgNPs may be due to different coating materials since other characteristics of AgNPs such as initial HDD, shape and zeta potential are not considerably different (Behra et al., 2013, Huynh and Chen, 2011). Also, no signs of NP aggregation in MilliQ water was observed within the same time duration. The aggregation of NPs in aquatic environments influences the fate and
transport of NPs, and hence the toxicity risk NPs pose to aquatic organisms (Zheng et al., 2015, Badawy et al., 2010). In general, the majority of studies support the idea that the aggregation of NPs reduces the toxicity to organisms (Kvitek et al., 2008, Lok et al., 2007) though some researchers claim highly aggregated NPs can stay as toxic as non-altered suspension to microalgae (Manier et al., 2013).

**Fig. 7.3:** Change in HDD of AgNPs with different coatings in M4 medium within 32 d. NPs were dispersed in M4 medium at Ag concentration of 5,000 µg L⁻¹. Error bars indicate the standard deviation from triplicates.

**Fig. 7.4:** Change in HDD of AgNPs with different coatings in MilliQ water within 32 d. NPs were dispersed in MilliQ water at Ag concentration of 5,000 µg L⁻¹. Error bars indicate the standard deviation from triplicates.
The percentage of ions released from T-AgNPs, E-AgNPs and C-AgNPs in M4 medium after 32 days were 0.67, 0.22 and 1.54% respectively (Table 7.1). Interestingly, the percentage dissolution in the MilliQ water was slightly higher with corresponding values of 2.18, 0.37 and 2.27% (Table 7.2). This may be due to the precipitation of Ag ions by halides (Cl\(^{-}\)) in the medium (Römer et al., 2011, Ellis et al., 2016a) which is not expected in MilliQ water. Halides may reduce the exposure of organisms to any free ions released from AgNPs. Also, the size of the AgNPs is one of deciding factors for the release of Ag\(^{+}\) ions (Zhang et al., 2011). Smaller particles have high surface to volume ratios and therefore, more atoms on the surface come into contact with oxidants in comparison to larger particles (Zhao and Wang, 2012b). Aggregation of NPs in M4 medium results in larger particles, with increased HDD reducing the surface area of particles available to release free Ag ions (Allen et al., 2010, Zhao and Wang, 2012a) in contrast to the NPs in MilliQ water. Liu et al. (2018a) observed mean dissolution rate of bovine serum albumin (BSA) and PEG coated AgNPs in the range of 0.01 and 0.04 µg L\(^{-1}\) per day in comparison to the 0.18 and 0.31 µg L\(^{-1}\) per day observed in this study. Studies have shown that far higher percentages of Ag\(^{+}\) ion release are commonly observed at low concentrations as a function of time while ion release of certain coated NPs is completely inhibited at higher concentrations (Liu et al., 2010). Therefore, the percentage of Ag\(^{+}\) ions released from the NPs in test vials may be higher than the values presented. Also, the adsorption of the ion to the culture vessels cannot be excluded (Dong et al., 2017, Sekine et al., 2015).

Table 7.1: The percentage of soluble Ag released from AgNPs with different coatings in the M4 medium within 32 d. NPs were dispersed in M4 medium at Ag concentration of 5,000 µg L\(^{-1}\). Standard deviations (± SD) are from triplicates.

<table>
<thead>
<tr>
<th>Substance</th>
<th>% dissolution</th>
<th>1 d</th>
<th>2 d</th>
<th>4 d</th>
<th>8 d</th>
<th>16 d</th>
<th>32 d</th>
</tr>
</thead>
<tbody>
<tr>
<td>T-AgNP</td>
<td>0.06 ± 0.14</td>
<td>0.15 ± 0.11</td>
<td>0.41 ± 0.25</td>
<td>0.55 ± 0.59</td>
<td>0.81 ± 0.15</td>
<td>0.94 ± 0.54</td>
<td></td>
</tr>
<tr>
<td>E-AgNP</td>
<td>0.16 ± 0.26</td>
<td>0.06 ± 0.21</td>
<td>0.27 ± 0.27</td>
<td>0.17 ± 0.10</td>
<td>0.21 ± 0.16</td>
<td>0.33 ± 0.47</td>
<td></td>
</tr>
<tr>
<td>C-AgNP</td>
<td>0.66 ± 0.81</td>
<td>1.36 ± 0.58</td>
<td>1.60 ± 0.78</td>
<td>1.54 ± 1.21</td>
<td>1.27 ± 0.46</td>
<td>1.47 ± 0.64</td>
<td></td>
</tr>
</tbody>
</table>
Table 7.2: The percentage of soluble Ag released from AgNPs with different coatings in MilliQ water within 32 d. NPs were dispersed in MilliQ water at Ag concentration of 5,000 µg L⁻¹. Standard deviations (± SD) are from triplicates.

<table>
<thead>
<tr>
<th>Substance</th>
<th>% dissolution</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1 d</td>
</tr>
<tr>
<td>T-AgNP</td>
<td>0.04 ± 0.07</td>
</tr>
<tr>
<td>E-AgNP</td>
<td>0.36 ± 0.10</td>
</tr>
<tr>
<td>C-AgNP</td>
<td>4.39 ± 0.52</td>
</tr>
</tbody>
</table>

A larger decrease in absorbance peaks was observed for T-AgNPs (~23%) and E-AgNPs (~28%) within a day while it changed less than ~7% in C-AgNPs (Fig. 7.5A, 7.5B and 7.5C). The SPR bands of T-AgNPs and E-AgNPs significantly broadened and flattened within 32 days which was not observed for C-AgNPs. Interestingly, a new absorbance peak appeared in T-AgNPs at a longer wavelength indicating scattered light from large aggregates (Tejamaya et al., 2012b). A broader absorbance peak and a large background signal in SPR spectra and increased HDD indicate aggregation of NPs (Stebounova et al., 2011, Tejamaya et al., 2012b, Jiménez-Lamana and Slaveykova, 2016). The absorbance reduction in SPR spectra for all AgNPs could be either due to the aggregation or dissolution of Ag⁺ ions (Jiménez-Lamana and Slaveykova, 2016). All three types of AgNPs showed the presence of NPs even after 32 days as indicated by the SPR bands; curcumin as the capping agent shows the most protective effect of the three different capping agents while tyrosine shows the least effective effect. However, the SPR bands of AgNPs over time do not fully agree with the observations made for HDD and zeta potential. The absorbance peak of E-AgNPs dropped faster than C-AgNPs though the latter showed higher zeta average size. The zeta potential values of all the AgNPs were quite stable during the ageing period, but the SPR curves were continuously flattening making it unclear which kind of transformation NPs have undergone. Therefore, overall, the techniques used were not sensitive enough to accurately discriminate between the stability of different coated AgNPs. Further specific work should be performed to fully address the effects of these coatings on the stability of AgNPs in media. The SPR bands of AgNPs did not change considerably in MilliQ water (Fig. 7.6A, 7.6B and 7.6C).
Fig. 7.5: The SPR curves of (A) T-AgNPs, (B) E-AgNPs, and (C) C-AgNPs in M4 medium.

Fig. 7.6: The SPR curves of (A) T-AgNPs, (B) E-AgNPs, and (C) C-AgNPs in MilliQ water.
This study shows that the coating material and the medium significantly influence the fate and behaviour of AgNPs. Change in NP characteristics such as HDD, zeta potential and dissolution with ageing may influence the bioavailability of NPs and hence, play an important role in determining toxicity (Jiang et al., 2009, Sager et al., 2007, Miao et al., 2010b, Tejamaya et al., 2012b). Also, culture medium impacted the behaviour and properties of AgNPs which may ultimately lead to various toxicological responses (Ji et al., 2011). Therefore, it is required to consider those in environmental risk assessment (Van Hoecke et al., 2008).

7.3.3. Sub-lethal toxicity of NPs with ageing

Oxidative stress and damage may occur upon exposure to xenobiotics at sublethal levels (Masteling et al., 2016). Increased ROS generation in algae is an important toxic mechanism (Liu et al., 2017). ROS generation in cells may be caused by particle properties at the nanoscale or by dissolved toxic metal ions from AgNPs (von Moos and Slaveykova, 2014). Here, oxidative stress was evaluated using the cell-permeable redox-sensitive dye H$_2$DCFDA that is converted to the highly fluorescent 2',7'-dichlorofluorescein upon cleavage of the acetate groups by intracellular esterases and oxidation in the presence of ROS. The ROS levels produced by *R. subcapitata* upon exposure to AgNPs are presented in terms of relative percentage increase compared to the control. The results showed that ROS formation in all the treatment groups were significantly higher than those in the control group ($p < 0.05$), which suggested that fresh and aged AgNPs caused increased oxidative stress (Fig. 7.7A). Fresh and aged T-AgNPs increased ROS levels by approximately 5 and 12 times respectively compared to the control, while E-AgNPs increased by 18 and 17 times and C-AgNPs increased by 10 and 40 times respectively. Based on the results obtained, we found a significant increase in ROS formation by aged T-AgNPs and C-AgNPs compared to their fresh forms while aged E-AgNPs did not cause a significant increase compared to its fresh form ($p < 0.05$). Aged C-AgNPs showed the strongest level of ROS formation which is approximately 2.5 times higher than the next highest, aged E-AgNPs. Several researchers have reported intracellular ROS increase in algae upon exposure to NPs (Xia et al., 2015, Dauda et al., 2017, Oukarroum et al., 2012, Costa et al., 2016, Dalai et al., 2013, Bhuvaneshwari et al., 2015). Oukarroum et al. (2012) reported ROS levels increased by 7 and 25 times in *Chlorella vulgaris* and *Dunaliella tertiolecta* respectively upon exposure to AgNPs at 10 mg L$^{-1}$. Increased ROS levels could cause toxicological injuries to algal cells such as oxidative deterioration of lipids and proteins, damage to genomic DNA, tissue damage and even cell death (Bagchi et al., 2001, Facchinetti
et al., 1998, Matés and Sánchez-Jiménez, 1999). The results obtained showed increased ROS formation in algae cells upon exposure to AgNPs while ROS levels are dependent on capping type and state of ageing of AgNPs.

Lipid peroxidation (LPO) is the oxidative degradation of lipids in cells by ROS, and therefore an indicator of oxidative stress. LPO levels are measured by measuring TBARS, which are a proxy for MDA, the end product of LPO. Increased MDA levels as a result of oxidative degradation of lipids cause membrane damage, impaired cellular function and cell lysis (Dauda et al., 2017, Rikans and Hornbrook, 1997). As depicted in Fig. 7.7B, the MDA levels in algae exposed to aged T-AgNPs and both fresh and aged E-AgNPs and C-AgNPs were significantly higher \((p < 0.05)\) compared with the control and positively correlates with increased ROS formation. Increased MDA levels in algae upon exposure to AgNPs (Oukarroum et al., 2012), CuO NPs (Melegari et al., 2013), CeO NPs (Rogers et al., 2010), TiO\(_2\) NPs (Xia et al., 2015, Dauda et al., 2017) are reported elsewhere. Dauda et al. (2017) reported increased MDA levels approximately from 50 to 1600 nM per 10\(^{10}\) cells in \(C.\ vulgaris\) when exposed to TiO\(_2\) NPs for 72 h where it was 34 to 1200 nM per 10\(^{10}\) cells in \(R.\ subcapitata\) in the current study when exposed to AgNPs. Oxidative stress caused by ROS formation leads to lipid peroxidation causing increased uptake of NPs into the algal cells due to membrane deformation and the formation of new pores making the cell membrane more permeable and less selective (Ozkaleli and Erdem, 2018, Melegari et al., 2013, Pacurari et al., 2012). Aged C-AgNPs caused a significant increase \((p < 0.05)\) in MDA levels compared with their fresh counterparts corroborating ROS levels. However, significant increase in MDA levels caused by aged E-AgNPs compared to fresh NPs does not corroborate with ROS levels observed where no significant difference was observed. Several researchers have reported a positive correlation between increased ROS and LPO levels in algae (Xia et al., 2015, Oukarroum et al., 2012, Ozkaleli and Erdem, 2018) upon exposure to NPs. Except for T-AgNPs, the results in the present study showed that AgNPs caused increased LPO levels and the effects were higher in algae exposed to aged NPs. Also, the level of increase depended on the type of coating.

Algae use several mechanisms to cope with ROS formation and increased CAT activity is attributed to an adaptation of algae to the stress induced by AgNPs (Melegari et al., 2013). The results showed that CAT activity in algae treated with aged T-AgNPs, fresh and aged E-AgNPs and C-AgNPs were significantly higher (approximately 5, 3, 9, 3 and 19 times respectively) than the relevant control group (Fig. 7.7C). This correlates with the increased
ROS levels by relevant treatments, though the magnitude of increase in CAT activity is less than the magnitude of increased ROS formation. Fresh T-AgNPs did not cause significant changes in CAT levels compared with the control though it caused a significant increase in ROS formation. In response to increased ROS levels, the organism responded by upregulating CAT; however, the increased TBARS show that CAT upregulation was insufficient to counteract the increased ROS and the organism was experiencing oxidative stress. Increased CAT activity was observed when the alga *C. reinhardtii* was exposed to CuO NPs (Melegari et al., 2013) and *Nitzchia closterium* was exposed to TiO$_2$ NPs (Xia et al., 2015).

Fig. 7.7: (A) Percentage ROS levels compared to the control, (B) intracellular TBARS level, and (C) CAT activity when *Raphidocelis subcapitata* was exposed to fresh and 29 d aged AgNPs. The error bars indicate the SD ($p < 0.05$, $n = 3$). The $p$-values for multiple pairwise comparisons were obtained from two-way ANOVA followed by Tukey’s test using Sigmplot. Letters denote comparison of $p$-values for effects of particles within fresh or aged NP treatment groups and control and effects of each type of fresh and aged NP treated algae.

The narrow scope of our enzyme selection reflects our experimental aims. Whilst the antioxidant system is comprised of numerous enzymes and antioxidant molecules (e.g., GSH, GSSG), our study did not aim to categorise the effect of nanoparticles on the full gamut of the
antioxidant system. Rather, we aimed to quantify one antioxidant pathway to demonstrate that nanoparticles can pose an oxidative challenge to organisms. We selected catalase as the most appropriate enzyme for our aims because catalase is the enzyme responsible for handling short-term, rapid increases of ROS in cells, which our ROS results proved is the type of oxidative challenge posed by our nanoparticle treatments (Pamplona and Costantini, 2011). Other antioxidant pathways which operate parallel to catalase (involving GPx, GRed, GSH and GSSH) were not assayed because this pathway, unlike the pathway involving catalase, is involved in responding to chronic elevated ROS concentrations, and was therefore inappropriate for our experimental aims.

Several studies have demonstrated ROS-mediated oxidative damage to cells by AgNPs. In general, increased ROS formation, lipid peroxidation and CAT activities in treatment groups compared with the control in this study show higher oxidative stress in *R. subcapitata* cells as reported by other researchers. The fate and behaviour of AgNPs in the medium and their sub-lethal effects towards *R. subcapitata* was found to depend on the type of coating and ageing of NPs. The potential sub-lethal effects induced by aged C-AgNPs was highest followed by E-AgNPs and T-AgNPs although stability tests do not provide data to elucidate the causes for the observed differences. Further, stability experiments were conducted at a higher concentration of AgNPs and may not reflect the actual behaviour of NPs at the concentration tested. For instance, the first order rate constant of ion release from AgNPs decreases with increased NP concentration (Liu and Hurt, 2010a). Besides, ion release rate in the natural environment might be slower than in the laboratory experiments due to the presence of natural organic matter (NOM), nutrients and salts (Lee et al., 2012b).

Aggregation, dissolution and oxidation of NPs are important characteristics that influence the effects of NPs in the aquatic environment. Aggregation causes increased precipitation of NPs making them less available to interact with cells (Römer et al., 2011). Also, smaller NPs have a higher potential to interact with algal cells (Bystrzejewska-Piotrowska et al., 2009) and to internalise compared to larger aggregates (Bhuvaneshwari et al., 2015). However, deformation of membrane structure as observed by increased TBARS levels in this study may result in higher uptake of NPs including larger aggregates as shown by several researchers. Moreover, the AgNPs tested in this study did not aggregate considerably in the medium while the zeta average HDD of T-AgNPs and C-AgNPs only doubled within 32 days. Manier et al. (2013) suggested aged NPs stayed as toxic as pristine particles since the
particles are linked in a loose manner leaving the particle surface area available for interaction with algal cells. Direct physical effects such as membrane disruption from closely adhered NPs or indirect effects such as shading, or the limitation of the nutrient intake cannot be excluded. We did not perform any standard toxicity tests such as growth inhibition in the current study. It is known that there are other mechanisms underpinning the toxicity of NPs to algae (Bhuvaneshwari et al., 2015, Nogueira et al., 2015) and therefore these results do not exclusively reflect the toxicity of AgNPs or the effects of coating or ageing of NPs. Further, the sub-lethal effects observed do not necessarily imply toxicity since cells mediate the negative impacts by certain cellular mechanisms. Overall, it is evident from this study that differently coated AgNPs have different behaviours in the culture media, leading to different physical and chemical alterations and therefore different bioavailability leading to biochemical reactions in algal cells. This dynamic interplay between physicochemical properties of NPs and the biological responses of organisms in the aquatic environment is an important factor in evaluating the risks to aquatic communities.

7.4. Conclusions

This study demonstrated that the coating material and ageing of AgNPs significantly influence the fate and behaviour of AgNPs and sub-lethal effects in algae upon exposure to AgNPs. AgNPs caused increased ROS formation compared with the control while aged T-AgNPs and C-AgNPs induced excessive ROS generation compared with their pristine forms. Both fresh and aged AgNPs caused higher lipid peroxidation while aged AgNPs did more damage. CAT activity was higher in the aged NP treatment groups in line with their increased ROS levels. The results obtained in the present study provide a better understanding of the environmental behaviour of differently coated AgNPs with ageing and their impacts on algae.
Chapter 8. The toxicity of non-aged and aged coated silver nanoparticles to the freshwater shrimp Paratya australiensis

This chapter has been submitted to a journal and is presented here with only minor modifications to adjust formatting to the requirements of the thesis.


Abstract

Nanoparticles (NPs) transform in the environment that alter their physicochemical properties compared to their pristine state. However, effects of NP ageing on their toxicity to aquatic organisms are scarcely investigated while contradictory results have been reported. Herein, the stability of differently coated silver nanoparticles (AgNPs) in the media and the effects of AgNP ageing on their toxicity to the freshwater shrimp Paratya australiensis were investigated. Our results showed that the stability of AgNPs in the medium was dependent on the coating of AgNPs. Also, coating dependent changes in AgNP stability were observed with ageing. Increased catalase (CAT) activity upon exposure to AgNPs indicated higher oxidative stress in P. australiensis. However, the sub-lethal toxicity of AgNPs was not significantly affected by type of coating or ageing of AgNPs.

8.1. Introduction

Nanoparticles (NPs) are increasingly used for many purposes due to their distinctive properties compared to their large size counterparts (Oomen et al., 2018). However, the unique characteristics of NPs result in different exposure scenarios and effects compared with other conventional contaminants. Nanosilver is one of most used NPs due to their excellent antimicrobial properties. The use of silver NPs (AgNPs) is increasing (Giese et al., 2018) and their environmental release (McGillicuddy et al., 2017), especially to the aquatic ecosystems (Vale et al., 2016) has raised concerns. Therefore, it is required to assess the risk of AgNPs to protect the organisms in the aquatic environment. Numerous studies were were carried out to investigate effects of NPs on aquatic organisms. The majority of the investigations were based on organisms exposed to pristine NPs (Manier et al., 2013). However, NPs undergo physicochemical modifications in the aquatic environment (McGillicuddy et al., 2017, Nguyen
et al., 2018) which may alter their toxic potential (Yang et al., 2019). However, coherent studies evaluating NP transformation with time in the aquatic environment and the resulting effects on organisms are limited.

Physicochemical characteristics of NPs influence their effects on organisms. Several studies have investigated the effects of size (Albanese et al., 2012, Ivask et al., 2014b), shape (Bacchetta et al., 2018, Albanese et al., 2012), coating (Badawy et al., 2010, Kwok et al., 2016, Bozich et al., 2014, Yung et al., 2017a), and charge (Nasser et al., 2016, Dominguez et al., 2015) of NPs. Capping agents are generally used to increase the colloidal stability (Baumann et al., 2014) of NPs while they can alter the transformation rate of NPs (Liu et al., 2018a). The behaviour of different AgNPs in the same medium depends on the type of coating (Bone et al., 2012). Biological effects such as uptake, accumulation, transformation and elimination are affected by surface functionalization (Kim et al., 2016, Park et al., 2015). Several materials are used as capping agents for AgNPs (Lekamge et al., 2018a) while research on organic based coatings is increasing.

Past studies show that NPs transform in the environment driving them away from their pristine state to different forms which have different physicochemical properties compared with freshly manufactured NPs (Ellis et al., 2018, Fang et al., 2017, Ellis et al., 2016b). Change of size, dissolution and zeta potential are certain essential processes occurring with transformation altering the effects of the NPs on organisms (Bacchetta et al., 2016b). NPs are bioavailable to organisms in their dissolved or nano-forms and the bioavailability depends on various factors including coating materials, aggregation and size of the NPs. Ecotoxicological exposure media influence the stability of NPs and the resulting toxicity (Römer et al., 2011, Hu et al., 2018). Therefore, evaluation of transformation and its effects on the fate and bioavailability to organisms in the environment is highly important (Amde et al., 2017, Angel et al., 2015).

Only a limited number of studies have focused on the effects of NP ageing on aquatic organisms and contradictory results are reported. Manier et al. (2013) observed no difference in toxicity to *Raphidocelis subcapitata* when the alga was exposed to fresh and 30 days aged CeO₂ NPs though the aged NPs had altered and highly agglomerated. Similarly, Cupi et al. (2015) did not observe a significant difference in toxicity between fresh and 48 h aged AgNPs to *Daphnia magna*. However, Phenrat et al. (2009) reported higher neurotoxicity of fresh nano zerovalent ion (nZVI) to mammalian cells in comparison to the aged (< 24 h) particles.
The potential effects of AgNPs were reported for many aquatic organisms including bacteria (Sheng and Liu, 2017), algae (Navarro et al., 2008b, Oukarroum et al., 2012), daphnids (Ribeiro et al., 2017, Conine and Frost, 2017), fish (Ribeiro et al., 2014a, Hoheisel et al., 2012, Bacchetta et al., 2016a), brine shrimps (Arulvasu et al., 2014, Becaro et al., 2015, Maurer-Jones et al., 2013b) and fairy shrimps (Blinova et al., 2013). However, there are no studies on AgNP toxicity to freshwater shrimps.

The aim of the current study was to assess the stability of NPs in media and the effects of ageing of differently coated AgNPs on the freshwater shrimp *Paratya australiensis*. AgNPs coated with Tyrosine, EGCG and Curcumin were used as test materials. Numerous studies have focused on using these ligands to improve the pharmacokinetics and biopharmaceutical value of NPs (Contino et al., 2016, El Khoury et al., 2015, Khaskel et al., 2015, Nune et al., 2009, Duse et al., 2017, Selvakannan et al., 2004, Dubey et al., 2015, Sherin et al., 2017). NPs are known to cause increased ROS formation in exposed cells and organisms in the aquatic environment (Dominguez et al., 2015, Von Moos et al., 2014). Organisms react by activating several antioxidant defence mechanisms such as increasing antioxidant enzyme activities and altering lipid peroxidation which are commonly used as biomarkers to index oxidative stress (Yuan et al., 2018).

Toxicity of TiO₂ NPs to the marine shrimp *Litopenaeus vannamei* was studied by Cordeiro et al. (2016) where an increase in the glutamate-cysteine-ligase (GCL) activity and the total antioxidant capacity was observed upon exposure to NPs. In the current study, the activity of two antioxidant enzymes, catalase (CAT) and glutathione S-transferase (GST) and thiobarbituric acid reactive substances (TBARS), a by-product of lipid peroxidation, were assessed as markers of toxic responses of test organisms. To the best of my knowledge, this is the first study on sub-lethal effects of NPs to *P. australiensis* though it has been used in toxicity studies and as a bioindicator for other contaminants in the Australian environments (Kumar et al., 2010a, Kumar et al., 2010b, Phyu et al., 2005). Further, this is the first study in its kind conducted on the effects of ageing of NPs on an aquatic macroorganism.

### 8.2. Materials and methods

**8.2.1. Test materials**

Please see *Chapter 3. NP Synthesis, characterisation and culturing organisms*, Section 3.1. *Synthesis of AgNPs*. 

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8.2.2. Characterization of AgNPs

Please see Chapter 3. NP Synthesis, characterisation and culturing organisms, Section 3.2. Characterisation of AgNPs.

8.2.3. Test organisms and culture conditions

Please see Chapter 3. NP Synthesis, characterisation and culturing organisms, Section 3.3. Culturing and maintenance of test species for information on culturing *P. australiensis* which was used in this study.

8.2.4. AgNP temporal stability and dissolution in the test medium

The stability of AgNPs in shrimp medium and MilliQ water was investigated for 32 days under the same environmental conditions used for the shrimp acute toxicity test (please see Section 8.2.5.), as previously described in Chapter 5. The toxicity of coated silver nanoparticles to the alga *Raphidocelis subcapitata*, Section 5.2.4. AgNP Temporal Stability and Dissolution in the Test Media. The measurements were taken at the beginning and after 1, 2, 4, 8, 16 and 32 days.

8.2.5. Sub-lethal toxicity

AgNP suspensions of 30 µg L⁻¹ were prepared in the dilution water by using stock solutions and aged for 28 days in beakers which were kept on shakers (100 rpm) (OM6, RATEK, Aus) under similar environmental conditions to that described above. Each test material including the blank control (containing only dilution water) was aged in triplicate. Fresh AgNP suspensions of 30 µg L⁻¹ were prepared just before exposure tests from the same NP stock suspensions in triplicate while fresh dilution water was used as the blank control. The physicochemical characteristics of NP stock suspensions were monitored, and no changes were observed over 28 days. All stock suspensions were sonicated before spiking into the test vessels for effective dispersion of AgNPs in media. After 28 days, shrimp were exposed to both fresh and aged NP suspension for 96 h under the same conditions used in the acute toxicity tests. pH was monitored throughout the testing period. Toxicity tests for aged NPs were performed in the same beakers used for NP ageing to avoid differences in interaction with the glass vessel. After 96 h, shrimps were snap frozen and stored at -80 °C for biochemical tests.
8.2.6. Biochemical tests

Antennae and tail end of the shrimps were removed using fine scissors and only head and abdominal segments of the body were used for biochemical tests. Each individual shrimp was homogenised in phosphate buffer (0.05 M, pH 7.8) in 2 mL lysing tubes (FastPrepTM, MP bio) using a FastPrep-24TM 5G Homogenizer, MP bio and centrifuged (Sigma 3-16KL centrifuge) at 9,000 g for 30 min at 4 °C for GST analysis and 3,000 g for 15 min at 4 °C for CAT and lipid peroxidation (LPO) analysis. The supernatant was collected and stored at -80 °C for further analysis.

CAT was assessed by measuring the decomposition of H₂O₂ as described by Hadwan (2016). When heated in the presence of H₂O₂, dichromate in acetic acid is reduced to chromic acetate. The concentration of chromic acetate measured colourimetrically at 570 nm is directly proportional to the H₂O₂ concentration. H₂O₂ was added to the supernatant and incubated in a water bath at 37 °C for 3 min. Dichromate/acetic acid solution was then added, further incubated for 10 min at 90 °C and centrifuged at 2500 g for 5 min to remove precipitated protein. The resulting supernatant was plated in a 96 well plate and the absorbance was read at 570 nm using a spectrophotometer (POLARstar Omega, BMG Labtech). CAT activity was calculated by modifying the equation presented by Hadwan (2016) and expressed as U mg⁻¹ protein, with one unit of CAT defined as the amount of CAT needed to reduce 1 µM of H₂O₂ per minute. Total protein concentration of samples was measured according to Bradford (1976) using a 96-well microplate (Nunc, Denmark) and absorbance reading at 595 nm in a microplate reader (POLARstar Omega, BMG Labtech). A calibration curve was obtained with bovine serum albumin standard. CAT activity was calculated as follows:

\[
\text{CAT activity (kU min}^{-1} \text{mg protein}^{-1}) = \frac{2.303 \times \log_{10} \left( \frac{St}{T-CT} \right) \times \frac{VT}{VS} \times \frac{1}{c}}{t}
\]

where, \( t \) is the time (min), \( St \) is the absorbance of the standard, \( T \) is the absorbance of the test, \( CT \) is the absorbance of the control test, \( VT \) is the total volume of reagents in the assay tube (mL), \( VS \) is the volume of the supernatant (mL) and \( c \) is the protein concentration (mg mL⁻¹).

GST activity was measured by the method described by Habig et al. (1974) adapted to a microplate by Frasco and Guilhermino (2002). This method measures the change of absorbance due to conjugation of the 1-chloro-2,4-dinitrobenzene (CDNB) with reduced glutathione (GSH). Samples were mixed with the reaction solution comprising CDNB and
GSH and the absorbance was read at one-minute intervals for 6 min. Blanks were run in the absence of sample. The enzymatic activity was expressed as micro moles (µM) of substrate hydrolysed per minute per milligram of protein.

Oxidative damage of lipids can lead to the formation of lipid hydroperoxides which further degrade into several types of aldehydes, including malondialdehyde (MDA). Oxidative damage by ROS was quantified by measuring TBARS which are a proxy for MDA levels, as described by Parrilla-Taylor et al. (2013). Briefly, the supernatant was incubated at 37 °C for 15 min in a shaking water bath and then the reaction was stopped by adding ice cold stopping solution (1.0 M HCl in 12.5% TCA and 1% TBA). The sample was incubated again at 90 °C for 10 min, cooled to room temperature, and centrifuged at 3,000 g for 10 min at 4 °C. The supernatant was collected, and its absorbance was read at 532 nm. The TBARS concentration was calculated using an extinction coefficient of 155 mM$^{-1}$ cm$^{-1}$ and expressed as nano moles (nM) TBARS per milligram of protein.

8.2.7 Data analysis

Data from bioassays were analysed by a two-way ANOVA followed by Tukey’s test for pairwise multiples comparison of means. Shapiro Wilk’s test was performed to check the normal distribution followed by Levene’s test for homogeneity of variance. All tests were performed at the 5% level of significance.

8.3 Results and discussion

8.3.1 Characteristics of AgNPs

Please see Chapter 3. NP Synthesis, characterisation and culturing organisms, Section 3.2. Characterisation of the AgNPs.

8.3.2 AgNP stability and dissolution

Visual observation revealed that the initial yellowish colour of all the AgNP suspensions were stable even after 32 days. The absolute zeta potential values of T-AgNP, E-AgNP and C-AgNPs in the dilution water sharply decreased by 16, 15 and 16 mV respectively within the first 24 h (Fig. 8.1). No significant changes were observed thereafter, while the values remained around -25 mV. Typically, an absolute zeta potential value below 30 mV is considered unstable (Rogers et al., 2010, Daima et al., 2014). However, constant zeta potential
values suggest that the surface corona of all three types of NPs became stable, resulting in stable NPs. Zeta potential values of AgNPs in the MilliQ water did not change significantly during the ageing process except for E-AgNPs, where the value dropped from -40 to -31 mV within the last 16 days (see Chapter 7, Section 7.3.2. AgNP stability and dissolution, Fig. 7.2). The observed decrease in the zeta potential in the shrimp medium compared with the MilliQ water may be due to the higher ionic strength of the medium (Hunter, 1981, Elimelech et al., 1995).

![Fig. 8.1: Change in zeta potential of AgNPs with different coatings in shrimp medium within 32 d. NPs were dispersed in shrimp medium at Ag concentration of 5,000 µg L⁻¹. Error bars indicate the standard deviation from triplicates.](image)

The z-average HDD of T-AgNPs and C-AgNPs did not change with ageing, while a 2-fold increase was observed for E-AgNPs after 16 days, decreasing to 1.5-fold after 32 days (Fig. 8.2). The HDD, shape and zeta potential of pristine AgNPs were not considerably different and therefore, the observed difference in HDD of E-AgNPs was attributed to the different coating material (Behra et al., 2013, Huynh and Chen, 2011). The percentage intensity distribution of NPs revealed that more than 90% of particles remained at the sub-micrometre level over the ageing period. Polydispersity index (pdi) values did not change significantly during the ageing period. An increase in HDD has previously been reported for citrate and PEG coated (Tejamaya et al., 2012b), alkane coated (Ribeiro et al., 2014a) and citrate coated (Sørensen and Baun, 2015) AgNPs. Aggregation of NPs affects the fate and transport of NPs in the environment (Zheng et al., 2015) and the toxicity to aquatic organisms (El Badawy et
al., 2010b). Increased size due to aggregation may favour deposition of particles making them less bioavailable (Quik et al., 2012, Römer et al., 2011). Aggregation may reduce the surface area of the NPs decreasing their chemical reactivity and toxicity (Bystrzejewska-Piotrowska et al., 2009). Also, increased size due to aggregation reduce the potential to internalise in to the cells (Bhuvaneshwari et al., 2015). However, Manier et al. (2013) showed that aggregated NPs could stay as toxic as non-aggregated particles. In contrast to the shrimp medium, none of the AgNPs increased in size in the MilliQ water during the ageing process (see Chapter 7, Section 7.3.2 AgNP stability and dissolution, Fig. 7.4).

![Graph](image)

**Fig. 8.2:** Change in HDD of AgNPs with different coatings in shrimp medium within 32 d. NPs were dispersed in shrimp medium at Ag concentration of 5,000 µg L\(^{-1}\). Error bars indicate the standard deviation from triplicates.

The percentage of ions released from T-AgNPs, E-AgNPs and C-AgNPs in shrimp medium after 32 days were 0.67, 0.22 and 1.54% respectively (Table 8.1). Interestingly, the percentage dissolution in the MilliQ water was slightly higher, with corresponding values of 2.18, 0.37 and 2.27% (see Chapter 7, Section 7.3.2 AgNP stability and dissolution, Table 7.2). As discussed in the previous chapter, this may be due to the precipitation of Ag ions by halides (e.g. Cl\(^{-}\)) and sulphides in the medium (Römer et al., 2011, Ellis et al., 2016a) which is not expected in MilliQ water. Precipitation of Ag\(^{+}\) ions by halides and sulfides may reduce the exposure to free ions released from AgNPs resulting in decreased toxicity to organisms (Garg et al., 2016, Lee et al., 2005). Liu et al. (2018a) observed dissolution rates of BSA and PEG coated AgNPs in the range 0.01 to 0.04 µg L\(^{-1}\) per day in comparison to the 0.11 and 0.47 µg
per day observed in this study. The dissolution experiments were conducted at higher AgNP concentrations in contrast to the exposure concentrations. The dissolution rate is higher at lower concentrations while it is completely inhibited at higher NP concentrations (Liu et al., 2010). Additionally, the glass vials may adsorb ions (Dong et al., 2017, Sekine et al., 2015). Therefore, the shrimp may have been exposed to higher Ag\(^+\) ion concentrations than observed through the dissolution experiments.

Table 8.1: The percentage of soluble Ag released from AgNPs with different coatings in shrimp medium within 32 d. NPs were dispersed in shrimp medium at Ag concentration of 5,000 µg L\(^{-1}\). Standard deviations (± SD) are from triplicates.

<table>
<thead>
<tr>
<th>Substance</th>
<th>1 d</th>
<th>2 d</th>
<th>4 d</th>
<th>8 d</th>
<th>16 d</th>
<th>32 d</th>
</tr>
</thead>
<tbody>
<tr>
<td>T-AgNP</td>
<td>0.06 ± 0.20</td>
<td>0.02 ± 0.09</td>
<td>0.13 ± 0.08</td>
<td>0.31 ± 0.58</td>
<td>0.39 ± 0.34</td>
<td>0.67 ± 0.41</td>
</tr>
<tr>
<td>E-AgNP</td>
<td>0.36 ± 0.15</td>
<td>0.15 ± 0.06</td>
<td>0.12 ± 0.07</td>
<td>0.05 ± 0.19</td>
<td>0.15 ± 0.05</td>
<td>0.22 ± 0.36</td>
</tr>
<tr>
<td>C-AgNP</td>
<td>1.46 ± 0.27</td>
<td>1.66 ± 1.47</td>
<td>1.73 ± 1.34</td>
<td>1.64 ± 0.34</td>
<td>1.24 ± 0.11</td>
<td>1.54 ± 0.66</td>
</tr>
</tbody>
</table>

The absorbance peaks of T-AgNPs and E-AgNPs decreased by 32% and 28% within 24 h respectively while it was only ~6% for C-AgNPs (Fig. 8.3A, 8.3B and 8.3C). The decrease in absorbance has been reported to be due to the aggregation or dissolution of NPs (Jiménez-Lamana and Slaveykova, 2016). The peaks of T-AgNPs and E-AgNPs further decreased, the SPR bands broadened and new absorbance peaks appeared at longer wavelengths which were not observed for C-AgNPs. New absorbance peaks are due to scattered light from large aggregates (Tejamaya et al., 2012b, Stebounova et al., 2011, Jiménez-Lamana and Slaveykova, 2016). The results show that curcumin provides the most protective effect as a coating agent compared with tyrosine and EGCG while SPR curves reveal that all three coated AgNPs showed the presence of AgNPs after 32 days. Interestingly, the SPR bands of AgNPs in shrimp medium over time do not fully agree with the values obtained for HDD and zeta potential. The absorbance peaks of both T-AgNPs and E-AgNPs continuously decreased and the SPR bands broadened though the zeta potential was quite stable after 24 h. No significant changes were observed in HDD of T-AgNPs. This may be due to the lack of sensitivity in techniques used to accurately discriminate the stability between different coated AgNPs. Therefore, further specific work is recommended to fully address the effects of these coatings on the stability of
AgNPs in shrimp media. The SPR bands of AgNPs did not change considerably in MilliQ water (please see Chapter 7, Section 7.3.2 AgNP stability and dissolution, Fig. 7.6).

![SPR curves of (A) T-AgNPs, (B) E-AgNPs, and (C) C-AgNPs in shrimp medium.](image)

Fig. 8.3: SPR curves of (A) T-AgNPs, (B) E-AgNPs, and (C) C-AgNPs in shrimp medium.

Change in NP characteristics such as HDD, zeta potential and dissolution with ageing may influence the bioavailability of NPs and hence, play an important role in determining toxicity (Jiang et al., 2009, Sager et al., 2007, Miao et al., 2010b, Tejamaya et al., 2012b). The current study shows that the above characteristics were dependent on the coating and the medium and therefore, these factors require careful attention in environmental risk assessment (Van Hoecke et al., 2008). Characterising NPs while testing for any adverse effects to organisms remains a challenge (Rasmussen et al., 2018) due to the complexity of the transformation processes and inadequacy of instrumental techniques (Lowry et al., 2012). The presence of natural organic matter (NOM), nutrients and salts in the environment may increase or decrease ion release from NPs (Lee et al., 2012b, Pokhrel et al., 2013, Pokhrel et al., 2014, Liu and Hurt, 2010b). The concentrations used for conducting stability experiments were much higher than the actual environmental concentrations of AgNPs in the natural aquatic environment. The first order rate constant of ion release from AgNPs decreases with increasing
NP concentration (Liu and Hurt, 2010a). Hence, laboratory experiments may not reflect the actual behaviour and impacts of AgNPs in the environment.

8.3.3. Sub-lethal toxicity of aged AgNPs to P. australiensis

LPO levels were indirectly determined by measuring TBARS levels, which are a proxy for MDA, the end product of LPO. Other than for aged C-AgNPs, TBARS levels in shrimps exposed to both fresh and aged AgNPs were not significantly different compared with the control ($p < 0.05$) (Fig. 8.4A). Further, aged C-AgNPs caused a significant increase in TBARS levels compared to its fresh counterpart and the other treatment groups. Increased ROS levels could cause toxicological injuries such as oxidative deterioration of lipids (Bagchi et al., 2001, Facchinetti et al., 1998, Matés and Sánchez-Jiménez, 1999) and therefore, lipid peroxidation (LPO) in cells is used as an indirect measurement to assess ROS levels (Melegari et al., 2013). Several researchers have reported that nanoscale particle properties or dissolved ions from AgNPs can cause ROS generation (von Moos and Slaveykova, 2014, Oukarroum et al., 2012, Li et al., 2013b, Mao et al., 2018). Increased MDA levels as a result of oxidative degradation of lipids cause membrane damage, impaired cellular function and cell lysis (Dauda et al., 2017, Rikans and Hornbrook, 1997). Also, LPO may increase the uptake of NPs into the cells due to membrane deformation (Ozkaleli and Erdem, 2018, Melegari et al., 2013, Pacurarari et al., 2012). Several studies have reported increase LPO in algae (Oukarroum et al., 2012, Melegari et al., 2013, Rogers et al., 2010, Xia et al., 2015, Dauda et al., 2017, Qian et al., 2016), bivalves (McCarthy et al., 2013, Gomes et al., 2014) and fish (Choi et al., 2010, Govindasamy and Rahuman, 2012, Martin et al., 2017) upon exposure to AgNPs. The present study showed that aged AgNPs caused increased LPO levels while the level of increase depended on the type of coating.

Organisms have well developed enzymatic and non-enzymatic defence systems to mediate such oxidative damages (Halliwell and Gutteridge, 2015). Organisms use several mechanisms to cope with ROS formation. For example, increased CAT activity is attributed to an adaptation to self-regulate the stress induced by ROS (Melegari et al., 2013, Qian et al., 2016). Except for aged C-AgNPs, the results showed that the CAT activity levels in shrimps treated with both fresh and aged AgNPs were significantly higher than those in the control group (Fig. 8.4B). However, no significant differences were observed between fresh and aged treatment groups. Upon exposure to AgNPs, increased CAT levels were reported in algae (Qian et al., 2016), daphnids (Ulm et al., 2015b), bivalves (Gomes et al., 2014, Buffet et al., 2013)
and fish (Govindasamy and Rahuman, 2012). Increased CAT levels in this study imply the organism can mediate the toxicity caused by the AgNPs. However, increased lipid peroxidation from aged C-AgNPs correlated with the inactivation of antioxidant system resulting in insufficient production of CAT to negate the effects.

![Fig. 8.4: (A) intracellular TBARS level, (B) CAT activity, and (C) GST activity when Paratya australiensis was exposed to fresh and 28 d aged AgNPs. The error bars indicate the SD ($p < 0.05, n = 3$). The $p$-values for multiple pairwise comparisons were obtained from two-way ANOVA followed by Tukey’s test using Sigmaplot. Letter sign denotes comparison of $p$-values for effects of particles within fresh or aged NP treatment groups and control and effects of each type of fresh and aged NP treated shrimps.](image)

GST can detoxify ROS and repair macromolecules oxidized by ROS and is therefore an indicator of the production of ROS (Angelucci et al., 2005, Dauda et al., 2017). GST is sensitive enough to reveal the stressors in the aquatic environment and studies have shown an induction of GST in organisms upon exposure to NPs (Buffet et al., 2013, Buffet et al., 2011, Dauda et al., 2017, Melegari et al., 2013). However, our results do not show a significant change in GST activity in treatment groups exposed to both fresh and aged AgNPs in comparison with the controls (Fig. 8.4C). GST enzyme production against oxidative stress
caused by the tested concentration may not be significant. However, higher AgNP concentrations may trigger increased production of enzymes to counteract the severe effects of the NPs (Lee et al., 2012a). Federici et al. (2007) observed that elevated TBARS levels were not sufficient to deplete tissue glutathione in the gill, intestine or brain in rainbow trout suggesting other antioxidative defences were available to cope with increased ROS upon exposure to TiO$_2$ NPs. Interestingly, the total glutathione was depleted in the liver with no increase in TBARS levels suggesting the liver used available enzymes to prevent oxidative stress.

8.4. Conclusions

The fate and behaviour of AgNPs in the dilution medium were found to depend on the type of coating and ageing of NPs. Also, the effects of coated AgNPs towards $P. australiensis$ was dependent on the type of coating. The observed high toxicity of AgNPs to $P. australiensis$ indicates that these organisms are vulnerable to AgNP exposure. Further, this study shows that $P. australiensis$ is well-suited for assessing the toxicity of AgNPs in the aquatic environment due to their high sensitivity to AgNPs. In general, increased CAT activity in treatment groups compared with the control showed higher oxidative stress in $P. australiensis$. However, sublethal effects of AgNPs were not significantly affected by type of coating or ageing of NPs other than for C-AgNPs. Better sub-lethal responses may be obtained by using specific target organs upon exposure to AgNPs in contrast to the whole body used in this study. Also, it is recommended that a large number of biomarkers are used, and genomics and transcriptomic studies are carried out to evaluate sublethal responses to NPs in future studies.
Chapter 9. General discussion

The application of nanotechnology to the health, food, home, automotive, electronics and computer industries over the last four decades has resulted in significant societal benefits. As a result, the production of engineered nanomaterials (ENMs) have increased significantly (Sudha et al., 2018). However, concerns are growing regarding the release of an ever-increasing quantity of nanoparticles (NPs) into the environment, largely due to their known and unknown potential in terms of toxicity to organisms. Aquatic environments are particularly at risk due to the substantial amount of NPs that ends up in such environments. Currently, thousands of different NPs are produced and on-going research, and development will discover more in the future (Tan et al., 2018). Although a number of risk assessment studies of NPs have been conducted, there remains many gaps in our understanding and further research is encouraged (Tang et al., 2018). Currently, risk assessment frameworks regarding the environmental health and safety of NPs (Oomen et al., 2018) are still being debated, with scientists, governmental and non-governmental organisations and regulators defending different interests and point of views (Maynard and Aitken, 2016, Monikh et al., 2018). As a consequence no comprehensive and commonly agreed regulatory framework or guidelines are currently available for NPs (Lai et al., 2018).

The purpose of this study was to investigate the potential effects of silver NPs (AgNPs) with different surface coatings (tyrosine, epigallocatechin gallate and curcumin) in the freshwater ecosystem by analyzing their effects on four freshwater organisms (hydra, algae, daphnia and shrimps) belonging to different trophic levels. From the study, it was found that the effects of AgNPs were dependent on several factors; the surrounding medium, ageing and type of coating all influenced the transformation and stability of NPs. Coating and dose-dependent morphological, behavioural, acute and sub-lethal effects on the organisms were observed. Differences in sensitivity and response among different organisms in the end-points tested were observed. Organisms also responded differently when exposed to aged AgNPs; compared with exposure to their pristine forms. The outcomes will enhance knowledge on the effects of NPs in the aquatic environment and contribute to the development of environmental guidelines for the safe use of AgNPs. The ecotoxicological experiments performed with the selected endpoints showed that AgNPs and Ag\(^+\) ions caused substantial deleterious effects on the test organisms. Divergences in sensitivity among different organisms and end-points were observed while coating, dose and time-dependent effects of AgNPs and Ag\(^+\) ions were evident.
The findings from the study are in accordance with the literature confirming that AgNPs cause acute effects (e.g. lethal, sublethal, bioaccumulation etc.) to freshwater organisms (Lekamge et al., 2018a).

9.1. Physicochemical characteristics and stability of NPs

Physicochemical characteristics such as shape, size and surface charge of NPs affect both the mode of action and the toxicokinetics of particles. Moreover, their effects are highly influenced by their interactions with the surrounding environment. The scientific community has reached a consensus on three classes of properties which should be assessed in order to assess the safety of NPs:

- NP characterisation: physicochemical properties such as size, shape and surface characteristics.

- Fate of NPs: biological and environmental fate including their transformation and dissolution in the environment.

- Activity of NPs: biological reactivity, toxico-dynamics, photoreactivity etc (Rasmussen et al., 2018).

Certain characteristics such as size, shape and surface chemistry and the dose are highlighted in the risk assessment of NPs in the scientific literature (Carnovale et al., 2016, Kim et al., 2015, Ivask et al., 2014b, Moon et al., 2018, Albanese et al., 2012, Allen et al., 2010). The experiments in this project were designed and conducted after taking above considerations into account. However, it should be noted that the use of certain attributes and approaches are still being debated. Schmid and Stoeger (2016) point out that the surface area is the most relevant biological dose metric for assessing pulmonary toxicity of biopersistent, spherical NPs. Also, certain limitations exist when performing measurements such as limitations of instruments (Aznar et al., 2017, Bundschuh et al., 2018). For example, instruments do not directly measure the relevant property, but convert the measurement to obtain the property of interest with certain assumptions which may lead to errors (Babick et al., 2016). Also, different preparation and measurement methods may yield different results for the properties of interest. This can be addressed by using a combination of multiple characterisation techniques to provide as many clues as possible on the nature of the NPs (Allen et al., 2010). This approach was taken as far as possible in this study. Further, the fate and
behaviour of NPs in a medium are influenced by the activity of organisms and their reactions. Certain organisms such as algae produce extracellular polymeric substances which play a significant role in mediating the toxicity of NPs (Adeleye and Keller, 2016b, Akhil and S. K, 2016, Miao et al., 2015). Adhesion of NPs to the outer surface of organisms such as crustaceans may change the speciation of silver (Blinova et al., 2013). Also, the behaviour of NPs in the real environment is affected by the presence of many other chemical substances such as dissolved organic matter (DOM). The bioavailability of NPs may increase in the presence of DOM (Akhil and Sudheer Khan, 2017, Amde et al., 2017). Overall, complex chemical and biological processes that constantly affect the fate and behaviour of NPs during the test make the analysis of the contribution of NPs to the net toxicity in a standard toxicity test complicated (Blinova et al., 2013).

Physicochemical characteristics of the individual NPs or their aggregates have the potential to cause toxicity (Adam et al., 2015b). Surface functionality is one of the main important determinants which dictates toxicity (Saei et al., 2017). The effects of coating on the behaviour of NPs and their toxicity have been studied by several researchers (Ellis et al., 2016b, Shoults-Wilson et al., 2011, Silva et al., 2014a, El Badawy et al., 2010b, Dominguez et al., 2015, Bozich et al., 2014, Baumann et al., 2014, Matzke et al., 2014, Zhao and Wang, 2012a, Li et al., 2013b). However, this thesis is the first comparative study which assessed the effects of differently coated AgNPs using tyrosine, curcumin and epigallocatechin gallate as coating agents. Each of these coatings has a different number of phenol structures which may affect their surface properties. In addition, this is the first comparative study where the effects of coated AgNPs were assessed using several organisms and endpoints in a single study.

Chemical (e.g. oxidative dissolution, photoreduction, sulfidation and chlorination) and physical (e.g. homo- and hetero-aggregation) transformation of AgNPs influence their toxicity (Zhang et al., 2018). Particle size, once considered as a highly influential characteristic of toxicity, has been shown to be less important. Rather, the size of aggregates, not particles, seems to be more important since the size of pristine particles rapidly change once released into the environment (Amde et al., 2017). The tested AgNPs aggregated depending on the coating and media. In daphnid medium, T-AgNPs and E-AgNPs increased approximately 10-fold in size after 32 days. Furthermore, T-AgNPs and C-AgNPs aggregated in algae resulting in an approximate 2-fold increase, while a 1.5-fold increase was observed for E-AgNPs. The size of E-AgNPs increased by 2-fold in shrimp medium, while the size of T-AgNPs and C-
AgNPs remained unchanged. None of the AgNPs aggregated in MilliQ water. Overall, the aggregation correlated with the ionic strength of the medium, with most of the aggregation occurring within 4 days after addition.

The surface charge of NPs affects the toxicity of NPs to organisms (Silva et al., 2014a, El Badawy et al., 2010a, Dominguez et al., 2015, Bozich et al., 2014, Baumann et al., 2014). The formation of agglomerates largely depends on the surface charge of NPs (Mikolajczyk et al., 2015). The zeta potential of tested AgNPs changed in all media but not in MilliQ water. Further, the magnitude of change was different in different media suggesting that the physicochemical characteristics of the surrounding media influences the zeta potential. However, the data do not suggest any influence of coating on the zeta potential. For all the NPs in all the media, the change of zeta potential occurred within the first 5 min and generally remained unchanged thereafter.

Changes in UV-Vis absorption spectra is also a measure of NP instability in a medium (Smékalová et al., 2018). In general, absorption peaks of T-AgNPs decreased significantly in all media while E-AgNPs and C-AgNPs showed less reduction in absorption peaks, implying that the former was least stable and the latter most stable. The observed decrease was higher in daphnid and algae media which have higher ionic strengths. The position of the maxima absorption of T-AgNPs and E-AgNPs in daphnid, algae and shrimp media shifted towards longer wavelengths with time indicating an increase in particle diameter from nanoscale to hundreds of nanometres or even millimetre levels which is in agreement with previous studies (Smékalová et al., 2018).

Physicochemical characteristics of the culture media also influence the toxicity of NPs (Aravantinou et al., 2015). Therefore, environmental test conditions such as media composition, pH and ionic strength are also important factors to consider in terms of the risk assessment of NPs (Adam et al., 2015b). As recommended by the organisation for economic co-operation and development (OECD) standard test guidelines, media parameters such as pH was kept within the recommended values and monitored throughout the acute tests. Thus, it was assumed that the influence of the media over time on the AgNPs in this study were minimal. Stability tests revealed that the fate and behaviour of AgNPs were dependent on different media with the stability of AgNPs decreasing with increasing ionic strength.
The dissolution state alters the toxicity of NP’s (Adam et al., 2015b), while the percentage dissolution depends on several factors such as coating, medium and particle size. The dissolution of differently coated AgNPs tested in this study did not differ significantly; the maximum percentage value observed was 1.47% after 32 days. There are various chemical approaches which are used for controlling the dissolution of ions from AgNPs. Surface modification is the most effective approach; these modifications may either inhibit or accelerate ion release (Liu et al., 2010). Since the dissolution of tested AgNPs in this study is comparatively low, it can be concluded that tyrosine, epigallocatechin-gallate and curcumin are capable of forming strong bonds which inhibit dissolution. The dissolution experiments were conducted at higher AgNP concentrations compared to the concentrations used for exposure assessments. The concentration of NPs may influence the dissolution of NPs (Liu et al., 2010) and therefore, the organisms in the exposure assessments might have been exposed to different concentrations of Ag$^+$ ions released from AgNPs than observed from dissolution experiments. The dissolution values observed for all the AgNPs tested in all media were less than the values observed in MilliQ water. This may be due to the precipitation of Ag ions by halides (e.g. Cl$^-$) in the medium (Römer et al., 2011) leaving less detectable ions in the solution. Halides may reduce the exposure of organisms to any free ions released from NPs. Also, the MilliQ water used was slightly acidic (pH: 6.44) compared to media which may have caused higher oxidation of AgNPs releasing more Ag$^+$ ions into the solution (Zhao and Wang, 2012a, Miao et al., 2010b).

There is a major uncertainty as to the state of many NPs following their release into the environment because much of current testing uses pristine NPs and therefore may be unsuitable for risk assessment purposes (Mitrano et al., 2015a). There have been numerous studies investigated the effects of NPs, however few investigated the effects of NP ageing on the subsequent effect on aquatic organisms (Manier et al., 2013, Cupi et al., 2015). In view of this, the effects of ageing of differently coated AgNPs on algae and shrimp were evaluated. This is the first comparative study which evaluated the effects of ageing of differently coated NPs. Previous studies also used growth inhibition (Manier et al., 2013) and immobilization (Cupi et al., 2015) as end points; in this study the sub-lethal effects in algae and shrimps were investigated. Conclusions on the effects of NP ageing in the literature are contradictory since increasing, decreasing and similar toxicity with ageing are reported (Su et al., 2018, Kittler et al., 2010, Pereira et al., 2011, Sørensen and Baun, 2015, Lei et al., 2016a, Manier et al., 2013, Seitz et al., 2015a). We observed similar or increased reactive oxygen species (ROS) and
antioxidative enzyme production from aged AgNPs as with pristine NPs. The effects were significant in algae for all the end points evaluated (ROS, catalase (CAT) and thiobarbituric acid reactive substances (TBARS) levels) while significant changes in shrimps were observed for CAT levels only.

Overall, physicochemical changes were observed for all tested AgNPs in the media depending on the type of coating and the characteristics of the media. Therefore, both the transformation process and the resulting products should be considered when assessing the environmental risk of AgNPs.

9.2. Toxicity of NPs

Freshwater ecosystems are among the most vulnerable of global environments due to multiple stressors present (Ormerod et al., 2010). The release of NPs into freshwater systems started a few decades ago and the quantities released are increasing every year (Lapresta-Fernández et al., 2012). Ag+ ions are known to cause extreme toxicity to aquatic organisms (Tappin et al., 2010); consequently AgNPs are among the most toxic NPs; (Haynes et al., 2017, Adam et al., 2015b, Garner et al., 2015b). The reported EC50 values of AgNPs and Ag+ ions for freshwater algae range from 8.0 to 1,200.0 µg L⁻¹ (Ribeiro et al., 2014a, Li et al., 2015b, Sørensen and Baun, 2015, Ivask et al., 2014a) and 5.0 to 34.0 µg L⁻¹ (Ribeiro et al., 2014a, Li et al., 2015b, Sørensen and Baun, 2015, Ivask et al., 2014a) respectively. The EC50 values of AgNPs for Daphnia spp. falls in the range of 0.26 to 236.3 µg L⁻¹ for AgNPs and 0.16 to 12.9 µg L⁻¹ for Ag+ ions (Zhao and Wang, 2011, Seo et al., 2014, Seitz et al., 2015b, Sakamoto et al., 2015, Becaro et al., 2015, Blinova et al., 2013). The LC50/EC50 values of AgNPs and Ag+ ions obtained for R. subcapitata (155.0 – 243.0 µg L⁻¹) and D. carinata (19.3 – 49.7 µg L⁻¹) in this study are in line with the above reported values. There are no previous studies related to the acute toxicity of AgNPs or Ag+ ions to freshwater shrimp. Usually, the reported acute toxicity values suggest that Daphnia spp. are more sensitive to NPs and metal salts than algal species. This was confirmed in this study (48 h LC50: 19.3 – 49.7 µg L⁻¹). The acute toxicity values for shrimp (48 h LC50: 55.3 – 317.1 µg L⁻¹) and alga (48 h LC50: 155.0 – 243.0 µg L⁻¹) indicated that both species show similar levels of sensitivity to AgNPs while daphnids were more sensitive (19.3 – 49.7 µg L⁻¹). However, the sensitivity of shrimp to Ag+ ions (48 h LC50: 0.1 – 2.2 µg L⁻¹) was similar to that of daphnids (48 h LC50: 0.69 – 1.21 µg L⁻¹). This implies that the modes of action of toxicity of AgNPs and Ag+ ions to the tested organisms vary.
Overall, daphnids appear to be the most sensitive model organisms among those tested. Since the LC$_{50}$ values for *P. australiensis* exposed to Ag$^+$ ions and AgNPs are of the same order of magnitude as those recorded for daphnids and alga, *P. australiensis* may be a suitable organism for use in risk assessment studies involving metallic ions and NPs. The suitability of *P. australiensis* for ecotoxicological studies on pesticides have been previously reported (Kumar et al., 2010a, Kumar et al., 2010b). Generally, there is a general lack of acute and chronic data for Australian native species. *P. australiensis* is a suitable test species to better understand the risks of NPs in the Australian freshwater environment.

In line with the majority of studies, the toxicity of Ag$^+$ ions were found to be higher than that of AgNPs for all individual organism tested in this study. One of the debates in the risk assessment of NPs is whether the effects of NPs are specifically related to NPs or the ions released with time. The majority of studies on AgNPs to date support the hypothesis that the effects are mainly from ions released into the aquatic environment (Shen et al., 2015, Sakamoto et al., 2015, Li et al., 2015b, Adam et al., 2015b) although a significant number of studies claim NP specific effects (Hoecke et al., 2009, Rogers et al., 2010). It is reported that the toxicity of certain NPs such as Zn and ZnO NPs were similar to their relevant bulk and metal salts (Adam et al., 2015b). Dissolution does not explain the toxicity of insoluble NPs such as TiO$_2$ and CeO$_2$ NPs. Angel et al. (2015) exposed *R. subcapitata* to CeO$_2$ NPs and observed that ROS generation was not the main toxic mechanism; in this case dissolution was negligible. Instead, toxicity correlated to the extent of NPs sorbed onto the algal cells. This correlates with the findings of Rogers et al. (2010) who concluded that membrane damage by CeO$_2$ NPs would likely be the cause. Also, the cause of toxicity depends on the mode of exposure to NPs. Nallanthighal et al. (2017), using a mouse model observed increased genotoxicity from AgNPs but not from the dissolved Ag$^+$ ions upon oral ingestion of NPs. The percentage dissolution of tested AgNPs in all the media in this study was less than 1.5%. The corresponding LC$_{50}$ values of Ag$^+$ ions, calculated based on the percentage dissolution of AgNPs were lower than the LC$_{50}$ values of Ag$^+$ ions observed upon exposure to Ag salt. This indicates that the NPs had specific toxic effects on tested organisms in addition to the toxicity caused by released ions.

The precise explanations for the mechanisms of toxicity of AgNPs remain in debate (Durán et al., 2016) while toxicity is partly attributed to particle-related and partly to ion-related mechanism (Zhang et al., 2018). The main proposed mechanisms for AgNP toxicity are oxidative stress, DNA damage, lipid peroxidation, membrane damage and mitochondrial
damage (Ivask et al., 2014a). The production of ROS is commonly believed to be one of the main effects occurring upon exposure to AgNPs (Ale et al., 2018). ROS are necessary for organisms at moderate levels (Du et al., 2018) while excessive production of ROS cause damage to cell functions and development leading to cell death and genotoxic effects (Fu et al., 2014). In response, organisms activate enzymatic defense mechanisms producing antioxidant enzymes such as peroxidases (POD), superoxide dismutase (SOD) and catalase to mitigate the damage by ROS. In this study, a change in concentrations of ROS levels was evaluated in algae and antioxidant enzymes, GST and CAT levels were evaluated in algae and shrimp following exposure to AgNPs and Ag\(^+\) ions. The concentration of ROS increased in algae with time (72 h) with the increase dependent on the coating. Increased GST activity over time was observed in algae for both AgNP and Ag\(^+\) ion exposure while no difference was observed in shrimp. The increase in GST activity correlated with exposure concentration up to 400 µg L\(^{-1}\) in algae while further increase resulted in a decrease in activity with exposure to some coated AgNPs. CAT activity increased significantly in both algae and shrimp compared with GST activity. CAT activity was also dependent on the coating and concentration of NPs.

Lipid peroxidation (LPO) is also commonly studied as a likely effect upon exposure to AgNPs (Ivask et al., 2014a). Lipids make up 30-80\% of biological membranes by mass and thus, peroxidation of lipids cause membrane damage (Harrison and Lunt, 1980). In this study, TBARS concentrations were measured in algae and shrimp as a proxy to MDA levels to assess LPO. A significant increase in TBARS was observed in algae upon exposure to AgNPs while the increase was dependent on the type of coating of the NPs. Only C-AgNPs resulted in an increase in TBARS concentration.

In summary, a significant increase in CAT levels were observed for all AgNPs in both algae and shrimp. Both GST and LPO levels were increased with exposure to AgNPs in algae while only C-AgNPs caused increased levels of LPO in shrimps. Increased CAT activity is recommended as a good biomarker for the exposure of both algae and shrimp to AgNPs while ROS levels, GST activity and LPO levels are recommended only for algae. The actual increase in ROS and LPO levels and GST activity was however dependent on the coating of the NPs and therefore, these biomarkers are good candidates for comparing the effects of differently coated AgNPs.

Reliable ecological risk assessments of NPs require information not only on the toxicity to organisms but also on their bioconcentration, bioaccumulation and biomagnification in the
aquatic food webs (Zhu et al., 2010b). NPs or their aggregates have a different mode of action compared to their metal ions (Adam et al., 2015b). Liberated ions from metallic NPs may affect the algae in three main ways. Firstly, NPs may release ions in the media, which diffuse to the algal surface causing toxicity. Secondly, NPs may attach to the cell surface, diffusing ions and causing toxicity. Thirdly, NPs may enter the cells and then liberate inside the cells, again resulting in toxicity. NPs entering the endoplasmic reticulum, Golgi and the endo-lysosomal system can act as foci for oxidative damage that cannot readily be expelled from the cell (Cid et al., 2015). Acute exposure of algae to AgNPs and Ag⁺ ions and trophic transfer studies from algae to daphnids reported in this thesis, showed that AgNPs and Ag⁺ ions bioaccumulate within the organisms, possibly influencing the effects observed. However, internal accumulation may impact over longer timescales (Federici et al., 2007); these effects cannot be studied in acute tests. Waterborne bioaccumulation was found to be dependent on concentration and coating of NPs. The trophic transfer of NPs has been reported in aquatic food chains (Lee et al., 2015, Bhuvaneshwari et al., 2017, Zhu et al., 2010b). However, this is the first study of its kind which evaluated the feeding behaviour and trophic transfer of differently coated AgNPs. The bioaccumulation profile of Ag in daphnids through trophic transfer did not correlate with the accumulation profiles of Ag in algal cells from differently coated AgNPs. Several factors, such as mode of entry and coating may affect AgNP accumulation profiles at different trophic levels. The mean feeding rates of daphnids over 5 days was highest for the control group followed by C-AgNP, E-AgNP and T-AgNP treated algae. However, a consistent difference was not observed for the daily feeding rate between each treatment group and therefore, the data obtained is not sufficient to prove any significant variations in feeding rates among daphnids based on algae treated with AgNPs with different coatings.

Only a few acute endpoints were assessed in this study. There are several other endpoints which could be evaluated to understand the effects and to assess the risks of NPs and metals. Other antioxidant enzymes such as GSH, GPx, SOD, and glutathione reductase (GR) are demonstrated to show significant responses upon exposure to NPs and metals (Kim et al., 2010, Lu et al., 2016b, Melegari et al., 2013). Further, organisms activate non-enzymatic antioxidant defence mechanisms producing molecules such as ascorbate, glutathione, flavonoids and carotenoids (Liu et al., 2017). Cell viability, granulosity, chlorophyll content, cell morphology, mitochondrial dysfunction, transcriptomics and metabolic profiling (Taylor et al., 2016b, Zhao et al., 2016, Li et al., 2015b) have all been used for assessing effects to algae. Gene expression, morphological changes (eg. body size, body length), swimming
behaviour, intake, assimilation, localisation, protein profiling and mechanical damage (Dominguez et al., 2015, Adam et al., 2015c, Bozich et al., 2014, Simon et al., 2015, Sakamoto et al., 2015, Lovern et al., 2008, Vijayakumar et al., 2016, Sá-Pereira et al., 2018, Tan et al., 2016a) have been studied for daphnids. Only a few selected biomarkers were used in the current study which focussed on comparison of differently coated NPs.

9.3. Test and water quality guidelines

As observed in this study, the fate and behaviour of NPs and their effects on organisms depend on various factors such as coating, media and the ageing process. Therefore, assessment of additional physicochemical properties compared to other conventional hazardous chemicals is required to assess the risks of NPs to protect organisms in the environment. However, current regulatory frameworks are based on certain characteristics of non-nanoscale properties and therefore are not equivalent to the effects of NPs (Rasmussen et al., 2018). Through its working party on manufactured nanomaterials, the OECD conducted a programme to evaluate the need for addressing additional physicochemical properties in OECD test guidelines (TGs) (OECD, 2015, OECD, 2016). They concluded that, in most instances, existing methods and approaches could be used to address the specific challenges posed by nanomaterials. However, adaptions in sample preparation and dosimetry may be necessary in certain situations. In response, the establishment of appropriate TGs has gone through several development stages as an ongoing commitment (OECD, 2018, Monikh et al., 2018) which is commendable.

As per the EU-Directive 93/67/EEC, contaminants are classified according to the lowest median LC/EC₅₀ value of the three key environmental organisms: algae, crustaceans and fish (CEC, 1996). Contaminants with the lowest median value below 1 mg L⁻¹ are classified as “very toxic” to aquatic organisms; between 1–10 mg L⁻¹ as “toxic” to aquatic organisms and between 10–100 mg L⁻¹ as “harmful” to aquatic organisms. From the current study, the values are available for algae and daphnids only and accordingly, the coated AgNPs tested in this study and Ag⁺ ions can be classified as very toxic. Currently no comprehensive framework for the regulation of NPs is available (Lai et al., 2018). Nanomaterials represent an emerging water quality contaminant issue which requires investigation in terms of the Australian Guidelines for Water Recycling (AGWR, 2018). None of the regulatory agencies in Australia provide any guidelines on NPs to protect freshwater bodies as of 2018. The National Industrial Chemical Notification and Assessment Scheme (NICNAS) reported no industrial uses of nanosilver in Australia based on a survey conducted in 2006 to 2008. They concluded that it was not possible
to assess the human health implications of NP in industrial applications. However, they reported that certain household appliances contain a nanosilver coating, but they did consider them as this was outside the regulatory remit of NICNAS. Further, the NICNAS reports that there were no nanoparticle-specific regulatory requirements in place for silver as of 2018; and they concluded that regulatory requirements applying to regular/bulk silver should also apply to nanosilver (NICNAS, 2018). Table 9.1 shows the guideline values for Ag in the Australian and New Zealand freshwater environments.

As reported by many researchers and the results of the current study, the risk of AgNPs to aquatic organisms do not exceed the risks of Ag\(^+\) ions. Therefore, the scientific community is reaching consensus that the guidelines and regulations for the counterpart ionic form of the relevant NPs are sufficient to address the issues with NPs (Ribeiro et al., 2014a). However, this needs to be treated cautiously since the unique behaviour of NPs in the environment and their effects on organisms compared to traditional contaminants has been clearly demonstrated. Also, the available information regarding NP concentrations in the natural environment is very limited; while there is little doubt that the NP concentrations in the environment will increases as a result of their many promising applications. Further, the risks of NPs are still not well understood. Therefore, the regulatory bodies and the public need to use a precautionary approach in addressing the issues related to NPs.

Table 9.1: Default guideline values (DGV) for silver for the Australian and New Zealand fresh waters. Source: Australian and New Zealand Guidelines for Fresh and Marine Water Quality (ANZECC, 2018)

<table>
<thead>
<tr>
<th>Level of species protection (%)</th>
<th>(\mu g\ L^{-1})</th>
<th>Specific comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>99</td>
<td>0.02</td>
<td>-</td>
</tr>
<tr>
<td>95</td>
<td>0.05</td>
<td>-</td>
</tr>
<tr>
<td>90</td>
<td>0.1</td>
<td>-</td>
</tr>
<tr>
<td>80</td>
<td>0.2</td>
<td>DGV may not protect key test species from chronic toxicity (this refers to experimental chronic values or geometric mean for species)</td>
</tr>
</tbody>
</table>
9.4. Conclusions and future directions

The purpose of this study was to investigate the potential effects of differently coated AgNPs in the freshwater ecosystem by analyzing their effects on organisms belonging to different trophic levels. From the results it can be concluded that the toxicity of NPs is coating dependent. However, there were divergences in sensitivity among different organisms and in the end-points tested. Coating- and dose-dependent morphological, behavioural, acute and sub-lethal effects on the organisms were observed. Despite the relatively high concentrations tested in the current study, the findings can contribute to better understand the potential risks of differently coated AgNPs to organisms in the freshwater environment.

Nanotechnology is a booming industry and more applications are continuously being developed. Therefore, release of NPs to the aquatic environment during their manufacture or use is unavoidable. There has been increasing interest in research on the fate and effects of NPs in the environment, but the scientific community has not been able to come to a general consensus on environmental risk assessment of NPs and to accurately design regulatory requirements or guidelines for NPs.

The effect of the particle properties of NPs on toxic responses has been heavily studied in the last decade, and it has been found that certain physicochemical properties such as size, shape and surface functionality of NPs influence toxicity. However, conflicting and inconsistent results demand further research in order to make sound conclusions and protect organisms from the adverse effects of NPs.

The fate and behaviour of AgNPs were dependent on the media while the stability decreased with increasing ionic strength of the media. Also, the available ion concentration released from AgNPs showed media dependency. This indicates that the surrounding medium largely influences the transformation of NPs once they are released into the aquatic environment. Therefore, the media should be considered as an important factor in designing test guidelines for NP risk assessment. Further, site-specific studies are recommended since mechanisms of transformation depend on the characteristics of any particular environment.

The stability tests in the present study revealed that the coating on the NPs significantly influenced the behaviour of AgNPs while NPs coated with curcumin are highly stable and tyrosine coated AgNPs were the least stable. This shows that the NPs change and evolve depending on the coating material. Additionally, AgNPs exerted different levels of toxicity on
each tested organism depending on the coating. However, a consistent trend was not observed between organisms which may be due to various reasons such as different modes of toxicity and the media constituents. Therefore, it is necessary to develop robust procedures to generate toxicity data with a high degree of credibility. e.g. standardisation of NP toxicity tests. Also, it is recommended that further research be conducted using computer models such as quantitative structure activity/toxicity relationship (QSAR/QSTR) models which correlate diverse properties of NPs and experimental toxicity values; to predict consistent and accurate toxicity values. Also, it is suggested to study representative groups of coatings with different degrees of lipophilicity and log K_{ow} that will help make generalisation for enhancing the predictive power to forecast the toxicity of differently coated AgNPs.

In general, the toxicity of AgNPs and Ag^{+} ions to the four species tested in this study followed the order: daphnids > shrimps > algae > hydra; and shrimps > daphnids > algae > hydra, respectively. This implies that in order to protect aquatic organisms from AgNP exposures, it is necessary to conduct risk assessments based on data gathered from toxicity studies relevant to species in such environments, and from different trophic levels; while the toxicity of AgNPs does not necessarily reflect the toxicity of Ag^{+} ions. Furthermore, it is recommended that toxicity tests be conducted with a broad range of taxa for regulatory purposes and for setting water quality guidelines.

The majority of research on NPs have been done on standard test species. However, due to their unique properties, the effects of NPs on standard test species cannot be generalized to the wider environment. For example, the sensitivity of shrimps and daphnia to Ag^{+} ions is quite similar, but their sensitivities to AgNPs are significantly different. Therefore, toxicity data on local native species are required and will be highly beneficial in developing site-specific risk assessments and water quality guidelines.

As demonstrated in this study, AgNPs caused higher oxidative stress in both *R. subcapitata* and *P. australiensis*. Compared with their pristine forms, aged AgNPs induced excessive ROS generation and caused higher lipid peroxidation in algae. CAT levels were higher in both algae and shrimps upon exposure to aged AgNPs. The biological effects of aged NPs compared with their pristine forms are still debated since increased, decreased or similar toxicity levels have been reported by various researchers. Therefore, further studies are recommended regarding the toxic effects of NPs after different ageing regimes.
The significantly lower LC$_{50}$/EC$_{50}$ value for Ag$^+$ ions in comparison to that for the AgNPs tested shows that ionic silver is much more toxic than AgNPs. However, as per the percentage dissolution of AgNPs, the computed concentrations of dissolved fractions of Ag$^+$ ions from AgNPs were below the toxicity values of Ag$^+$ ions. The toxicity may therefore not exclusively come from the Ag$^+$ ions released from AgNPs; and that NPs may have different toxicity. Whether NPs, ions released from NPs, or a combination of both causes toxicity is still an issue in understanding the nature of the toxicity of metallic NPs as well as their toxicity mechanisms. Therefore, it is necessary that more comparative research be conducted by exposing organisms to both nanoparticles and to the ions released from those NPs as separate experiments. This will help to better understand the toxic potential of metallic NPs for accurate risk assessments.

ROS-mediated oxidative damage to organisms by AgNPs was evident in this study in line with several previous studies. In general, increased CAT and GST activity in treatment groups compared with the control in this study indicated higher oxidative stress caused by AgNPs. The underlying mechanisms for the toxic interactions of AgNPs are complex and other toxicity mechanisms include membrane damage, protein denaturation, DNA damage, behavioural effects, physical damage, etc. which were not investigated in the current study due to limitations of time. Improved understanding on the mechanisms of NP toxicity is crucial in risk assessment of NPs since conventional toxicity tests may not reflect the risks associated with NPs. Better sub-lethal responses may be obtained by using specific target organs upon exposure to AgNPs in contrast to the whole body used in this study. Also, measuring a large number of end points are recommended for future research.

Coating-dependent Ag accumulation profiles were observed in algae and daphnids. However, the accumulation profile in algae does not mirror that of daphnids. NPs can be ingested and accumulated inside the organism or adsorbed onto the surface which may lead to trophic transfer of NPs through the food web. The transfer of AgNPs along the aquatic food chain could have adverse implications and therefore there is a need to take this into consideration in protecting aquatic organisms. In doing so, great caution must be taken when assessing the risk of differently coated NPs.

Daphnid feeding rate decreased when they were fed with AgNP contaminated algae. This indicates that the AgNPs pose indirect negative effects other than direct toxic effects. Attention to such tests is still lacking, and further research is recommended. Furthermore, due
to the low NP concentrations in field conditions, the toxicity or any other physiological effects in organisms are unlikely to be prominent enough for detection. Behavioural effects may be more sensitive and would be efficient in certain situations to evaluate toxic effects. Also, behavioural toxicity tests are fast and cheaper which could be helpful in assessing the toxicity of ever-increasing varieties of NPs. Additionally, behavioural tests may be more relevant in addressing challenges posed by NPs such as transformation and aggregation.

Due to the complexity of risk assessment of NPs, a multi-disciplinary approach is recommended as the way forward to effectively understand the effects of NPs. In the current study, knowledge generated by using various tools from different disciplines such as toxicology, ecology, physiology, chemistry and physical science were integrated to obtain desired outcomes. Transferability and analysis of data from many fields are challenging and it is necessary to explore the possibilities of using new approaches and promote the use of advanced tools such as artificial intelligence, data mining and big data analysis.

The exact concentrations of NPs in water bodies are yet to be assessed and only limited predicted data are available with various assumptions since there is also a lack of published data on NP-containing products. Efforts have been taken to assess the flow of NPs into the environment and the exposure levels. Recent developments in material flow modelling are noteworthy. Also, recent efforts to accurately measure the environmental concentrations of NPs by analytical methods are a positive sign since they can offer support in verifying the values predicted by models. However, factors such as the complexity of real sample matrices, transformation and aggregation of NPs once released into the environment and limitations in the analytical methods present a huge challenge in accurately measuring the environmental concentrations, while there is considerable uncertainty in models resulting in a lack of reliable data. Therefore, estimates of more refined environmental levels of NPs are needed, and further research is needed for determination of actual environmental concentrations of NPs for reliable risk assessment and for regulating the NP industry.

Finally, continued efforts to understand the behaviour and effects of NPs in the environment are recommended.
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