# Silver nanoparticles in the environment: sources, detection and ecotoxicology

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**Table of Contents**

**Abstract**

The environmental impact of silver nanoparticles (AgNP) has become a topic of interest recently, this is due to the fact that AgNPs have been included in numerous consumer products including textiles, medical products, domestic appliances, food containers, cosmetics, paints and nano-functionalised plastics. The production, use and disposal of these AgNP containing products are potential routes for environmental exposure. These concerns have led to a number of studies investigating the release of particles from nano-functionalised products, the detection of the particles in the aquatic environment and the potential environmental toxicology of these AgNPs to aquatic organisms. This review examines numerous studies including; AgNP synthesis methods, the application of AgNPs, the AgNP release from nano-functionalised products, the fate of the AgNPs in the aquatic environment, techniques used to detect AgNPs in aquatic environments including methods for the capture of the particles, techniques for the characterisation and chemical quantification along with studies investigating the environmental toxicology of AgNPs to aquatic organisms.

**1.0 Nanoparticles**

Nanoparticles (NP) are defined as natural, incidental or manufactured material containing particles, in an unbound state or as an aggregate or as an agglomerate and where, for 50% or more of the particles in the number size distribution, one or more external dimensions is in the size range 1nm – 100nm (Hood, 2004; The European Commission, 2011). Silver nanoparticles (AgNP) are among the engineered nanomaterials most often incorporated in nano-functionalised consumer products (Zhang et al., 2016). A primary function of AgNP is as a biocide, attributed to the well-documented antimicrobial properties of silver (Bone et al., 2012; Cleveland et al., 2012; Maillard and Hartemann, 2012). Additionally the unique physicochemical properties of AgNPs, including a high electrical and thermal conductivity, are increasingly being applied in the areas of microelectronics and medical imaging (Fabrega et al., 2011; Zhang et al., 2016). The ever increasing range of applications of AgNPs,

including medicine, consumer products, electronics and in the food industry, make it imperative that their environmental impact is studied.

This review follows the full life cycle of AgNPs from synthesis to applications to end of life discharge, assessing existing knowledge and identifying knowledge gaps where gaps exist

There has been an explosion of scientific interest in AgNP as evidenced by the exponential growth in peer review journal publications as shown in Figure 1. This figure depicts the number of peer reviewed journal articles published each year found using the keyword "silver nanoparticles" as listed on Scopus over the last 25 years.
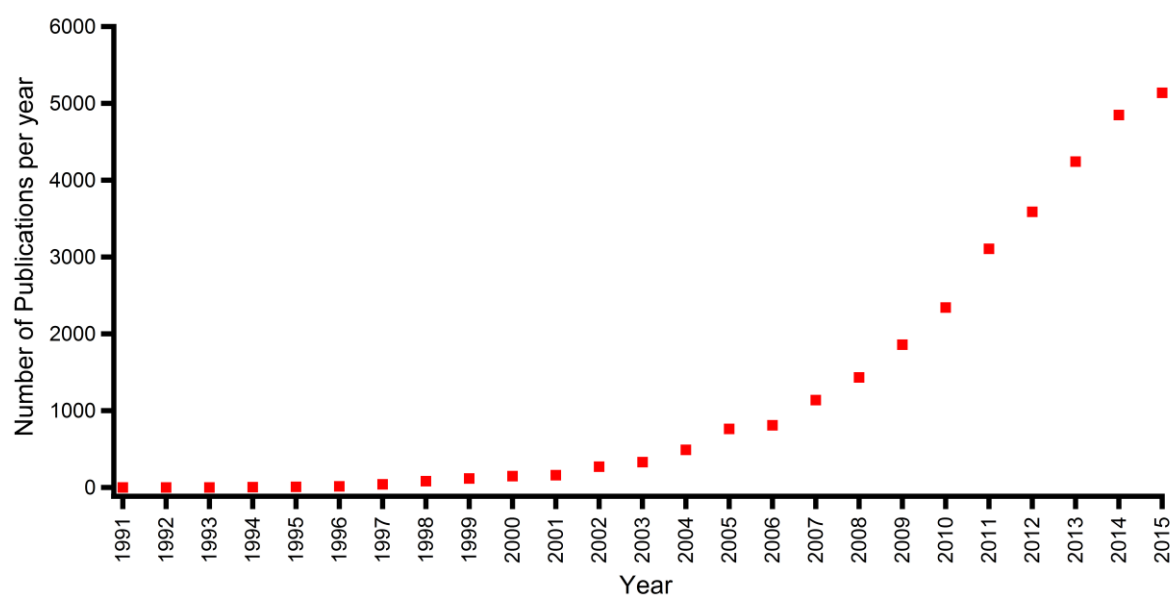


**Figure 1**: Number of publications per year on Scopus.com for the keywords "silver nanoparticles".

## 2.0 History of silver applications

The use of silver for its antimicrobial properties, in bulk and colloidal forms, is not a recent development (Nowack et al., 2011). The antibacterial properties of silver have been used for centuries to purify potable water by storage in silver containers (Amato et al., 2011). Herodotus wrote in 425 B.C., that Persian kings such as Cyrus the Great carried water supplies in silver vessels to prevent it from becoming contaminated when going to war. There is anecdotal evidence for the use of nanosilver in ancient Egypt and Rome (Reidy et al., 2013). The Macedonians used silver plates to improve wound healing and Hippocrates used

silver in the treatment of ulcers (Alexander, 2009). C.S.F. Crédé is credited with the first scientific publication to describe the medical use of silver in the late nineteenth century (Alexander, 2009; Maillard and Hartemann, 2012; Schaller and Klauss, 2001). Credé used eye drops containing one-percent silver nitrate solution to treat eye infections in new-borns (Maillard and Hartemann, 2012; Schaller and Klauss, 2001). It was noted historically that the purity of liquids such as; water, wine, vinegar and milk was increased if they were stored in silver vessels (Alexander, 2009). Later silver dollars were placed into buckets of milk by American pioneers to prevent milk from spoiling (Alexander, 2009). In the United States colloidal nanosilver, suspensions of silver particles in liquid, which was registered in 1954 as a biocidal material, (Nowack et al., 2011; Reidy et al., 2013), has been used in medications for nearly one hundred years (Flory, 2012). This long history and varied applications for nanosilver has led to a significant amount of scientific research on its chemical and environmental properties (Nowack et al., 2011).

## 3.0 Silver nanoparticles: current applications

The current worldwide consumption of AgNPs has been estimated in a number of studies, Piccinno et al., (2012) calculated that the median global consumption of AgNPs was 55 ton/year with other estimates from Future Markets estimating AgNP consumption to be as high as 360 tons to 450 ton/year (Lazareva and Keller, 2014; Zhang et al., 2016). Actual figures for the global consumption of AgNPs are to the knowledge of the authors unavailable. Currently, examples of where AgNPs are used include water filters, paints, cosmetics, deodorants, clothing, textiles, food packaging, functionalised plastics, medical devices, wound dressings, electrical appliances such as washing machines and refrigerators, detergents, biosensors and biomedical products (Abou El-Nour et al., 2010; Benn and Westerhoff, 2008; Blaser et al., 2008; Cunningham and Joshi, 2015; Cushen et al., 2012; Dubas et al., 2006; Etheridge et al., 2013; Farkas et al., 2011b; Goswami et al., 2014; Hadioui et al., 2013; Jain and Pradeep, 2005; Kaegi et al., 2010; Kumar et al., 2008; Jingyu Y Liu and Hurt, 2010; Lodeiro et al., 2016; Maneerung et al., 2008; Reidy et al., 2013; SCENIHR, 2014; Silver et al., 2006; Wasmuth et al., 2016; Yin et al., 2015; Zhang et al., 2016) Silver can be utilised in medical devices, it can be used at lower concentrations than organic compounds, the silver fraction can also be incorporated into plastics whereas the organics may not survive plastic incorporation due to temperatures used in the manufacturing process. (Nowack et al., 2011; Reidy et al., 2013). The ever increasing range of applications for

AgNPs is likely to result in an increase in synthesis of NPs and engineered nanomaterials (ENMs). Production of ENMs has been highlighted as a potential source of human and environmental exposure to NPs (Cunningham and Joshi, 2015).

## 3.1 Inventories of AgNP containing products

Globally a number of databases have been created cataloguing products containing ENMs. Vance et al. (2015) compiled a list of these in a review of consumer product inventories, while recently Foss Hansen et al., (2016) examined the availability of such information in Europe. Both of these studies are the result of two different projects developing databases of nano products. The Vance et al. (2015) review was a result of the Project on Emerging Nanotechnologies which also created the Nanotechnology Consumer Products Inventory (Vance et al., 2015). Currently this inventory lists 442 products as containing silver out of a total 1827 listed products ("Project on Emerging Nanotechnologies Consumer Products Inventory," 2013). That study also found silver to be the element most often combined with other nanomaterials in consumer products, with a total of 35 combined products (Vance et al., 2015). The work of Foss Hansen et al., (2016) stemmed from the formation in 2012 of "The Nanodatabase" in Denmark, which is an inventory of commercially available products that claim to contain ENMs and are available in the European consumer market (http://nanodb.dk/). This inventory is updated daily, (Foss Hansen et al., 2016), and currently lists 2329 products of which 340 are registered as silver containing products ("The Nanodatabase," 2016).

Further to the consumer products inventories listed above the two reviews, (Foss Hansen et al., 2016; Vance et al., 2015), listed several other databases that have been created to collect information on nano-functionalised products that are available. Two European consumer organizations, BEUC and ANEC: compiled an inventory of AgNP containing consumer products (ANEC/BEUC, 2013). The most recent inventory available on the website http://www.beuc.eu/safety/nanotechnology is from February 2013 and it lists 141 products containing AgNPs including; appliances, automotive products, clothes & textiles, cosmetics and personal care products, cross cutting , food & drink, home & garden and products for children (ANEC/BEUC, 2013).

Foss Hansen et al., (2016) listed another inventory of consumer products containing ENMs currently available in Germany. The BUND database (Friends of the Earth Germany) is

currently searchable, and is available online, in German, at the website: http://www.bund.net/themen_und_projekte/nanotechnologie/nanoproduktdatenbank/

The creation and maintenance of up to date inventories of products containing nanoscale components is difficult. The Scientific Committee on Emerging and Newly Identified Health Risks (SCENIHR) EU report 2014 on the safety of AgNPs acknowledged that tracking of products containing AgNPs is difficult. This is because the products are marketed under numerous brand names and current labelling regulations, with the exceptions of cosmetic products (Regulation (EC) No 1223/2009), the regulation on the provision of food information to consumers; (Regulation (EU) No. 1169/2011) and a regulation on plastic materials and articles intended to come into contact with food (Regulation (EU) No. 10/2011), do not require nanoparticles to be listed as a component (SCENIHR, 2014). Interestingly several studies found that some fabrics did not contain detectable levels of Ag despite claims from manufacturers of nano-functionalisation (Benn and Westerhoff, 2008; Kulthong et al., 2010; Lorenz et al., 2012). Therefore, relying on the claims made by manufacturers may not be an accurate method in determining whether a product is nano-functionalised.

The review by Vance et al. (2015) also refers to separate inventories including: a German database of nanotechnology products Nano Products and Technologies, which listed 586 products in 2014 however this inventory is no longer accessible (Vance et al., 2015). Japan has also compiled a database of ENP containing products. The Japanese National Institute of Advanced Industrial Science and Technology (http://www.aist-riss.jp/) compiled a list of consumer products that claimed to incorporate nanotechnology in 2007 listing 541 product lines and 1241 products (Vance et al., 2015).

In terms of governmental response to the development of ENM containing products in France, companies producing, distributing and importing substances at nanoscale are required to report them to ANSES (The French agency for food safety, the environment and labour) (Ministry of Ecology, Sustainable Development, 2012). Belgium, Italy and Denmark have also initiated similar inventories of products while Germany, the United Kingdom and Sweden are proposing introducing some form of regulation (Anses, 2014; Foss Hansen et al., 2016; KEMI, 2015).

## 4.0 Synthesis of nanoparticles

Several physical and chemical methods have been described for the manufacture of NPs (Fabrega et al., 2011). Synthesis of AgNPs can be divided into top-down and bottom-up methods (Tolaymat et al., 2010). Top down techniques involve mechanically reducing bulk silver by techniques such as lithography or laser ablation. These methods tends to generate powders, however this approach provides less control over important physical parameters, such as the size and shape of the particles (Fabrega et al., 2011). The bottom-up methods involve the dissolution of silver salts, such as silver nitrate into a solvent, followed by the addition of a reducing agent (e.g., $NaBH_4$), possibly supplemented by a stabilizing agent, like citrate, to prevent NP agglomeration (Reidy et al., 2013). The choice of stabilizing agent has a profound effect on the surface changing behaviour, particle size and the aggregation potential of AgNPs (El Badawy et al., 2010). It is suggested that in environments such as landfills, wastewater, soils, surface, and groundwater uncoated and electrostatically stabilized AgNPs will likely settle out of suspension due to particle aggregation. The solvents and reducing agents used in the synthesis of AgNPs also affect the physical characteristics of the AgNPs generated (Tolaymat et al., 2010). AgNPs can also be generated through electrochemical reduction, irradiation or biological reduction (Zhang et al., 2016). Biological reduction or green synthesis methods, using microorganisms and plant extracts, are gaining increased interest as they are potentially less costly and less toxic than physical and chemical methods (Prasad, 2014; Vijayan et al., 2016). Production of AgNPs have been observed in a variety of photosynthetic plants (Prasad, 2014) and in fungi such as *Phoma glomerata* and *Fusarium* species (Fabrega et al., 2011) and by a variety of microorganisms such as Proteobacteria, cyanobacteria and microalgae (Kumar and Poornachandra, 2015; Patel et al., 2015).

Seaweed has also been used in the green synthesis of AgNPs (Vijayan et al., 2016). Extracts from the brown and green seaweeds, *Sargassum plagiophyllum, Ulva reticulata* and *Enteromorpha compressa*, have been used in the green synthesis of AgNPs (Dhanalakshmi et al., 2012) AgNPs with an average diameter of 20 nm were produced when a solution containing extracts from the dried seaweed was mixed with a solution of $AgNO_3$ and kept in a water bath at 60 ºC in the dark (Dhanalakshmi et al., 2012). Extracts from wet and dry seaweed, *Codium capitatum* have been used in the synthesis of AgNPs (Kannan et al., 2013). The seaweed extracts were mixed with $AgNO_3$ solution at room temperature in the dark. The AgNPs produced had an average diameter of 30 nm (3 to 44 nm) (Kannan et al., 2013).

*Sargassum wightii* seaweed extract has been used in the synthesis of AgNPs by mixing the extract with AgNO$_3$ solution at 28ºC for 24 hr, the AgNPs resulting were in the range 5–22 nm and were found to inhibit bacterial growth in human pathogens with the strongest effect noted for *S. aureus* (Shanmugam et al., 2014). *Gracilaria corticata* extract produced AgNPs by the reduction of AgNO$_3$ after heating to 60 ºC for 20 minutes, TEM analysis found the AgNPs to be in the range 18 to 46 nm and were found to have antifungal activity inhibiting both *Candida albicans* and *Candida glabrata* (Kumar et al., 2013). Extracts from the red seaweed *Gracilaria birdiae* were used in the production of AgNPs the diameter of the AgNPs ranged from 20 nm to 95 nm with varying extract concentration and pH. The AgNPs were found to have antibacterial activity particularly against the Gram negative *E. coli* (de Aragão et al., 2016). AgNPs were synthesised by adding a solution of AgNO$_3$ to a suspension containing ground *Kappaphycus alvarezii,* the mixture was then exposed to high intensity irradiation, the AgNPs produced had varying average diameters 16 nm to 12 nm with varying periods of irradiation from 120 to 720 min (Faried et al., 2016)

AgNPs are manufactured to a wide variety of sizes and forms including spheres, rods, cubes, wires and triangles (Reidy et al., 2013; Zhang et al., 2016). AgNPs with different shapes have increasing applications in different areas such as photonics and medicine (Khodashenas and Ghorbani, 2015). The addition of different coatings or capping agents to particles (e.g. citrate, polymers, peptides and sugars)  helps to control the shape of the particles leading to the functionalising of their surface while helping to reduce particle growth (Cunningham and Joshi, 2015; Fabrega et al., 2011; Reidy et al., 2013). Capping agents include polymers and surfactants used in the synthesis of AgNPs, NP aggregation is prevented by the electrostatic repulsion, steric repulsion or both caused by the addition of the capping agents to the NP surface (El Badawy et al., 2010). The most commonly used capping agents for AgNPs include citrate, sodium borohydride (NaBH$_4$), and polyvinylpyrrolidone (PVP) (El Badawy et al., 2010). Positively and negatively charged AgNPs are generated via the use of different reducing agents in synthesis (e.g citrate results in negative particles while branched polyethyleneimine (BPEI) produce positively charged particles). The different particle charge can result in different behaviour (Tan et al., 2007; Tolaymat et al., 2010).

**5.0 Silver nanoparticles in the environment**

Historically the photographic industry contributed significantly to large emission loads of silver into the environment (Fabrega et al., 2011; Purcell and Peters, 1998) but the advent of digital photography resulted in a rapid decrease in percentage of usage for this application (Cunningham and Joshi, 2015). Natural leaching from bedrock and mining activities also contributed to silver contamination in surface waters, however a low level of silver mining occurs in Europe with 1580 Mg(Ag) mined/year (Lanzano et al., 2006) with a reliance more on silver imports and the recycling of scrap in production and fabrication. In natural and contaminated waters the measured concentrations of silver are in the ng/L range (Purcell and Peters, 1998).

The global use of AgNPs containing products suggests that individuals and the environment may be exposed to AgNPs (Bone et al., 2012; Cunningham and Joshi, 2015; Ebeling et al., 2013; Farkas et al., 2011b; Reidy et al., 2013; SCENIHR, 2014). This exposure may occur over several phases of the lifespan of the AgNPs, from synthesis and manufacturing, distribution, end-product use and end-of-life disposal of everyday consumer products which are nano-functionalised including; textiles and fabrics, food-contact materials such as containers, kitchen appliances, cosmetics, deodorants, water filters, toys and alternative health supplements (Blaser et al., 2008; Cunningham and Joshi, 2015; Hong et al., 2014; Mueller and Nowack, 2008). AgNPs may be released into the environment via wastewater streams and effluent. It is predicted that in wastewater treatment plants (WWTPs) AgNPs are retained in sewage sludge (Blaser et al., 2008; Doolette et al., 2015; Kaegi et al., 2013). Due to regulations prohibiting the dumping of sewage sludge at sea in both Europe (EU Directive 91/27/EEC; EEC, 1991) and in the USA (Ocean Dumping Ban Act of 1998), sewage sludge generated in WWPTs is often used as a fertiliser for agricultural soils, which with surface runoff can then transfer, if present, AgNPs from the sludge to the aquatic system, WWTP sludge is also sent to landfill which, by leaching, can result in AgNPs entering the aquatic system (Blaser et al., 2008). In the EU, where the use of sewage sludge in agriculture is governed by the sewage sludge directive (86/278/EEC), it has been reported that in 2013 18% of the sewage sludge produced was used as fertiliser (Eurostat, 2016). End-of-life disposal can also result in AgNPs being emitted into the environment by leaching into the groundwater or subsoil from landfill (Blaser et al., 2008). According to the SCENIHR report 2014, in the environment AgNPs could deliver silver to organisms in soil, water and

sediment. Over an extended period of time these AgNPs may act as sources of ionic silver. (SCENIHR, 2014).

Klaine et al. (2008) in a critical review of nanomaterials in the environment posed a series of questions to be addressed to determine the environmental fate of ENMs in order to inform regulations to adequately protect the environment. These factors include possible effects on the NPs size and structure, how the environmental physicochemical paramaters will affect them and are they likely to be modified by existing biota (Klaine et al., 2008)

## 5.1 Predicted environmental concentration (PEC) of AgNPs

Due to their low expected concentration when compared with total silver, and dearth of available capture methods there has been little to no analytical studies carried out to quantify the specific contribution of AgNPs to the presence of silver in the environment. (Bone et al., 2012; Cunningham and Joshi, 2015; Fabrega et al., 2011; Gottschalk et al., 2009). The concentrations of total silver in different environmental compartments have been estimated to be in the range of ng/L for water and mg/kg for soil and sediments (Blaser et al., 2008). Based on modelling Gottschalk et al. (2009), reported the expected concentrations of AgNPs in U.S. surface waters are between 0.09 and 0.43 ng/L and levels between 0.59 and 2.16 ng/L in European surface waters, while the predicted total Ag concentrations in the river Rhine, were modelled to be from 40 to 320 ng/L. (Blaser et al., 2008; Gottschalk et al., 2009; Nowack et al., 2011). Due to the uncertainty surrounding the concentration of AgNPs in the environment and the lack of understanding of their environmental impact, the increasing use of AgNPs in many different applications has raised concern over their likely release into aquatic ecosystems (Bone et al., 2012; Klaine et al., 2008). It is not possible to assume that AgNPs have little or no environmental impact as there is uncertainty surrounding the level at which AgNPs are present in the environment (Tiede et al. 2009 Cunningham and Joshi, 2015).

## 6.0 Nanoparticle release from consumer products

Exposure to AgNPs and ENMs in general may occur during several phases of a consumer products life-cycle; during the initial synthesis, at production and use of a consumer product, release by industrial emissions, product degradation and disposal of consumer products. (Cunningham and Joshi, 2015). A number of studies have examined the speciation and release of $Ag^+$ from Ag-functionalized commercial products (Lombi et al., 2014). The release of silver from functionalized textiles and fabrics (Benn and Westerhoff, 2008; Geranio et al.,

2009; Kulthong et al., 2010; Lombi et al., 2014), medical devices (Sussman et al., 2015), paint (Kaegi et al., 2010) and a commercial washing machine (Farkas et al., 2011b) have all been investigated. For functionalized products, the form of Ag and the method of incorporation was found to affect the amount of Ag released (Geranio et al., 2009).

Benn & Westerhoff (2008) investigated the Ag released from six commercially available Ag containing socks into water, that study also investigated the fate of the released Ag in WWTPs. The concentration of Ag in the different socks was measured and ranged from below the detection limit to 1360 $\mu g/g$. The socks that did release Ag into water during the study leached Ag in both the particulate and ionic phase. The socks leached from 1.5 up to 650 $\mu g$ of Ag in 500 mL of distilled water and were found to leach variable amounts of the total Ag content from <1% to almost 100% suggesting that fabric manufacturing process influences the release of silver from the sock (Benn and Westerhoff, 2008). Geranio et al. (2009) investigated the release of Ag from nine fabrics that had differing methods of silver incorporation. The release of silver from the fabrics in the tests ranged from undetectable to about 45%. At pH 10 (mimicking the conditions during washing) the dissolution of the AgNPs was found to be 10 times lower than at pH 7. When bleaching agents (e.g. hydrogen peroxide or peracetic acid) were used the dissolution of Ag was observed to be greatly accelerated. The concentration and physical form of the Ag released from the fabrics as ionic ($Ag^+$) and particulate Ag (in the size fraction above and below 450 nm) was dependent on the Ag-incorporation method used on the fabric. The size distribution of silver released from a conventional silver fabric was not significantly different from a nano-functionalised Ag textile. While in the tests carried out at pH 10, a silver nano-sock released up to 25% of its silver content as ionic Ag, $Ag^+$. It was also found that in simulated washing conditions the majority of particles released were greater than 450 nm suggesting that the mechanical stress was the predominant factor causing the release under these conditions. These findings are important as it suggests that under washing conditions the Ag particles released are in the coarse fraction which will have an impact on risk assessments of Ag containing nano-functionalised textiles and also for AgNP environmental impact studies. The release of Ag from textiles may be enhanced due to the mechanical stress of the washing along with the effects of the chemical agents employed. A study by Walser et al. (2011) suggested that in a life-cycle assessment of a nano-functionalised T-shirt the production phase, and not the washing and end of life disposal, is the significant source of Ag released due to toxic Ag emissions during mining. Kulthong et al. (2010) studied the release of Ag from nano-silver

treated fabrics into four different standard artificial sweat preparations (ISO, EN, AATCC) with varying pH from 4.3 to 8.0. The amount of Ag leached into the artificial sweat was found to be dependent on several factors including: the initial amount of Ag coating, the fabric quality, pH and the artificial sweat formulations. Separately, Lorenzz et al. (2012) examined the Ag release from eight commercial Ag-textiles during a washing and rinsing cycle (Lorenz et al., 2012). While four of the textiles didn't declare the method of Ag incorporation three contained nano-sized silver and in one the Ag was incorporated using an Ag wire. The total Ag content of the textiles ranged from 1.5 to 2925 mg Ag/kg. Detectable amounts of silver were leached from four of the textiles (LOD 1.25 mg/kg), of which 34-80% was released as particles >450 nm. Different forms of Ag were found to be released from the different Ag textiles during washing and that AgCl was the most commonly observed chemical form of Ag in the wash-water (Lorenz et al., 2012). Interestingly several studies found that some fabrics did not contain detectable levels of Ag despite claims from manufacturers of nano-functionalisation (Benn and Westerhoff, 2008; Kulthong et al., 2010; Lorenz et al., 2012).

Kaegi et al., (2010) investigated the leaching of AgNPs into the environment from exterior paints. Following one year of exposure about 30% of the AgNPs initially contained in the paint were lost. Of the 30% lost 80% of this was found to be leached in the first two months of the study. The AgNPs were released as composite colloids and were attached to organic binders from the paint. It was suggested that the leached AgNPs are likely transformed to forms such as $Ag_2S$ which are considered less toxic. (Kaegi et al., 2010)

The effluent from a commercially available nano-functionalised washing machine was analysed for Ag content (Farkas et al., 2011b). The average Ag concentration in the effluent was 11 μg/L, measured by Inductively Coupled Plasma Mass Spectrometry (ICP-MS). The AgNP number concentration in the effluent analysed by single particle ICP-MS (SP-ICP-MS) was measured to be about 80 million AgNPs $mL^{-1}$ with the majority of the measured particles being below 20 nm in diameter. The average size of the AgNPs was found to be 10 nm when measured with TEM while using nanoparticle tracking analysis (NTA) found the size to be 60 – 130 nm. Differences in results generated by NTA and TEM methods have previously been noted and it was suggested that this may be due to smaller particles produce lower scattering limiting their measurement by NTA and that NTA may measure agglomerates of particles as a single particle (Farkas et al., 2010) The effluent was also shown to negatively affect the natural bacterial community. This study suggests that if AgNP containing washing machines

become commercially successful their effluent may contain significant loadings of silver which may enter the environment (Farkas et al., 2011b)

In a recent study it was found that commercially available AgNP functionalised toothbrushes could leach AgNPs, and are potential exposure routes for both consumers and the environment (Mackevica et al., 2016). This study found that nano-functionalised toothbrushes for both children and adults release AgNPs into tap water after they were submerged for 24 hours while exposed to friction. The median diameter of particles released from the toothbrushes varied between 42 – 47 nm. Higher levels of AgNPs were released from the adult toothbrushes emitting between 9.4 and 20.3 x$10^6$ particles/L compared to 3.6 to 6.6 x $10^6$ particles/L released by the children's toothbrush, however the adult toothbrushes did originally contain higher levels of silver than the children's toothbrush. The particulate emissions were found to only represent 1 to 3% of the total silver released, smaller particles may not have been counted as part of the particulate emissions as they may have been below the limit measured by SP-ICP-MS which in this case was found to be 35 nm. The total levels of Ag released from the toothbrushes was in the ng/L range. While the authors stated that human and environmental exposure risks from the toothbrushes alone were negligible, they did note that uncertainty still remains with regard to human and environmental risk from AgNP exposure (Mackevica et al., 2016).

Many studies have looked at the AgNP functionalised plastics used in the food packaging industry. Whilst it has been found that this poses little toxic risk to humans from studies identifying silver leaching into chicken meat, (Cushen et al., 2014a, 2013; Fröhlich and Roblegg, 2012; Hannon et al., 2015), this has potential to be a secondary risk factor. As recycling of solid waste and packaging increases, these packaging materials are frequently washed out adding to the load of AgNPs in the receiving environment. However Reidy et al., (2013) stated that food contact materials containing AgNPs are not approved for sale in Europe as the European Food Safety Authority (EFSA) currently doesn't assess nanoscaled silver products (Reidy et al., 2013).

A study by Sussman et al. (2015) investigated release of Ag from five Ag containing medical devices. Three of the medical devices contained AgNPs while the other two were labelled as containing ionic silver. While the devices were found to release silver in levels from the limit of detection (1 x $10^{-1}$ ng/cm$^2$) to 1 x $10^6$ ng/cm$^2$, only one of the medical devices tested was found to release observable AgNPs levels in all assays (water, saline and human plasma), it is therefore possible that not all nano-functionalised devices containing AgNPs will release

them in observable amounts in aqueous environments. It was also noted that the extraction conditions (extraction medium, time, and temperature) strongly influenced the amount of silver that could be extracted from the devices (Sussman et al., 2015).

## 7.0 Nanoparticle speciation in the environment

In the environment AgNPs may; stay in suspension, aggregate or agglomerate, dissolve or react with different species present in the aquatic system (Cunningham and Joshi, 2015; Luoma, 2008; SCENIHR, 2014). The form that AgNPs assume once they are released into the environment is of interest as the environmental impact can be related to their speciation. The speciation of the AgNPs in aquatic system is governed by complex reactions and relationships, and is affected by several factors including; organic matter content, concentration of ligands, pH and ionic strength (Luoma, 2008). In a recent study Zhang et al. (2016) outlined the complex nature of the physicochemical behaviour of AgNPs in the aquatic environment and suggested that much more research is needed in order to fully understand the kinetics of NP dissolution. They noted that current assumptions surrounding Gibbs free energy of formation of AgNPs in aquatic environments and release of silver ions through NP dissolution do not fully describe actual fate of the AgNPs (Zhang et al., 2016).

Silver may exist in different oxidation states; $Ag^0$ (metallic silver), $Ag^{+1}$, $Ag^{+2}$ and $Ag^{+3}$, of which the former two are the most common and latter two are unstable in aquatic environments (Cunningham and Joshi, 2015; Reidy et al., 2013; Wijnhoven et al., 2009). While the reactivity of $Ag^0$ is low, $Ag^{+1}$ will associate with negatively charged ligands or ions and can therefore react with any such ligands or ions (e.g. sulphide and chloride) present in the environment (Luoma, 2008).

In the aquatic environment different physicochemical parameters will influence the fate of the AgNPs. Dissolution of AgNPs has been shown to be influenced by several different factors (Zhang et al., 2016). The dissolution of AgNPs involves an oxidation step from $Ag^0$ to $Ag^{+1}$. This process is complicated and can be effected by; particle size, particle concentration, temperature, dissolved oxygen levels, pH, presence of ligands and ionic strength (He et al., 2013; Jingyu Y Liu and Hurt, 2010; Zhang et al., 2016). Smaller AgNPs have higher solubility than larger particles (Ma et al. 2012). It has been noted that particle aggregation reduces surface area and therefore slows the rate of dissolution, with dissolution ceasing when the particle size becomes large enough (Jingyu Y Liu and Hurt, 2010; Zhang et al.,

2016). High NP concentrations reduce the dissolution of AgNPs, due to the fact that at higher concentrations particles are likely to aggregate faster than at lower concentrations, aggregation then inhibits dissolution (Zhang et al., 2016, 2011). Higher temperatures have been shown to increase the amount of dissolution of AgNPs (Liu and Hurt, 2010). The pH of the water influences the rate of particle dissolution with dissolution rates decreasing with increasing pH (Liu and Hurt, 2010; Zhang et al., 2016). The type of coating present on the particles also influences particle dissolution. Citrate coated AgNPs were found to have faster dissolution rates than PVP coated particles in both freshwater and seawater (Angel et al. 2013). The presence of ligands can influence the dissolution. Sigg and Lindauer (2015) found that there was an initial increase of Ag dissolution in the presence of cysteine, while AgNP dissolution was not greatly affected by the presence of chloride and fulvic acids.

AgNPs can undergo aggregation or agglomeration in natural waters (Luoma, 2008). It has previously been calculated that the aggregation of AgNPs is thermodynamically favourable (Zhang et al., 2016). The aggregation of NPs is often modeled using the Derjaguine-Landaue-Verweye-Overbeek (DLVO) theory (Lodeiro et al., 2016). In the DLVO model the aggregation of NPs is dependent on the balance between the attractive, van der Waals forces, repulsive and electrostatic, forces (Dwivedi et al., 2015; Lodeiro et al., 2016; Zhang et al., 2016) The aggregation of NPs is influenced by several factors including particle size, concentration, particle coating, dissolved oxygen levels, pH, dissolved organic matter and ionic strength (Cunningham and Joshi, 2015; El Badawy et al., 2010; Luoma, 2008; Yin et al., 2015; Zhang et al., 2016). Coatings are added to AgNPs in order to stabilize the particles and to prevent aggregation. El Badawy et al. (2012) investigated the effect several stabilizing agents had on particle aggregation, they found that sterically (PVP) and electrosterically (BEPI) stabilized AgNPs are less prone to aggregation compared to the electrostatically ($H_2$ and Citrate) stabilized AgNPs (El Badawy et al., 2012). It has been noted previously that increasing ionic strength, especially the presence of divalent cations such as $Ca^{2+}$ and $Mg^{2+}$, increases the rate of particle aggregation. (Yin et al., 2015). The presence of dissolved organic matter inhibited the aggregation of AgNPs (Yin et al., 2015). The rate of aggregation of citrate coated AgNPs was found to increase when dissolved oxygen was present in the water (Zhang et al., 2011). The pH of the solution can strongly affect the aggregation of citrate coated AgNPs in acidic environment (pH 3) while the aggregation of PVP coated particles was unaffected by changing pH (El Badawy et al., 2010). The aggregation and agglomeration of AgNPs makes the particles more likely to settle out of the liquid phase into

the sediment than individual NPs (Luoma, 2008). It has been demonstrated that humic acid can induce NP formation (Dubas and Pimpan, 2008; Gunsolus et al., 2015). Akaighe et al., (2011) describe the induction of NP formation under environmentally relevant conditions by humic acid. This example of "natural interference" further underpins the overarching need to understand the full material life cycle of the pollutant of concern as it can be influenced by extrinsic factors such as local geography, humic acids being introduced to the aquatic ecosystem by peat rich areas and forestry for example.

Upon dissolution $Ag^+$ can undergo speciation through reactions with ligands naturally occurring in the environment. The important silver species that are formed in the aquatic environment include; $AgCl$, $Ag_2S$, $Ag_2O$, and silver(I) complexes (Zhang et al., 2016). Silver, at the surface of sediments, where oxygen is present and where sulfides are largely absent, predominately forms complexes with organic material whereas in deeper sediments, where oxygen is not available, Ag tends to form strong sulfide complexes (Luoma, 2008). $Silver^+$ has a strong affinity with thiol (-SH) ligands and with sulfide ligands (Adams and Kramer, 1999). The speciation of Ag in the aquatic system is of great importance as its toxicity is strongly related to its form (Nowack et al., 2011). It has also been suggested that in natural waters $Ag^+$ tends to bind to particulate matter suspended in the water (Blaser et al., 2008; Cunningham and Joshi, 2015; Luoma, 2008).

A number of studies have been undertaken to establish the fate of AgNPs once they have been introduced into the environment. Impellitteri et al. (2009) suggested that any Ag ions that are leached from nano functionalized fabrics during washing, with a hypochlorite/detergent, solution rapidly react with any chloride present in the wash water to form AgCl. It was also suggested that any silver that does reach a WWTP after being released from Ag-containing materials is likely be present as AgCl and it was suggested that it should remain in that form in biosolids (Impellitteri et al., 2009). However it has been found that under the high pH conditions, typically employed during laundry washing, little dissolution of Ag occurred from functionalised textiles, instead larger Ag particles (> 450 nm) were released through mechanical stress of the fabric (Geranio et al., 2009). In wastewaters it has been estimated that > 90% of AgNP present are captured by clearing sludge in WWTPs (Tiede et al., 2010). In a field study investigating the sewage sludge in a municipal WWTP Kim et al., (2010) found Ag to be in the form of α-$Ag_2S$ NPs. It was suggested that in the reduced, sulphur rich conditions of the WWTP α-$Ag_2S$ NPs are being formed from ionic and nano silver present in the water (Kim et al., 2010). The bioavailability of silver can be limited

by being bound into silver complexes e.g. Ag sulfide complexes are insoluble (Nowack, 2010). While the majority of the AgNPs may be captured in the sewage sludge the potential for their release to receiving water remains and is dependent on the fraction of wastewater adequately treated (Blaser et al., 2008).

In waters with higher salinity AgNPs, and ENMs, generally are more likely to aggregate or agglomerate (Luoma, 2008). In marine waters it has been suggested that, NP aggregates may sink to the ocean floor very slowly, similar to freshwater environments however whether the NPs accumulate at the thermocline, the transition between warmer and cooler water, or are recycled by biota is uncertain. (Klaine et al., 2008).

## 8.0 Capture, detection and characterisation of silver nanoparticles in aquatic environments

In order to gain an understanding of the impact that AgNPs have on the environment suitable sampling and measurement techniques must first be developed in order to detect, quantify, and characterise AgNPs in natural waters. The parameters of interest include measurement of NP concentration and particle size distribution, (Heithmar, 2011). There are a number of challenges associated with the capture and detection of AgNPs from the environment (Poda et al., 2011; Sadik et al., 2014; Zook et al., 2011). The capture of any NPs is problematic due to their small size. Conventional membrane filters cannot be used due to their large pore sizes which exceed the NP dimensions (Sadik et al., 2014). The ability to trap NPs doesn't necessarily translate to successful detection (Sadik et al 2014) The development of nanostructured materials for environmental applications, e.g. membranes, provide for the detection and separation of ENPs, however, inconsistent range in pore size means that some extremely small NPs can still penetrate the pores of these filters (Sadik et al., 2014). Equally in natural environments detection and quantification of ENMs is generally problematic due to background interference. For AgNPs in particular sampling difficulties are associated with the speciation effects; dissolution and complexing with other ions (e.g. AgCl) making the measurement of the NP fraction of silver most challenging (Neale et al., 2013; Zook et al., 2011). A number of studies use ICP-MS to detect AgNPs. The high sensitivity of ICP-MS for elemental detection makes it an appropriate detection approach (Liu et al., 2012). However, the particle size distribution cannot be determined by most element-selective detection techniques, including ICP-MS (Heithmar, 2011). Several different techniques have been

investigated to determine if they are suitable for capturing or concentrating ENMs from suspension including; activated charcoal, nanofilters and magnetic particles, cloud point extraction, ultrafiltration (Gicheva and Yordanov, 2013; Liang et al., 2010; Liu et al., 2012, 2009; Mwilu et al., 2014; Okello et al., 2011; Sadik et al., 2014; Trefry et al., 2010).

## 8.1 Capture of AgNPs

Due to the numerous nanometer scale pollutant particles that have to be removed during water treatment there is a demand for fibrous filters with nanometer scale pores (Liang et al., 2010). The fabrication of carbonaceous nanofiber filtration membranes, with a very narrow pore size distribution which is capable of quickly filtering nanometer-scale particles from suspension has been described (Liang et al., 2010). These filters were flexible and robust enough to separate NPs from the suspension even when a high pressure is applied. The pore size of the filters provided for very effective size selection, the capture of 25 nm Ag particles by the CNF-50 filters (generated using CNFs with a diameter of 50 nm) was almost 100%, whereas when a mixture of 5 and 25 nm Au particles was filtered by the CNF-50 filter the 25 nm particles were captured and the 5 nm particle passed through the membrane. Membranes generated from CNFs of different diameters resulted in filters which captured different diameters of NP (Liang et al., 2010).

Okello et al., (2011) used nanostructured poly(amic acid) [nPAA] as nanofilters to capture, isolate and measure the concentration of AgNPs in water. These AgNPs were then quantified by attaching the PAA membrane to gold electrodes which were then used for electrochemical measurements. Preliminary results demonstrated the capacity of the nPAA membranes to capture AgNPs from suspension and to quantify their abundance on the membranes. Using this process, concentrations of AgNPs near the toxic threshold of silver were successfully detected (Okello et al., 2011; Sadik et al., 2014)

Activated carbon has been successfully used to capture AgNPs from suspension (Gicheva and Yordanov, 2013). Gicheva and Yordanov (2013) demonstrated 60 nm citrate coated AgNPs were removed from aqueous dispersions using mesoporous activated carbon (Norit® CA1). It was suggested that NPs were adsorbed in pores in the carbon corresponding to the particle size. The deposition of AgNPs onto the activated carbon was increased by the presence of electrolytes (such as NaCl, KCl, $NaNO_3$, $KNO_3$) with the AgNPs being completely removed from the aqueous phase, and aggregating on the carbon surface, when the electrolytes were present at levels above their critical coagulation concentration. The authors suggested that

this method could be suitable for the removal of metal NPs from polluted aqueous media, however the volumes of electrolytes required may be a drawback to this technique (Gicheva and Yordanov, 2013).

Surface functionalized magnetic iron oxide NPs are a functional material with large surface-to volume ratios and high surface energies (Wu et al., 2008). Mwilu et al. (2014) used synthesized magnetic iron NPs coated with dopamine or glutathione as a pre-concentration media for the separation of trace levels of AgNPs from water dispersions. The AgNPs that adsorbed to the magnetic particles were then separated from the liquid matrix using an external magnetic field. These particles were analysed by ICP-MS and SEM. For initial laboratory studies it was found that >99% of the AgNPs were recovered from the samples, while for spiking experiments using environmental water samples recoveries higher than 97 % for tap water, fresh and saline surface water were observed. This method also had the ability to separate the particles and the ionic $Ag^+$ ion present in solution (Mwilu et al., 2014).

Cloud point extraction (CPE) has been used for the pre-concentration of AgNPs prior to analysis (Liu et al., 2009). CPE involves the partitioning of the AgNPs into a surfactant (e.g. Triton X–114) phase. The surfactant is added to the sample above its critical micellar concentration, the sample is then heated above the surfactants cloud point which then traps non-polar components, including AgNPs, in the micclles generated in the surfactant. The surfactant phase can then be separated from the sample (Hartmann et al., 2013). AgNPs have been concentrated successfully for samples without altering the particle size and shape characteristics (Liu et al., 2009). CPE has been used to concentrate AgNPs in environmental water samples, (Chao et al., 2011; Hartmann et al., 2013; Liu et al., 2009) tap water, bottled water and sea water samples (López-García et al., 2014). The amount of AgNPs concentrated into the surfactant phase from environmental water samples was found to vary form 57-116% when environmental sample were analysed (Liu et al., 2009).

Ultrafiltration membranes have been used to separate AgNPs from suspensions (Liu et al., 2012). Trefry et al., (2010) utilised tangential flow ultrafiltration as a method to size separate a polydisperse sample of AgNPs. This method pumped the sample through a 50 nm filter followed by a 100 kDa filter, it was found to provide greater control over particle size, aggregation and concentration when compared with ultracentrifugation (Trefry et al., 2010). Cross-flow ultrafiltration was used to separate AgNPs in a study investigating AgNPs effect on juvenile salmon. (Farmen et al., 2012). The size distribution of the colloidal samples was

characterised using ultrafiltration, the samples were firstly filtered with a 0.22 µm filter followed by 10 kDa hollow-fibre cross- flow ultrafiltration.

Other methods have been utilized to separate AgNPs from their matrices in order to prepare them for physical or chemical chacterisation (Liu et al., 2012). These include: Ultrafiltration (Trefry et al., 2010) (El Badawy et al., 2011)(Liu and Hurt, 2010), Hydrodynamic chromatography (Tiede et al., 2009a), ultracentrifugation (Kennedy et al., 2010), tangential flow filtration (Maurer et al., 2014) and field flow fractionation (Cumberland and Lead, 2009).

## 8.2 Size characterisation of AgNPs

The characterisation of the size distribution of AgNP is generally carried out using techniques including: Electron Microscopy (EM), Atomic Force Microscopy (AFM), or Dynamic Light Scattering (DLS) (Liu et al., 2012; Tiede et al., 2008).

### 8.2.1 Electron Microscopy

Both Scanning Electron Microscopy (SEM) and Transmission Electron Microscopy (TEM) are commonly used in the detection and characterisation of ENMs generally. With SEM the image is generated by scanning the surface of the sample with a low-energy electron beam. The characterisation of the NPs is through the detection of secondary or back-scattered electrons (Dudkiewicz et al., 2011; Liu et al., 2012). Samples for SEM must be conductive otherwise the sample may accumulate a static charge. In order to prevent this the sample can be coated in a conducting material however this can result in the loss of some surface information (Tiede et al., 2008). TEM characterisation involves the transmission of a high energy electron beam through the sample. TEM images can provide information on particle size, shape, morphology and aggregation state. As TEM involves the transmission of the beam through the sample very thin samples must be prepared for the analysis (Dudkiewicz et al., 2011; Liu et al., 2012). For both TEM and SEM the imaging is carried out under vacuum conditions, therefore it is not possible to introduce liquid samples into the EMs while other samples must be prepared (e.g. dehydration) before being introduced which can lead to alteration of the sample. The development of Environmental SEM (ESEM) has allowed the imaging of samples in their natural state. In ESEM the electron beam is generated under vacuum and is separated from the sample chamber by pressure limiting apertures allowing the sample to be introduced in its natural state (Doucet et al., 2005; Tiede et al., 2008). While EM techniques can characterise NPs size and shape sufficient quantities of the particles have

to be analysed in order to generate results that are representative of the full sample (Tiede et al., 2008). Energy-dispersive X-ray spectroscopy (EDS) can be coupled with EM in order to generate elemental spectra of the particles by analysing the characteristic X-rays emitted by the elements in the sample allowing the elemental composition of the particle to be determined (Hassellöv et al., 2008). Studies that have utilised EM techniques include: (Benn and Westerhoff, 2008; Cumberland and Lead, 2009; Gicheva and Yordanov, 2013; Kaegi et al., 2013; Liu et al., 2009; Ma et al., 2012).

8.2.2 Other characterisation techniques

Atomic Force Microscopy (AFM), which consists of an oscillating cantilever arm that measures electrostatic forces between the tip of the arm and the sample surface as the arm scans across the sample, has the ability to characterise AgNPs (Chinnapongse et al., 2011; Tiede et al., 2008). AFM analysis can give size, shape, surface and aggregation information about the samples and can be used to analyse wet samples however liquid samples have to be immobilised to prevent interference with the probe (Tiede et al., 2008). AFM has been successfully used to characterise natural aquatic colloidal samples (Lead et al., 2005; Muirhead and Lead, 2003) and has also been applied to the study of the temporal stability of AgNPs in natural water, synthetic seawater and simulated estuarine water (Chinnapongse et al., 2011), to characterise AgNPs and monitor their effect on marine diatoms (Pletikapić et al., 2012) and the effect of thiol containing amino acids on AgNPs (Ravindran et al., 2012).

Dynamic Light Scattering (DLS) is a useful tool for determining the hydrodynamic diameter and particle size distribution in suspension. The limitation of DLS is that the scattering intensity can be affected by the presence of other suspended material, such as dust, in the sample which can therefore interfere with the size measurements (Tiede et al., 2008). DLS has however successfully been applied to the study of the size of AgNPs in solution. In a time resolved fashion it has been used to monitor the aggregation of AgNPs (Huynh and Chen, 2011), the stability of AgNPs in different media (Chinnapongse et al., 2011) and AgNP fate in environmental water (Li and Lenhart, 2012; Yin et al., 2015). Lists of the many different techniques that have been utilised in the separation, capture and characterisation of ENM in different media is available elsewhere (Liu et al., 2012; Tiede et al., 2008).

**8.3 Quantification of AgNPs**

8.3.1 Inductively Coupled Plasma Mass Spectrometry (ICP-MS)

The most common methods used for the chemical quantification of AgNPs are element-selective detection techniques that are suitable for the analysis of AgNPs as they offer high speed, precision, sensitivity and large linear range (Heithmar, 2011; Liu et al., 2012). Inductively Coupled Plasma Mass Spectrometry (ICP-MS) and ICP-Optical Emission Spectrometry (ICP-OES) are the most common element selective techniques that have been used to quantitatively determine total Ag levels in studies of AgNPs (Liu et al., 2012). In both ICP-MS and ICP-OES the sample is introduced into a plasma flame where the analyte is ionised, in MS the ions generated in the plasma are then introduced into a mass spectrometer for quantification while in OES the radiation emitted at the characteristic wavelength of the element being analysed is measured by an optical emission spectrometer. The number of ions released and the intensity of the radiation emitted are both related to the concentration of the element of interest in the sample and it is therefore quantified by the MS or OES (Baalousha et al., 2011). ICP-MS has previously been used in numerous studies that investigate AgNPs. These include: the leaching of AgNPs from consumer products and their effect on the biota present in a mesocosm (Cleveland et al., 2012), the release of silver from a nanosilver washing machine (Farkas et al., 2011b), total silver in a study of silver dissolution in different media (Zook et al., 2011), release of AgNPs from food containers (Echegoyen and Nerín, 2013), AgNP release from paints (Kaegi et al., 2010), to quantify AgNPs after concentration using magnetic particles (Mwilu et al., 2014), silver release from medical devices (Sussman et al., 2015), the effect of AgNPs on bacterial communities (Das et al., 2012) and AgNPs fate in WWTP (Kaegi et al., 2013, 2011). ICP-OES has been used to: quantify total silver in an aggregation and dissolution study of AgNPs in natural surface water (Li and Lenhart, 2012), WWTP sludge (Kiser et al., 2010; Yang et al., 2012) and a study of the effect of pH on AgNP transport through porous media (Flory et al., 2013).

8.3.2 ICP-MS combined with chromatographic techniques

While the use of ICP-(MS and OES) for the chemical quantification of AgNPs is beneficial it can only provide results for the total Ag content of the suspension and is unable to distinguish the NPs from the ionic or complexed silver (Heithmar, 2011). In order to characterise NPs ICP-MS has been coupled with chromatographic techniques. These "hyphenated" techniques have been used to separate the NPs in the sample based on their size in order to measure the

element distribution across the size range of the sample (Liu et al., 2012). The chromatographic techniques that have been coupled to ICP-MS include; hydrodynamic chromatography HDC-ICP-MS and field flow fractionation (FFF-ICP-MS), (including; flow-FFF, and asymmetric flow FFF ($AF^4$)). In HDC-ICP-MS the particles are separated by their size by passing the particles through a column packed with non-porous beads in an eluent under laminar flow conditions (Heithmar, 2011; Tiede et al., 2009a). In HDC larger particles elute more quickly than smaller particles (Tiede et al., 2009a). HDC-ICP-MS has been used to study the behaviour of ENMs in different media and mixtures of ENPs (Tiede et al., 2009a) and in a study examining the fate of AgNPs in sewage sludge (Tiede et al., 2010). In FFF the chromatographic separation is produced by the application of a force field orthogonally to a laminar flow of eluent carrying the sample (Heithmar, 2011). The force field applied, forces the NPs toward the channel wall, the balance of the applied force field and particle diffusivities, based on size, results in size separation. In FFF small particles elute faster than larger particles (Heithmar, 2011). FFFF-ICP-MS has been deployed to characterise AgNPs (Poda et al., 2011). $AF^4$-ICP-MS has been used in a study of AgNPs in suspension (Mitrano et al., 2012),to measure AgNP aggregation under different conditions in a study of AgNP interactions in an aquatic microcosm (Bone et al., 2012) and AgNPs in chicken meat (Loeschner et al., 2015).

### 8.3.3 Single particle ICP-MS

The development of Single Particle ICP-MS has allowed the chemical characterisation of individual AgNPs and the differentiation of the particles from the dissolved ions. In SP-ICP-MS the signal is monitored at a higher time resolution than standard ICP-MS, this allows the discrimination between the ion plumes caused by a particle and the background analyte. The intensity of the ion plume is related to the mass of metal in the NP (Heithmar, 2011). The theory of operation and application of SP-ICP-MS to the study of colloids and NPs is outlined elsewhere (Degueldre et al., 2006, 2004; Degueldre and Favarger, 2004). SP-ICP-MS has been used in a study of AgNPs in suspension (Heithmar, 2011; Mitrano et al., 2012; Tuoriniemi et al., 2012), the study of AgNP uptake by biota (Gray et al., 2013) and a study of AgNPs in human digestion models (Walczak et al., 2013). Using SP-ICP-MS AgNPs have been characterised down to a size limit of about  20 nm below which it is difficult to discriminate from the dissolved  (Mitrano et al., 2012; Tuoriniemi et al., 2012)

### 8.3.4 Raman Spectroscopy

The SERS activity of AgNPs has previously been demonstrated (Creighton et al., 1979; Munro et al., 1995). In a study of Au and Ag sols Lee and Meisel, (1982) adsorbed negatively charged dyes onto the particles and then demonstrated a strong surface enhancement of its Raman spectra. As a method for examining nanoparticles SERS has previously been used to characterise nanoparticles in different environments. Guo et al., (2016, 2015) used SERS to analyse the concentration of AgNPs in antimicrobial products (Guo et al., 2015) and aqueous samples (Guo et al., 2016). For both investigations the AgNPs were pre-concentrated using centrifugation for the antibacterial products and coagulation followed by filtration for the aqueous samples. The SERS analysis was then carried out by first conjugating an indicator, ferric dimethyl-dithiocarbamate (ferbam), which binds strongly to AgNPs. The SERS was then run using a 780 nm laser. (Guo et al., 2016, 2015). In the study using filtration to detect AgNPs in water, (Guo et al., 2016), the AgNPs were first aggregated using $CaCl_2$ or $AlCl_3$ to increase the particle size to >100 nm, the particles were then captured using filter membrane with a pore size of 0.1 μm. Using this method samples containing 5 μg/L AgNPs were successfully concentrated and quantified using SERS. This technique was also used to measure 10 μg/L AgNPs spiked in environmental (pond) water samples (Guo et al., 2016). Badireddy et al., (2012) used a Raman spectrometer to examine spectral differences between AgNPs with no coating, citrate, PVP and GA coatings. The spectrometer for the study was operated at a wavelength of 632.8 nm. It was found that for the coated particles the peak maxima was red-shifted compared to the uncoated AgNPs citrate coated and uncoated AgNPs were found to have similar spectra while for the PVP and GA coated particles the presence of secondary peaks to the left of the peak maxima were noted (Badireddy et al., 2012).

The interaction of AgNPs with dissolved organic matter (DOM) was studied using Raman spectroscopy (Yin et al., 2015). The AgNPs were spiked into river water and water containing electrolytes and DOM. The confocal Raman spectroscopy was undertaken using a 5 mW 785 nm laser. It was found that in the presence of DOM the Raman spectra peaks associated with the coating decreased as a new peak appeared which was attributed to the replacement of the PVP coating molecules with DOM. The presence of cations in the solution was found to increase the PVP peak due to the cations inducing aggregation. Divalent cations were found to have a stronger influence on aggregation than monovalent cations, the introduction of DOM prevented this aggregation (Yin et al., 2015).

Trefry et al., (2010) used the SERS capability of AgNPs in order to compare two different separation method namely; ultracentrifugation and tangential flow ultrafiltration. Both the ultracentrifugation and ultrafiltration concentrated the AgNPs successfully and SERS analysis at 632.8 nm, using a standard solution of rhodamine 6G (R6G), showed that both concentration methods had analytical (AEF) and surface enhancement factors (SEF) 1000 times the AEF and SEF of the original mixture. The findings suggested that the tangential flow ultrafiltration may be the more beneficial method as it provided grater size concentration and aggregation control compared with the ultracentrifugation method (Trefry et al., 2010).

The Raman Effect has previously been used in a study of the cellular uptake of AgNP. Haase et al., (2011) used confocal Raman microscopy with a 532 nm Nd:YAG laser, 60x objective a 600 gratings/mm CCD detector in order to confirm the uptake of peptide coated AgNPs by a THP-1 cell line (Haase et al., 2011).

8.3.5 Preparation of solid samples for analysis

In order to successfully quantify the concentration of silver in solid samples they must firstly be prepared for chemical analysis. Several different methods have been used in the preparation of these solid samples. In order to measure the total silver concentration present in sewage sludge Kim et al., (2010) used microwave assisted nitric acid ($HNO_3$) digestion. To specifically examine Ag-S NPs in the sewage sludge, the sludge was first freeze dried and then ground. 0.5 g of the ground sludge was then made into a slurry with 10 ml of deionised $H_2O$ which was, following sonication, diluted 200 fold with methanol. The methanol fraction was then analysed by ICP-OES and TEM to examine the presence of Ag-S NPs in the sludge (Kim et al., 2010). When examining the release of AgNPs from nano-functionalised paints Kaegi et al., (2010) digested samples of; paint, dried paint on wax paper, and paint from the façade both before and after exposure for ICP-MS analysis, using microwave assisted digestion with 2 ml of hydrofluoric acid (HF) and 5 ml $HNO_3$ following the initial digestion a boric acid solution was added to complex the remaining HF the vessels were then returned to the microwave for a further digestion run (Kaegi et al., 2010). In studies of nano-functionalised textiles, fractions of the textiles under investigation were prepared for chemical analysis using microwave assisted digestion with $HNO_3$ and Hydrogen peroxide $H_2O_2$ (Lombi et al., 2014; Lorenz et al., 2012). Benn and Westerhoff, (2008) analysed the Ag content in nano-functionalised socks using acid digestion to prepare samples by first heating

samples of the socks with $HNO_3$ and deionised water and then adding $H_2O_2$ to the digestion vessel and reheating the sample. Galotto and Ulloa, (2010) prepared a nano-functionalised food bag for ICP-MS analysis by microwave assisted digestion using $HNO_3$ and $H_2O_2$.

Methods of digestion have also been developed for analysing Ag in biological matrices. To study the uptake of AgNPs by biological tissues Gray et al., (2013) used tetramethylammonium hydroxide (TMAH) to digest beef samples, *Daphnia magna* and *Lumbriculus variegatus* in order to prepare the samples for SP-ICP-MS analysis. Using the TMAH digestion both mass and particle number recoveries were found to be from 83 – 121% for AgNPs. A total metals digestion was also carried out as part of a comparison using microwave digestion and nitric acid. The TMAH digestion method found to be comparable to the total metal digestion method (Gray et al., 2013). Chicken meat that was formed into a paste and mixed with a solution containing AgNPs was prepared for characterisation by enzymatic digestion, using Proteinase K from *Engyodontium album* allowing the AgNPs to be characterised using $AF^4$-ICP-MS or SP-ICP-MS (Loeschner et al., 2015, 2013). Cushen et al., (2014b), in a study of the migration of AgNPs from nano-functionalised plastic into chicken, digested the samples using $HNO_3$ and Hydrochloric acid (HCl) which was added to the sample in a round bottomed flask which was heated to 95ºC under reflux the samples were then analysed using ICP-OES (Cushen et al., 2014b).


**9.0 Environmental Toxicology: Toxicity of AgNP to freshwater biota**

The behaviour of nanotoxins including AgNP in the aquatic environment will have a substantial influence on the toxic effects induced. As previously discussed, this speciation of silver can occur at virtually any stage of the life cycle from synthesis to discharge and consequently at the interface with target matrices, biologics and organisms. This speciation and concomitant interference is of particular note to researchers attempting to risk assess AgNPs. Test matrices can influence bioavailability and sensitivity.

Given the immense number of different nanoparticles available, the dearth of knowledge in actual environmental concentrations, characteristics of AgNP in common usage, the substantial interferences from extrinsic factors both in the environment and in laboratory conditions and the variety of tests available, a diverse set of existing eco-toxicological data has been published on a range of aquatic organisms (Asghari et al., 2012; Binaeian et al., 2012; Blinova et al., 2013; Bondarenko et al., 2013; Fabrega et al., 2011; Griffitt et al., 2008).

Several studies look at AgNP toxicity to primary producers using algal growth inhibition bioassays. These studies employ traditional toxicological endpoints which are defined as follows: $LC_{50}$ Median lethal concentration, $EC_{50}$ Median effective concentration, $IC_{50}$ Median Immobilising Concentration. Griffitt et al. (2008) reported the $EC_{50}$ for *Pseudokirchneriella subcapitata* to be 190 µg/L AgNP for 20-30 nm nominal nanoparticle size. Ivask et al. (2014) using the same algae report an $EC_{50}$ of 180-1140 µg/L AgNP in a range of citrate coated nanoparticle sizes of 10-80 nm.  Kennedy et al., (2010) investigated the effects of silver nanoparticle coatings (citrate, PVP, EDTA and others) and reported an $EC_{50}$ for *Pseudokirchneriella subcapitata* to be in the region of 9 µg/L AgNP, while Ribeiro et al., (2014) reported an $EC_{50}$ of 32 µg/L AgNP.

A substantial number of studies assess the toxicity of silver nanoparticles to secondary consumers (e.g freshwater invertebrates). Colloidal silver nanoparticles yielded mean $EC_{50}$ values of 2-4 µg/L compared to 187 µg/L for suspended powder AgNPs to *Daphnia magna* (Asghari et al., 2012). Blinova et al., (2013)  reported toxicity from PVP-coated-AgNP (<10 nm) as $IC_{50}$ 15-17  µg/L to *Daphnia magna*  and 20-27 µg/L to *Thamnocephalus platyurus.* Metal oxide coated 20-30 nm AgNP toxicity to *Daphnia pulex* was demonstrated by Griffitt et al., (2008) who observed an  $IC_{50}$ 40 µg/L. Park and Choi, (2010) observed AgNP <100nm toxicity of $IC_{50}$ 1.5 µg/L to *Daphnia magna* with genotoxic effect detected by the comet assay at 1 µg/L.  Alkane coated AgNP 3-8 nm yielded an $IC_{50}$ value of 11.02 µg/L in *Daphnia magna* (Ribeiro et al., 2014) whilst, AgNP and Ag-nanowires yielded $IC_{50}$ values of 7 µg/L and 117 µg/L respectively (Sohn et al., 2015). PVP coated nanosilver NM-300 <20 nm was observed by Völker et al., (2013) to yield $IC_{50}$ values of 8.95 µg/L, 13.9 µg/L and 121 µg/L to *Daphnia pulex, Daphnia galeata* and *Daphnia magna* respectively. An $EC_{50}$ value of 27 µg/L was observed in the freshwater shrimp *Gammarus fossarum* by Funck et al., (2013).

Toxicity assessments have employed *in vivo* fish studies (Fabrega et al., 2011; Ribeiro et al., 2014; Yoo-Iam et al., 2014) using silver barbs, zebra fish and goldfish.  With respect to the three R's of toxicology (Replacement, Reduction and Refinement) and the ethical issues with vertebrate testing, a trend towards *in vitro* piscine cells is evident. Aquatic ecotoxicological studies utilise primary fish cells and continuous fish cell lines. Connolly et al. (2015) compared the piscine cytotoxicity of AgNPs to primary cell cultures and selected continuous piscine cell lines i.e. RTL-W1, RTH-149, RTG-2 and primary hepatocytes. The continuous cell lines were found to be useful indicators of toxicity coupled with the assays employed however, cell culture media was noted to have a substantial effect on AgNP behaviour and

concomitant cytotoxicity of AgNPs. Two separate studies by Farkas *et al*., (2010, 2011a) examined the cytotoxic effects and $Ag^+$ uptake of silver nanoparticles to primary rainbow trout gill cells and hepatocytes. Glutathione interference and increased reactive oxygen species are widely accepted as important cellular effects of AgNPs. Katsumiti et al., (2015) detected effects on mussel haemocytes and gill cells in the 1-100 mg/L range and in rainbow trout hepatocytes and gill cells at >19 and 5 mg/L respectively. $Na^+ / K^+$ ATPase interference is reported by Massarsky *et al.*, (2014) who detected effects at 3.1-31 µg/L AgNP in primary rainbow trout hepatocytes.

In addition to the traditional acute ecotoxicity data described above, more recent papers focus on AgNP risk assessment which takes into account the effects of Ag speciation and concomitant effects on the bioavailability to test biota. The freshwater algae *Pseudokirchneriella subcapitata* yielded $EC_{50}$ values of 0.18 – 1.14 mg/L and the crustacean *Daphnia magna* $IC_{50}$ values of 0.010 – 0.218 mg/L (Ivask et al., 2014). This study also expresses the $EC_{50}$ corrected to Ag dissolution in terms of ionic Ag, possibly alluding to the potential for crossover in toxicity between the two silver species. Size dependant toxicity was investigated by Ivask et al., (2014). Silver toxicity from a range of AgNP sizes (10, 20, 40, 60 & 80nm) was observed to be dose responsive on a multitrophic test battery. In that study, the smaller AgNPs were observed to be more toxic than larger AgNPs although ionic silver (from silver $AgNO_3$) was found to be more toxic than any of the tested AgNPs. The effect of particle size / agglomeration / dissolution on the toxicity to aquatic organisms is not fully understood. It would be expected that smaller particle size would increase the effective surface areas and concomitant toxicity however Ivask et al. (2014) concluded that the toxicity was dependant on dissolution in 20-80nm but not 10nm. Whilst size dependant toxicity is shown, correlation does not imply causation and it is clear that a full understanding of the interactions and behaviours of silver based on water chemistry and extrinsic factors is critical. Kalbassi et al. (2013) demonstrated that the powdered suspensions of AgNP leads to increased agglomeration and reduced toxicity by investigating the effect of agglomeration on the toxicity to Rainbow Trout (*Oncorhynchus mykiss*). This study characterised the sizes of AgNPs in two forms by a number of methods and concluded that suspended powder AgNPs agglomerate and precipitate to a greater extent than colloidal silver. Rainbow trout alevins do not actively swim and are more sensitive to the increased exposure of benthic toxicants which sediment from the water column. Kalbassi et al., (2013) demonstrated a particle size influenced, dose dependant toxicity on two day old Rainbow Trout (*Oncorhynchus mykiss*)

alevin and juveniles (15.47g ±0.83g) treated for 4 days with colloidal silver and AgNPs in suspended powder forms. The colloidal silver nanoparticle were observed by TEM to be smaller than the agglomerates formed by the suspended powdered form (Colloidal: 65.4% 1-13nm, 2.28% <100nm; dry powder: 85.97% 1-45nm, 1.34% >100nm). The colloidal silver was found to be 60-100 times more toxic (depending on exposure time) than the dry powder form with average $LC_{50}$ values of 0.25-2.75 mg/L and 28.25-186.42 mg/L reported respectively in alevins. Juveniles were found to be less sensitive. Gill pathologies were also observed including the trapping of agglomerates under the gill operculum and in the oral cavity. The colloidal silver was reported to have been well dispersed throughout the test matrix, whereas the suspended dry powdered silver was observed to rapidly sediment as agglomerates were formed.

The toxicity of suspended powdered AgNPs was compared to $Ag^+$ on four aquatic organisms by Yoo-Iam et al., (2014). $EC_{50}$ for *Chlorella sp.* algae was found to be 0.89mg/L (AgNP) and 0.39mg/L ($Ag^+$). AgNP $LC_{50}$ for *Moina macrocopa, Chironomus spp.* and *Barbonymus gonionotus* were reported to be 1.11, 1.08 and 1.76 mg/L respectively whilst, $Ag^+$ $LC_{50}$ values of 0.026, 0.042 and 0.057 mg/L demonstrate comparatively increased toxicity from silver ions. Silver concentrations within the organisms were also recorded. The algae and *Chironomus spp.* contained more silver from the AgNP tests than $Ag^+$, however, the inverse was reported for the other test species. This suggests that the tested algae and worms would bioaccumulate $Ag^+$ more readily than AgNP whereas the invertebrates and fish would bioaccumulate AgNP more readily.

Investigating the uptake of silver nanoparticles to rainbow trout gill cells, Farkas et al., (2011a) compared the toxicity of $Ag^+$ (from $AgNO_3$) with PVP and larger citrate coated AgNP in monolayer and multilayer primary cell cultures . Uptake of the citrate coated AgNP was shown to be higher than PVP through monolayer epithelia whereas the smaller PVP AgNP transported readily through cultured multilayers. Cytotoxicity was evident for all treatments. Glutathione levels were noted to be elevated post treatment which is hypothesised to be an overcompensation reaction to oxidative stress.

Several studies have looked at the influence of various physical characteristics of AgNP such as size, coating and charge on their toxicity (Baumann et al., 2014; Blinova et al., 2013; Choi and Hu, 2008; El Badawy et al., 2011; Ivask et al., 2014; Kalbassi et al., 2013; Silva et al., 2014).  Research into the toxicity of AgNP in the aquatic environment appear to demonstrate

that whilst the effects of AgNP on the receiving environment are important, the effects of the environment on AgNP (and silver) is also a critical factor in understanding the toxicity. In the assessment of AgNP toxicity, there are a substantial number of variables to consider, notably bioavailability. Walser et al., (2011) state that the bioavailable fraction of silver is influenced by water chemistry. Chinnapongse et al. (2011) found that AgNPs are more stable in freshwater than saltwater due to the reduced concentrations of chloride (NaCl) thus limiting precipitation of AgCl. A review by Fabrega et al., (2011) which includes data from Blaser et al. (2008) reported that concentrations of silver nanoparticles in surface waters are most likely in the ng/L range and also predict the most likely effect is the partitioning to sewage sludge. This view is further substantiated by Levard et al. (2012) who say that AgNPs are transformed in WWTPs to silver sulfate which is largely non-toxic and eluted to surface waters. The effects of several factors affecting the toxicity of silver to aquatic organisms were studied under laboratory conditions by Erickson et al., (1998) who reported that increased pH, alkalinity, hardness, sodium sulfate and organic carbon decreased the toxicity of silver, whereas an increased sodium chloride concentration increased toxicity to fathead minnow (*Pimephales promelas*) and *Daphnia magna*. El Badawy et al., (2010) reported the effects of environmental conditions such as pH, ionic strength and electrolytes on AgNP suspensions by influencing their surface charge. The stabilising influence of capping agents (coatings) such as PVP, citrate etc. were also examined. The coatings available on AgNP tend to be reported to have a greater influence on particle stability rather than toxicity. PVP coated were found to be the most stable, whereas other coatings and uncoated AgNPs were found to aggregate more readily at higher ionic strengths and acidic pH. $Ca^{2+}$ was also noted to increase aggregation. Tejamaya et al., (2012) reports that in a comparison of monodisperse citrate, PVP and PEG coated AgNPs in a diluted OECD *Daphnia* test media PVP was the most stable over 21 days and thus would be most suitable for chronic studies. Chloride in the media was also noted to have an effect on particle shape and dissolution. As previously mentioned, the degree of dissolution, can greatly influence the exposure index and hence toxicity.

In a report by the US EPA on the detection and characterization of ENM in the environment, Montano et al., (2014) describes the key characteristics that effect the environmental fate and hence bioavailability of ENM's. These include reactivity and persistence as influenced by alterations to organic coatings, dissolution and ligation, redox reactions, aggregation and biological transformations.

Angel et al. (2013) investigated the impact of size on the fate and toxicity of AgNP in aquatic systems and found that ionic silver was more toxic to the freshwater organisms *Pseudokirchneriella subcapitata* and *Ceriodaphnia dubia* than AgNP with micron-scaled silver particles less toxic. This study added another layer of complexity to the influence of humic acid reported by Akaighe et al., (2011). Humic acid was found to impair the aggregation of citrate coated AgNPs. PVP coated AgNP's were not found to aggregate to a significant extent in any of the test matrices, AgNP dissolution increased with chloride concentrations which may prove important in estuarine, marine and transitionary waters.

Several studies use ionic silver as a proxy to assess the toxicity of AgNP's. This may well prove to be the most prudent approach for acute risk assessment. Kennedy et al., (2010) compares the toxicity of silver in terms of ionic silver ($Ag^+$), AgNP and fractionated silver to *Daphnia magna,* fathead minnow (*Pimephales promelas)* and *Pseudokirchneriella subcapitata* and reports that $Ag^+$ may play a critical role in the toxicity of silver. Beer et al. (2012) observed that the proportion of $Ag^+$ present in AgNP suspensions plays a substantial role in toxicity, however $Ag^+$ may also cause interference with biologics and electrolytes such as thiol residues, glucose and chloride as well as components of cell culture media (Connolly et al., 2015; Hansen and Thünemann, 2016; Levard et al., 2012). Cysteine was shown to reduce AgNP toxicity to algae (Navarro et al., 2008). Several papers have been published on the optimisation of culture media and test matrices to improve the sensitivity of AgNP toxicity risk assessment (Connolly et al., 2015; Lee et al., 2005; Loza et al., 2014).

A variety of analytical techniques previously described in this review are employed to characterise the AgNP investigated in toxicity studies however, there is a need to standardise characterisation and toxicity testing methods to facilitate true replication and comparisons (Barnard, 2006; Behra and Krug, 2008; Hegde et al., 2016; Morris et al., 2011)

## 10.0    Conclusion

It is clear that AgNPs, due to their many applications are likely to be emitted into the environment. The levels in the environment are difficult to determine as they are present at low concentration and AgNPs undergo complicated reactions which change their speciation including; dissolution, aggregation and chemical complexation. In order to measure AgNP levels in the aquatic environment there is a need for the development of appropriate methods for their capture. Appropriate capture methods would allow the chemical quantification of the

particulate silver present in the environment. The proliferation in the use of AgNPs in different applications hastens the need for the development of inventories of AgNP containing products at national and international levels.

The fluctuating nature of metals in aqueous solution and their speciation to different forms confuses the issue substantially. Dissolution of AgNPs into ionic silver can also lead to an increased toxic effect, however this is likely to be time dependant as the dissolution takes place over time and can be influenced by many factors (Liu and Hurt, 2010). The most likely scenario is that the toxic effects of small NPs, <25nm are due to the inherent toxicity of the NPs themselves, however larger particles and agglomerates will in time shed ionic silver yielding further time and water chemistry dependant toxicity. These agglomerates by their nature are most likely less bioavailable than their smaller counterparts but in time will give rise to toxicity from dissolution processes producing mixed toxicity silver species. This demonstrates the importance of understanding the full chemical life cycle in the risk assessment of AgNPs. In the absence of actual environmental concentrations or silver speciation data the research community must adapt a systematic approach not only in the optimisation of test matrices but also in the choice of analyte, selecting the most recalcitrant silver species based on the water chemistry in the geographical area of interest.

Evidently, a substantial amount of research has gone into the assessment of the risks associated with silver nanomaterials in the freshwater aquatic environment confirmative evidence of toxicity or safety remains elusive (SCENIHR, 2014). Complications and uncertainty may stem from the variation in results, the plethora of different types of ENM / AgNPs already in the market and likelihood of legal ramifications in condemning existing market products without concrete evidence of risk.

The recent landmark passage of H.R. 2576 in the US House of Representatives will revamp the archaic 1976 Toxic Substances Control Act (TSCA) and shift the chemical risk assessment from requiring proof of harm to requiring proof of safety thus giving the EPA(US) substantially more impact in chemical safety oversight (Hogue, 2016).
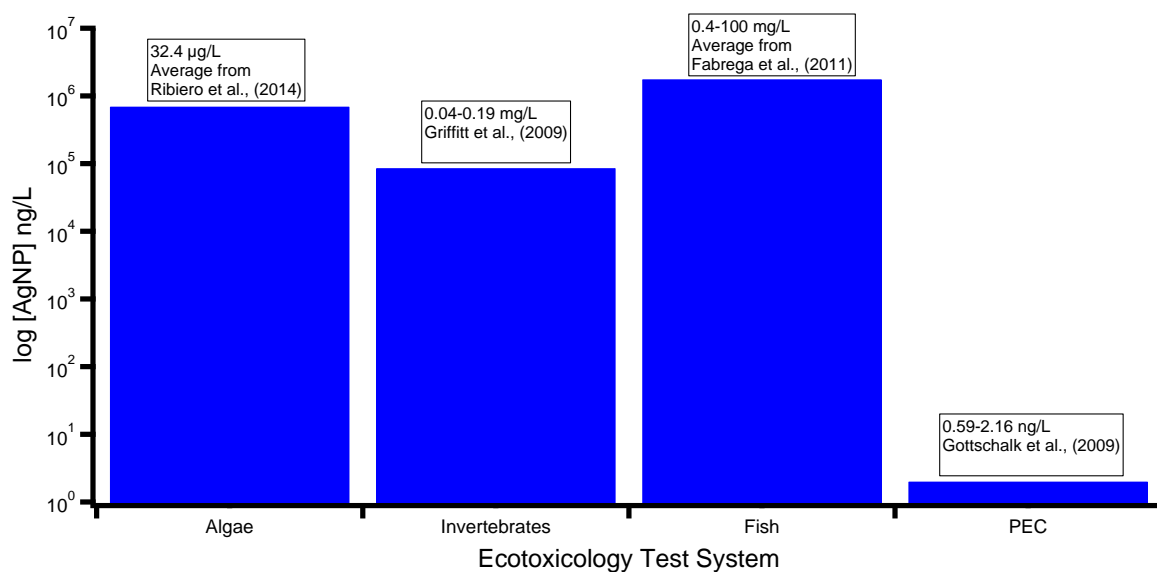
**Fig 2:** Comparison of ectox test sensitivity to PEC's

The PECs of AgNP have been reported in the ng/L range (Blaser et al., 2008; Gottschalk et al., 2009), however, the acute toxicity data values reported in the literature and mentioned above are generally in the µg/L range. This disparity is highlighted in Figure 2. The relative average sensitivities of typical standard test organisms to AgNP compared to the PEC highlights the urgent need to focus on sub-lethal effects at environmentally relevant concentrations. Further research and clear quantification of the concentrations of silver in all of its forms in the environment is urgently needed to facilitate ecologically relevant toxicity assessment. If proven true, the PEC's seem to indicate that primary toxicity to the tested organisms are not of high concern, however, bioconcentration up trophic levels, reproductive and chronic studies are perhaps more urgently needed to ensure a thorough risk assessment.

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## 11.0  References

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