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Assessment of exposure of marine and freshwater model organisms to metallic nanoparticles

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Assessment of exposure of marine and freshwater model organisms to metallic nanoparticles

Project Title

Assessment of exposure to metallic nanoparticles (NPs), focusing on silver (AgNPs) on marine and freshwater model organisms at a cellular and genetic level

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The EPA Research Programme addresses the need for research in Ireland to inform policymakers and other stakeholders on a range of questions in relation to environmental protection. These reports are intended as contributions to the necessary debate on the protection of the environment.

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Executive Summary

Silver nanoparticles (AgNPs) continue to be utilised in a wide range of everyday products due to their unique physicochemical attributes, extending from silver's historical use in medicinal and industrial applications. Silver, as a bulk chemical, is well understood and characterised, its exposure risk known and its toxicity determined. However, there are new and unknown risks associated with materials which have been synthesised or manufactured as engineered nanomaterials (ENMs), where individual particles have one angle measurement within the nanometre (nm) range. It is of importance to note that naturally-occurring particles within the nm range, i.e., nanoparticles (NPs), can and are created under natural conditions within the environment, such as by erosion. However, naturally-occurring NPs are typically slow-released from their source at extremely low point concentrations and are typically inert, with low reactivity to their surrounding environment.

With the advent of industrial utilisation of ENMs, it is now of the utmost importance that exposure risk scenarios in relation to increasing and unnatural NPs, where their function and interaction with their environment is unpredictable, are monitored, assessed and, if required, that regulatory guidelines are implemented early in their application. Of all ENMs currently in use, AgNPs are contained in greater than 40% of listed 'nano'-containing consumer products. AgNPs lend themselves to broad applications, as detailed in Section 1, and present a credibility exposure risk and a suitable model for risk assessment of exposure. Within this project, assessment of exposure to metallic NPs, focussing on AgNPs on marine and freshwater model organisms, has been taken. Standardised toxicity systems and protocols are utilised, highlighting areas of concern or adaption for utilisation as NP assessment protocols. A novel marine invertebrate system has also been investigated as a potential alternative organism for animal testing.

The principal findings of the research are:

• Information regarding the stability due to stabilising agents and the risk of AgNP dissolution has indicated that the potential toxicity of AgNPs can, in part, be attributable to ionic form and its reactivity. This, in turn, will also lead to the accumulation of environmental silver in conjunction with other elements, such as silver sulphate complexes AgS in sludge.

• Physicochemical characterisation and profiling is required throughout the life-cycle of ENMs. Properties playing key roles include the shape and surface area – both are key, in terms of rate of dissolution and the release/transformation into ionic forms. This includes the characterisation within the 'test' environment. Attributing factors which should be detailed are the capping/surface stabilisation materials utilised for all ENMs.

• Reference samples of ENMs, including AgNPs, are needed for cross-study tabulation, to permit larger datasets to be built to understand the full biological risk of these materials. Currently, no such standard particle set exists.

• A need for a review and modification of current biological assessments is required for utilisation in the assessment of AgNPs. Standardised International Organisation for Standardisation (ISO), Organisation for Economic Co-operation and Development (OECD) and Clinical and Laboratory Standards Institute (CLSI) protocols, which are routinely used for assessment of compounds and fine chemicals, were found in this report not to be suitable for ENM risk assessment, reference ISO 11384-1, -2, -3. Observations of accelerated dissolution rates, subject to media composition, demonstrate a need for uniform comparison and testing to be applied across all testing. Exposure assessment requires a multi-system testing to be performed. This is especially important for studies where only a single bacterial species is tested, as reported and indicated within this work.

• *Danio rerio*, Zebrafish, are a suitable model for exposure study assessment, developmental monitoring, and the identification of potential exposure indicators. Zebrafish embryos lend themselves to high throughput screening programmes, with outcomes potentially known within five days.

• A need for suitable non-animal models, developed for exposure assessment and testing, requires further investigation and development. Within this work, *Hydractinia echinata,* as an invertebrate marine model, was investigated. Though early in this model's adaption for this purpose, it has demonstrated that it could be developed for such assessment.

The type of information generated in this project can be used to assess the impact of AgNPs on marine and aquatic systems, and with the systems used to monitor these effects, this can be interpolated to water quality and human health. Exposure to AgNPs and other ENMs is an increasing risk worldwide. A clear understanding of the physicochemical properties and their associated biological risk is needed, to enable policy makers and legislators to make informed decisions in reducing the impact of nanotechnology at both national and European Union level.

1. Introduction

Nanoparticles (NPs) and nanomaterials (NMs) are broadly defined as substances which have at least one critical dimension less than 100 nanometres (nm) (Hood, 2004). Engineered nanoparticles (ENPs) and engineered nanomaterials (ENMs) are intentionally made, as opposed to being an incidental by-product of combustion or a natural process, such as erosion. These materials can, in theory, be engineered from nearly any chemical substance, and due to their physicochemical properties and composition, they exhibit unique physical, electrical, thermal, mechanical, imaging and biological properties, making them highly desirable for wide-ranging applications. However, the comparatively high reactivity, mobility and other properties that come with nanoscale size are also likely to impart undesirable properties (Nel et al., 2006; Xia et al., 2009). As with all engineered and technological developments, EMNs offer huge potential for beneficial use, though unknown exposure risk remains to be determined (Balbus et al., 2007; Tolaymat et al., 2010; Warheit & Donner, 2010). ENMs can be categorised based on the core material from which they are made, such as copper, gold, aluminium or silver, to name but a few materials in the case of metals. However, such simplistic categorisation does not reflect their ranging physicochemical parameters or their potential unique properties, be they advantageous or deleterious. ENMs can also be further manipulated and differentiated by the addition of surface coatings or capping agents, leading to the functionalising of their surface. Thus, two particles of the same material but of different size and shape may have very different physicochemical properties, applications and, indeed, exposure impact (risk) on surrounding environmental biological systems (Boverhof & David, 2010; Hassellöv et al., 2008; Osborne et al., 2012).

Definition:

> ***ENMs**: As used in this report, ENMs refer to both engineered nanoparticles (ENPs) as individual particle types, and nanomaterials (NMs) where they may be supplemented by other materials, chemicals or agents for application.*

The assessment of the potential benefits, and of both proposed and latent risks, relies on the information collection and presentation, characterisation, testing and profiling of each ENM independently. Given the nascent state of nanotechnology, much remains to be learned about the characteristics and impacts of ENMs to support environmental and health assessments (Casals et al., 2010; Mueller & Nowack, 2008; Oberdörster et al., 2005a). Reports from the U.S. Food and Drug Administration Nanotechnology Task Force in July 2007 highlighted that nanoscale materials can be more biologically active than non-nanoscale materials, and that basic research on bio-accumulation, bio-persistence, ecotoxicology, interactions with cellular and sub-cellular structures, dose/concentration usage and exposure

(occupational and environmental) is required[1]. Both the European Union (Look-forward Initiative on Nano-medicine, 2003) and the European Science Foundation (Towards a European Strategy for Nanotechnology, COM (2004) 338, EC Communications, Brussels) have continuing research activities, mapping the applications and associated risks of nanotechnology with agencies and committees commissioned to explore this field (SCENHIR[2], REACH[3] and ECETOC[4]). The research reported in this work has endeavoured to build upon current knowledge and, more importantly, add new data that could be of value in assessing exposure outcome in marine and aquatic settings. In this study, the focus has been specifically aimed towards the risk of exposure to engineered nanoparticle silver (AgNPs).

1.1 Silver in the Environment

1.1.1 Conventional Silver in the Environment

Silver (Ag) is rarely found in its pure, free form, but instead is more commonly found as an alloy with other metals or associated with minerals, the predominant form of which is argentite (Ag_2S). Ag can exist in its metallic state Ag^0 (zero valency) and in three cationic states (Ag^+, Ag^{2+}, and Ag^{3+}), with Ag^0 and Ag^+ being most common. Silver is naturally released into the environment by wind and water erosion of soils and rocks containing silver. Earth's crust contains approximately 0.1 part per million (ppm) of silver, and soils contain approximately 0.07 mg/kg (Taylor, 1964). Silver sulphide (Ag_2S), silver nitrate ($AgNO_3$), and silver chloride (AgCl) are the most abundant silver compounds found in the environment (Wiberg et al., 2001). Water concentrations of 0.2 microgrammes per litre (µg/L) in freshwater and 0.25 µg/L in seawater have been reported. Environmental background silver has also been found in biota at levels of microgrammes per gramme (µg/g) of tissue, particularly in fish and shellfish.

1.1.2 Historic and Current Uses of Silver and Silver Compounds

The use of silver compounds for therapeutic purposes has a long history (Nowack et al., 2011). In the 18th century, $AgNO_3$, often called lunar caustic, was moulded into pencil-like forms and used to remove granulation tissue from wounds and to lance abscesses, while powdered $AgNO_3$ was used to kill impurities and to dry open wounds (Klasen, 2000; Rai et al., 2009). In the 19th century, physicians used $AgNO_3$ to promote the healing of burns and other wounds. It was during this century that research into the bactericidal effects of silver was initiated. As early as 1880, physicians used $AgNO_3$ eye-drop solution to prevent gonococcal conjunctivitis in newborns. In the early 20th century, surgeons used silver foil, silver sutures, and other silver dressings to prevent infection in surgical wounds. The use of silver-containing products was

[1] http://www.fda.gov/nanotechnology/taskforce/report2007.html

[2] http://ec.europa.eu/health/scientific_committees/emerging/index_en.htm (Verified 09/2014)

[3] http://ec.europa.eu/enterprise/sectors/chemicals/reach/index_en.htm (Verified 09/2014)

[4] http://www.ecetoc.org/ (Verified 09/2014)

surpassed in popularity and application with the introduction of penicillin, around the period of the Second World War (Chopra, 2007; Rai et al., 2009). Other applications of note include Louis-Jacques-Mandé's (1839) introduction of a photography technique, which used a silver-plated sheet of copper, sensitised with iodide vapour, to make the silver react with light, and a salt solution to permanently set the image on the sheet. Silver is still used in photography today, but with the advent of digital photography, the percentage of its total usage continues to decline, dropping from 39% in 1979 to 13% in 2008 (GFMS Limited, 2009).

Of any pure metal, Ag has the highest thermal and electrical conductivities across a wide range of temperatures (Nordberg & Gerhardsson, 1988). Owing to this, it has found use in industrial applications, such as household switches, switch panels in electrical appliances, batteries, and superconductors (The Silver Institute, 2009)[5]. In 2008, industrial applications of silver accounted for 54% of the total Ag used in manufacturing,), with jewellery (19%), silverware (7%), and coins (8%) accounting for the remaining silver used in manufacturing (GFMS Limited, 2009). Other industrial uses of Ag include as coatings on mirrors and compact discs, in water purification systems, as antibacterial disinfectants, and in rear window defrosters in automobiles (The Silver Institute, 2009).

[5] https://www.silverinstitute.org/ (Verified 09/2014)

1.1.3 Historic Environmental Silver Levels

Industrial processes, such as smelting and mining, photography, and jewellery manufacturing, have led to elevated levels of silver being released into the environment (U.S. Environmental Protection Agency, 1987). Typically, areas of elevated silver concentrations occur near sewage outfalls, electroplating plants, mine waste sites, and silver iodide-seeded areas (Eisler, 1996). Runoff from silver disposal sites can transport silver away from these locations, and subsequent human activities, such as dredging and construction can further extend the transport of this silver (Purcell & Peters, 1998). Ecological and toxicological effects have been linked to silver concentrations in the environment in the ng/L (ppb) range, as demonstrated by several field and dietary toxicity studies summarised by Fabrega et al. (2011) and Luoma (2008). Biochemical signs of stress, including most notably the failure of a clam species (*Corbula amurensis*) to reproduce on a mudflat two kilometres from a domestic-sewage outfall in South San Francisco Bay, USA, was previously documented (Hornberger et al., 2000). Over a 30-year period, the amount of silver in waste delivered to this sewage facility decreased with improved waste facility treatment processes, resulting in reproductive capabilities of the clams returning (Hornberger et al., 2000).

With respect to water quality criteria in protecting aquatic life, there is now a need for regulatory determination and limit-setting, based upon aquatic life chronic criteria concentrations. A number of states across the USA have initiated such classification, ranging from 0.06 mg/L to 10 mg/L (North

Carolina Department of Environment and Natural Resources, 2007). Within the European Union, there is a lower level of silver mine production (~1600 tonne silver/year), and instead it relies on silver imports and the recycling of scrap in production and fabrication to make up ~ 60% of silver for use (Lanzano et al., 2006). The release and waste flow of Europe's silver usage has previously been detailed by Lanzano et al. (2006).

1.2 Silver in the 'Nano' Size Range

Human and environmental exposure to ENMs from consumer products may occur during several phases of the product life-cycle: during the synthesis, at production and use of a consumer product, release by industrial emissions, product degradation and end-of-life disposal of consumer products. Variations in routes of exposure and expected concentrations levels will vary greatly. However, the use and application of sub-micron sized silver is not a new discovery, as often illustrated with reference to The Lycurgus Cup from the 4th century AD. Nanoscale silver has historically been used in craft works and stained glass (Kelly et al., 2003). Reports in literature support the existence of products containing colloidal silver over a long period of time. Though not supported by scientific findings, applications including potential therapeutic agents have been documented (Bottomly et al., 1909; Nowack et al., 2011). A number of these silver preparations may have contained particles within the AgNP-defined range, employed long before their use in these applications was recorded. The Woodrow Wilson Centre's Project on Emerging Nanotechnologies (PEN) has compiled an inventory of consumer products reported by their manufacturers to contain nanomaterials[6], showing both the increasing application of ENMs in consumer products, as well as providing information which demonstrates that AgNPs are amongst the most common NMs used today in manufactured consumer products, with 437 known products as of September 2014. This is a marked increase from 26 products in 2006 and the 313 recorded products in 2011[7].

PEN has divided nanoscale materials into three classes based on their origins:
(i) Naturally-occurring nanosized particles include, for example, particles that originate from volcanic explosions, ocean spray, soil and sediment weathering, and biomineralisation processes.
(ii) Incidental nanosized particles, which are generated as by-products of processes, such as combustion, cooking, or welding.
(iii) ENMs purposely generated for a specific function, e.g. carbon nanotubes used in tennis rackets to make them lighter and stronger, and AgNPs utilised in new antibacterial materials and surgical devices.

Recently, PEN has also introduced a proposed classification system to provide a subjective confidence level in the "nano" claims gathered for consumer products[8].

[6] Project on Emerging Nanotechnologies, Consumer Products inventory; (Verified 09/2014)

[7] http://www.nanotechproject.org/cpi/browse/nanomaterials/silver-nanoparticle/ (Verified 09/2014)

[8] http://www.nanotechproject.org/cpi/browse/claims/ (Verified 09/2014)

Products progress from one category to another, as greater information and supportive data is validated regarding claims:

(i) Category 1 (Extensively verified claim)
(ii) Category 2 (Verified claim)
(iii) Category 3 (Manufacturer-supported claim)
(iv) Category 4 (Unsupported claim)
(v) Category 5 (Not advertised by manufacturer).

Of the 1,317 consumer products included in the March 2011 PEN inventory of ENM-based consumer products, greater than 40% were listed as containing AgNPs. The highest number of products to date are produced in the United States of America (USA), despite silver being classified as a toxic element under Section 313 of the Emergency Planning and Community Right-to-Know Act of 1987 (U.S. Environmental Protection Agency, 1987). Consumer products containing AgNPs represent a significant fraction of 'nano' end-product categories examined for the PEN inventory.

From March 2006 to March 2014, the number of products reported to contain AgNPs increased from 26 to 437, a 16.8 fold increase. These consumer product numbers are based upon manufacturer literature and related internet searches, and have not all been independently or scientifically validated. With this consideration, it is important to note that unreported products may also be available which contain AgNPs at consumer level.

Of the 437 identified AgNPs containing consumer products, their end-use applications encompass:

(i) Disinfectants for the elimination of viruses, fungal, bacteria, and their related odours (Cho et al., 2005; Huang et al., 2008; Yoon et al., 2008)
(ii) Dietary supplements (Martirosyan & Schneider, 2014)
(iii) Laundry detergent, body soap, toothpaste
(iv) Construction materials, wall paint, lightweight composite materials[5]
(v) Functionalised plastics (kitchen utensils, food preservative film), polymers (filters of air and water purification systems) and fabrics (clothing, socks, fabrics, and shoe soles)[6]
(vi) Medical products, wound dressings, contraceptives, surgical instruments, bone prostheses, and cardiac catheters (Ahamed et al., 2010)
(vii) Biosensors, medical imaging, tissue engineering (Doria et al., 2012; Sagadevan & Periasamy, 2014).

The rapidly-developing field of ENMs and expanding applications of AgNPs in the consumer market would suggest that an increased exposure risk to AgNPs is inevitable. Exposure routes are widespread, based upon point of origin, the vast array of consumer product types, end-of-life waste, and discharge during use. This validates the need for risk assessment studies to determine any deleterious effects of these NPs, in this case focussing on AgNPs on living systems. The behaviour of AgNPs in the environment, background concentrations of AgNPs, and also of bulk Ag in some environmental media, could represent additional sources of long-term and incremental exposure to both humans and biota.

Definition:

AgNP: *As used in this report, AGNP refers to preparations of engineered silver nanoparticles which adhere to the size-based definition of having at least one dimension in the order of less than 100 nm. When reading this document, it is important to understand that the general use of this term encompasses specific preparations which can display a range of characteristics and behaviours depending on physicochemical particle parameters and the test conditions.*
Where information is not specific to AgNP, the term silver (Ag) is used without the "nano" prefix.

Bulk silver: *To distinguish between the nanoscale silver and other forms of silver not adhering to the size-based definition of having at least one dimension in the order of less than 100 nm, the term "bulk" is used.*

2. Nanoparticles in the Environment

Naturally-occurring colloids ranging in diameter from 1nm to 1μm, now termed natural nanoparticles, have always been present as a key component in our ecosystem, with our ability to detect them only arising within the last century (Hood, 2004; Lead & Wilkinson, 2006). They are produced by natural processes, including microbial processes, sedimentation, natural combustion and environmental erosion (Simonet & Valcárcel, 2008).

The introduction and environmental impact of ENMs is, as yet, unknown. Existing knowledge of environmental and health effects of ultrafine particles, however, gives an indication of the potential effects ENMs could cause on ecosystems. For example, ultrafine particles in the atmosphere have been linked to detrimental health effects and environmental impacts (Maynard et al., 2006; Oberdörster et al., 2005; Stone et al., 2007; Xia et al., 2009). For the determination of exposure effects, potential toxicity, and bioavailability of any ENM, it is necessary to have information regarding concentration, presentation, persistence, and potential bioaccumulation in the environment. However, detection of ENMs in natural systems is not easily achievable due to current technical limitations and, indeed, the ability to discriminate against naturally-occurring NPs. To date, there has been no direct quantification of (i) naturally-occurring and /or (ii) released ENMs (Maynard et al., 2006; Mueller & Nowack, 2008; Som et al., 2010).

2.1 Air, Terrestrial and Aquatic System Risk

With industrial production of ENMs in increasing quantities, a predicted global market share for nanotechnology-enabled or containing products of 4% of general manufactured products in 2014, with ENMs in 100% of the PCs, in 85% of consumer electronics, in 23% of the pharmaceuticals and in 21% of the automobiles, not including agricultural or food industry applications as ingredients, preservatives or packaging, has been forecast. Given the vast potential use of these materials, and the high turnover of the consumer goods which they are incorporated into, both intentional and unintentional release can be anticipated into air, soil, and water (Meyer et al., 2010; Wiesner et al., 2006). Water, marine and aquatic environments are at risk from contamination due to runoff and drainage via sediments or disposal, while groundwater may be impacted through leaching or transport via mobile colloids. It is uncertain whether the potential environmental risks of ENMs may outweigh their established benefits. AgNPs released to air, soil or water could then be transported or transformed through chemical, physical, and biological processes. Presently, the fundamental properties governing the environmental fate of ENMs, in general, are not thoroughly understood.

2.1.1 Air

ENMs can potentially be introduced and suspended in air during manufacturing (synthesis, mixing, grinding, or agitation), distribution, or application (Grassian, 2009).

The potential introduction at point of disposal via incineration is also a concern. Factors influencing the fate of airborne particles in both indoor and outdoor environments include physicochemical properties, chemical characteristics, the nature of interactions with other airborne particles, residence time in the air, and distance travelled prior to deposition (Dankovich & Gray, 2011; U.S. Environmental Protection Agency, 2007). The fate of airborne ENMs can be further influenced by climatic factors, such as the magnitude of air currents, temperature and relative humidity (Navarro et al., 2008). It has been speculated that individual ENM particles will likely follow the laws of gaseous diffusion when released to air, with their diffusion rate inversely related to their diameter (U.S. Environmental Protection Agency, 2007). If this holds true, due to their high diffusion coefficients, ENMs based on particle diameter should be much more highly mobile than micrometre-sized (µm) particles (Aitken et al., 2004; Seaton et al., 2010). Particle aggregation, agglomeration and dispersion in air has been discussed in detail elsewhere in literature (Biswas & Wu, 2005; Dasgupta et al., 2009; Ma-Hock et al., 2007; Maynard et al., 2006; Seaton et al., 2010).

Diffusion is likely to occur according to a concentration gradient, from high-concentration to low-concentration zones. Single particles have short residence times in air due to their rapid diffusion, deposition to surfaces, and association with larger particles. In general, particles in the 100 nm to 10 µm range have the longest residence time in the atmosphere (Biswas & Wu, 2005). Longer residence time in the atmosphere allows more time for the particles to be mobilised by wind and climatic factors; therefore, atmospheric transport of ENMs over greater distance is possible (Wiesner et al., 2006).

Eventually, all particles in the ambient air are deposited or washed out to aquatic or terrestrial systems (e.g., soil and plants) (Mueller & Nowack, 2008). ENMs and AgNPs, which have been deposited, may further experience secondary transport within aquatic and terrestrial systems.

2.1.2 Terrestrial Systems

ENMs and AgNPs can be introduced into the terrestrial system during manufacturing, distribution, end-product use and end-of-life disposal. Indirectly, they may be transported into the terrestrial system via air or aquatic sources (Mueller & Nowack, 2008). End-of-life disposal via incineration, as mentioned previously, may also result in deposition on soil and plants. Solid waste landfill may also present deposition risk, with landfill sewage sludge potentially leading to AgNP release into subsoils and groundwater (Blaser et al., 2008a). The use of sewage sludge from wastewater treatment process is sometimes used as a fertiliser for agricultural soils (Blaser et al., 2008a). Therefore, AgNPs may be released into soil over a wide area. Runoff flowing along the ground surface could transfer ENMs in the sewage sludge to nearby terrestrial systems or aquatic systems (Blaser et al. 2008a; O'Brien & Cummins 2010). To date, information on the fate and transport of ENMs in terrestrial systems is limited within literature and regulatory reports, with no direct studies of silver yet reported.

2.1.2.1 Soil

ENMs' fate in soil will vary greatly, depending on their unique physicochemical properties and the complex characteristics of the soil environment. Climatic conditions are key factors in potential runoff, drainage and leaching (AFSSET, 2006). Due to their size range, ENMs are potentially mobile in soils (AFSSET, 2006). ENMs are small enough to migrate between soil particles, and the depth they migrate to before becoming trapped in the soil matrix has not been determined. Properties attributed to coating and surface modification of ENMs can lead to increased absorption to soil (O'Brien & Cummins, 2009; U.S. Environmental Protection Agency, 2007). Properties of the soil environment (e.g., soil type, soil organic matter, pH, ionic strength, presence of other pollutants) will also affect ENM transport.

2.1.2.2 Plants

Plants may be exposed to AgNPs (and ENMs in general) via air, water, and soil (Navarro et al., 2008). The extent of risk may depend on factors, such as leaf area surface, type of plant/tree (e.g., deciduous versus coniferous), season and evapotranspiration. Once on the foliage surface or presented at the roots, ENMs could be translocated to different tissues of the plant. The mobility of ENMs in pore water is an essential condition for interactions with plant roots or fungal hyphae (Harris & Bali, 2007). In the presence of certain organic compounds, ENMs will have improved mobility in soils, and could thus interact more efficiently with plant roots (Harris & Bali, 2007; Navarro et al., 2008).

2.1.3 Aquatic Systems

ENMs can potentially be introduced into the aquatic system by the same gateways as air - during manufacturing, product fabrication, distribution, end-product application and/or disposal (Grassian, 2009). Indeed, products containing AgNPs could be discharged into the municipal water supply unknown to the user, via household discharges. Sewage sludge (separated during the wastewater treatment process) is sometimes applied as a fertiliser to agricultural soils (Blaser et al., 2008a). Therefore, AgNPs might be released into soil via sewage sludge. Runoff flowing along the ground surface could transfer AgNPs in the sewage sludge to nearby waterways (Blaser et al., 2008a; O'Brien & Cummins, 2009). Overall, few studies are available on the fate and transport of AgNPs in natural aquatic systems. Information obtained from the literature provides a general description of the behaviour of ENMs in water and sediments, although information specific to AgNPs is limited. Aquatic organisms are highly susceptible to silver ion (Ag^+) toxicity in natural waters. The behaviour of AgNPs in water will strongly influence whether significant exposure and toxicity to aquatic organisms can be anticipated and predicted.

2.2 Environmental Factors affecting Exposure Risk

In terrestrial and aquatic systems, ENMs can be affected by abiotic factors including and not limited to (i) organic matter content, (ii) concentrations of ligands, (iii) pH and (iv) ionic strength. A further factor is the purity and composition of the ENMs upon release, as the presence of chemicals or substances used in manufacturing can modify these

interactions. Abiotic factors alter the chemistry that determines the extent of aggregation and agglomeration, and may ultimately influence toxicity (Cumberland & Lead, 2009; Handy et al., 2008a; Johnston et al., 2010; Wang et al., 2009).

(i) **Organic Matter**: Ag^+ readily binds to soil and particles. Organic matter (containing negatively-charged humic and fulvic acids) can also coat the surface of ENMs, resulting in particles that disperse rather than form clusters (Handy et al., 2008a,b). Steric repulsion effectively stabilises ENM suspensions and results in a remarkable drop in the rate of particle aggregation and agglomeration.

(ii) **Presence of Ligands**: Inorganic and organic ligands are commonly present in the environment (Choi et al., 2009). Ag^+ tends to bind readily to particulate matter (Blaser et al., 2008a; Luoma, 2008). Strong complexes with organic material predominate at the sediment surface, where oxygen is usually present and sulphides typically are not; in deeper sediments, where oxygen is absent, silver forms stable complexes with sulphide (Luoma, 2008). Ag^+ forms especially strong complexes with free thiol (-SH) ligands and with the sulphide ligands that are present in natural organic materials dissolved in water (Adams & Kramer, 1999).

(iii) **pH**: pH can change the surface charge of particles, thereby affecting the size distribution, and be causative of aggregation and agglomeration under specific conditions (Guzman et al., 2006). As the pH of the system increases, the number of negatively-charged sites in the system increases, elevating the potential for adsorption of the AgNPs to negatively-charged species (O'Brien & Cummins, 2009). The mobility of Ag^+ increases under conditions of increased acidification (Adams & Kramer, 1999; Luoma, 2008). The pH further influences the rate of aggregation and agglomeration, depending on the surface charge of the particles involved (Handy et al., 2008a,b).

(iv) **Ionic Strength**: The rate and extent of particle aggregation and agglomeration depend in part on ionic strength and ionic composition. In general, increasing ionic strength and the presence of divalent cations, such as calcium (Ca^{2+}) and magnesium (Mg^{2+}), increase the rate and extent of clustering, and can affect the stable size of the clusters formed (Cumberland & Lead, 2009; Handy et al., 2008b; Levard et al., 2012).

(v) **Water Hardness**: Is critical in determining rates of particle aggregation and agglomeration (Handy et al., 2008b). Hard water (as opposed to soft water) has a high mineral content, which is associated with water ionic strength. ENMs' surface charge effects on particle behaviour can be influenced by the concentrations of competing cations, such as Ca^{2+} and Mg^{2+}. It is conceivable that AgNPs' and ENMs' dispersion in aquatic systems may be controlled at local levels by free cation concentration (Handy et al., 2008a,b).

2.2.1 Factors Influencing Fate and Transport of AgNPs

Once released into the environment, AgNPs and ENMs would generally be expected to behave as follows (Luoma, 2008); (i) stay in suspension as individual particles, (ii) form clusters with other particles (and potentially deposit or undergo facilitated transport); (iii) dissolve in a liquid; or (iv) be chemically transformed, based on reactions with natural organic matter or other particles. The

distribution and fate of AgNPs within the environment depends on the physical and chemical processes that occur in the environment and the characteristics of the environmental system, as well as characteristics of the particles (Boxall et al., 2007).

2.2.2 Persistence

Although Ag can accumulate in water, sediments, soils, and organisms, Ag^+ ions and AgNPs are fundamentally different. Free Ag^+ ions can associate with other ions, but the ion itself is intrinsically persistent, although it can be converted to other ion species. In contrast, AgNP is not necessarily persistent. Dependent upon physicochemical parameters, AgNPs can dissolve or physically transform; they will not necessarily re-form, losing the properties of the primary particle. The persistence of AgNPs in aquatic systems has been demonstrated to be determined to some extent by dissolved oxygen (DO) levels (Liu & Hurt, 2010). Other factors, including pH levels of natural organic matter, have been shown to be linked with rates of decay. In deoxygenated water systems, however, dissolution of AgNPs is greatly inhibited. AgNPs engineered to have charged functional groups or surface coatings can remain dispersed. In general, particles that remain dispersed will tend to exhibit greater persistence in the environment than particles that are not treated (Luoma, 2008).

2.2.3 Particle Aggregation and Agglomeration

Translocation of AgNPs depends largely on their size - for this reason, clusters of AgNPs will behave quite differently compared to single AgNPs (Ma-Hock et al., 2007). Generally, aggregated or agglomerated AgNPs are less mobile (Nowack & Bucheli, 2007). The extent of clustering depends on the physicochemical properties of the particle, including particle shape, size, surface area, surface charge (measured within this work as zeta potential) and the characteristics of the environmental system (Handy et al., 2008a; Tiede et al., 2009b). Aggregation and agglomeration also affect the potential release of AgNPs from material products into environmental media (Wijnhoven et al., 2009). Coatings can be implemented to reduce aggregation and agglomeration, so that high surface reactivity can be exploited (Kandlikar et al., 2006).

Deposition refers to the settling of a mobile particle onto a land or water surface. AgNPs, and ENMs in general, which associate with dissolved materials or particles in the environment, will likely be deposited in sediments or soils (Luoma, 2008), although this outcome can be complicated by processes, such as dissociation or re-suspension, due to changes in aquatic or soil chemistry, and physical disturbance and activity of aquatic organisms. Deposition and sedimentation directly affect the concentration of bioavailable AgNPs, and ENMs in general, in the water column. Although these aggregated and/or agglomerated ENMs become less mobile, uptake by soil or sediment dwelling organisms or filter feeders is still conceivable (Nowack & Bucheli, 2007).

2.2.4 Transport, Fate and Exposure

Once ENMs are released into the environment, their transport is a critical factor in assessing their impact and ultimate fate in the environment. Generally, ENM transport on the molecular or particle scale is dominated by Brownian motion. Weaker forces, such as London or van der Waals, are responsible for attachment behaviours that ultimately determine particle mobility (Biswas & Wu, 2005). Transport or mobility potential of AgNPs is affected by the physicochemical characteristics, associated chemical agents from manufacturing or product formulation, and the environmental medium (O'Brien & Cummins, 2009; O'Brien & Cummins, 2010). The potential of the AgNP to adsorb onto a surface is influenced by its surface area, surface charge, degree of aggregation and agglomeration, and the presence of surface coatings (O'Brien & Cummins, 2009). Because of their high surface area-to-volume ratio and complex capability, AgNPs have the ability to adsorb biological and chemical agents, which alters their ability to be transported within systems, their solvent solubility and their bioavailability and persistence (Navarro et al., 2008).

AgNPs can also readily undergo transformation physically, chemically, or biologically, once released to the environment. Transformation can affect size, shape, and surface chemistry of the particles and their coatings, and this process will affect their ultimate distribution, persistence, and toxicity in the environment (Lowry & Casman, 2009). Transformation can lead to substances that present a very different hazard than does the untransformed material that was originally released (Hansen et al., 2008; Maynard, 2006). Particles can dissolve or disassemble, for example, which means they essentially transform, altering the properties of the initial particle (Luoma, 2008).

2.3 Key Environmental Factors affecting Natural Waters and Aquatic System Exposure Risk

Environmental factors influencing the dispersion and deposition behaviour of AgNPs and ENMs in general, include salinity (ionic strength), the presence of capping agents or surface coatings, pH, and water hardness (Gao et al., 2009). Typical aquatic environments, including rivers, lakes, and estuaries, contain monovalent and divalent salts, as well as organic matter (Saleh et al., 2008). Particles of all dimensions are more likely to associate as salinity increases (Luoma, 2008). Thus, ENMs will tend to aggregate and agglomerate to a greater degree in saltwater (owing to high ionic strength) than in freshwater (Klaine et al., 2008; Lowry & Casman, 2009). Even small increases in salinity above that of freshwater can cause a rapid loss of free particles through aggregation and precipitation processes (Stolpe & Hassellöv, 2007).

2.3.1 Particle Aggregation and Agglomeration

In water, or in any altered modified conditions from those at point of synthesis, the physicochemical properties of the AgNPs are altered (AFSSET, 2006). The formation of aggregates and agglomeration significantly affect the transport of particles in aquatic systems. Clusters of ENMs which settle can be expected to accumulate in sediments. Those that do not settle can travel without limitations in the water column

from the point of release (Lowry & Casman, 2009). AgNP aggregates and agglomerates are less bioavailable than free single AgNPs (Navarro et al., 2008). The formation of aggregates that are trapped through sedimentation affects the concentration which is bioavailable to organisms. Although aggregates, agglomerates, or adsorbed AgNPs have reduced mobility, they can still be taken up by sediment-dwelling animals or filter feeders (Nowack & Bucheli, 2007).

2.3.2 Surface Properties

The surface properties of AgNPs and ENMs, in general, are among the most critical determinants governing mobility and fate in aquatic systems. The settling rate of particulate in suspension depends on factors, such as particle size, density, and shape. Waterborne NPs generally settle more slowly than larger particles of the same substance. The high surface area-to-mass ratios enable NPs to have the ability to adsorb to sediment particles and become removed from the water column (Oberdörster et al., 2005a; U.S. Environmental Protection Agency, 2007). Surface properties of AgNPs govern their stability, potential mobility in solution as suspensions, and aggregates and agglomeration in aquatic systems (Navarro et al., 2008).

Modification of surface coating and altering physicochemical properties also affects water solubility and suspension characteristics. Surface coating of ENMs can occur at the point of synthesis by design, or can be acquired upon release to an environment by natural organic matter (Nowack & Bucheli, 2007). The interactions between AgNPs and natural organic matter influence particle fate and transport in aquatic systems. The formation aggregates and agglomerates by high-molecular-weight natural organic matter compounds favour precipitation of the particles into sediments, decreasing their bioavailability. Solubilisation by lower-molecular-weight natural organic matter compounds, however, might increase their mobility and their bioavailability (Navarro et al., 2008).

2.3.3 Ionic Ag (Ag^+) and Ag Complexes in Water

The mechanisms of action that govern toxicity of AgNPs or Ag^+ have not yet been defined. Indeed, the potential toxicities of AgNP preparations have not yet been determined, i.e., if they are solely due to 'nano' and their intrinsic properties (independent of ion), or if they are due directly to the release of Ag^+. Further to this, exposure effects may be attributable to a synergistic combination of both 'nano' and Ag^+ (Lubick, 2008; Navarro et al., 2008). The inhibitory effects of AgNPs, Ag^+, AgCl and $AgNO_3$ have been studied on microbial systems, indicating accumulation of AgNPs could detrimentally affect microorganisms within the exposure ecosystem (Choi et al., 2008). Choi et al. (2008) demonstrated experimental differences of AgNPs and Ag^+, where levels of inhibition of bacterial growth were measured at 86 ± 3% and 42 ± 7% respectively. Ag^+ has a strong tendency to associate with negatively-charged ligands in natural waters to achieve stability. Ligands can occur in solution, on particle surfaces, or on dissolved organic matter (Luoma, 2008).

The distribution of Ag^+ and silver complexes depends on the concentration of silver,

concentrations of the charged ligands, and bond strength between ligand and the Ag^+ (Blaser et al., 2008b; Choi et al., 2009; Luoma, 2008). A low level of total dissolved silver in water has been observed to remain as Ag^+, and forms other than Ag^+ are predominant in the aquatic environment (Blaser et al., 2008b; Luoma, 2008).

Ag^+ might also interact strongly with the chloride anion (Cl^-), although the nature of that reaction differs depending on freshwater or seawater. Concentrations of Cl^-, is typically low in freshwater systems. Reaction of Ag^+ with Cl^- produces AgCl, which in theory precipitates from solution under normal conditions. Dissolved sulphides, organic materials, and Cl^- will complex essentially all the Ag^+ in fresh waters, subject to concentration, reducing bioavailability and greatly reducing Ag^+ to very low levels (Luoma, 2008). In seawater, Cl^- occurs in very high concentrations, permitting saturation of Ag^+ by multiple Cl^-, forming dense complexes which remain in suspension. Sulphide complexes have also been found in seawater (Luoma, 2008).

2.4 Wastewaters

The particle aggregation and agglomeration, surface charge, and surface area of AgNPs, as well the broad spectrum of physicochemical attributes and properties, all contribute to the fate of AgNPs in wastewater (O'Brien & Cummins, 2009). At water processing and treatment facilities, water-loaded ENMs will undergo absorption processes and chemical reactions. Reports have detected concentrations of AgNPs entrapped in sludge flocs. Although wastewater treatment plants may remove a proportion of contained AgNPs and associated Ag^+, some silver will survive treatment and ultimately be discharged into water bodies. Further to this, sludge and floc from treatment processes could potentially release AgNPs into soil via sewage sludge, which is sometimes applied as a fertiliser to agricultural soils depending on regulations (Benn & Westerhoff, 2008; Blaser et al., 2008a). Runoff from ground water could potentially return AgNPs, or indeed any loaded ENMs, to nearby terrestrial systems or waterways (Blaser et al., 2008a; O'Brien & Cummins, 2009).

3. Experimental Setup

Literature and regulatory requirements support the need for the greater, more refined assessment of ENMs (Drobne, 2007; Handy, et al., 2008b; Hansen, 2010; Nowack et al., 2014). Studies on bacteria, algae, water fleas (*Daphnia magna*) and zebrafish (*Danio rerio*) have produced mixed results, depending on the ENMs and test procedures. Across these studies, the potential for harmful effects to occur from exposure have been highlighted and the need for further investigation demonstrated. *In vitro* data has indicated that surface structure and water solubility are important properties, with respect to interactions of ENMs and NPs with biological systems. In addition, synergism of the effects of NPs and metals or organic compounds has been suggested. With growing numbers of ENM type and application, a single risk assessment encompassing all potential exposures at environmental, human or biota level is impossible to apply across nanomaterials.

The focus of this project has been refined to examine a number of AgNP preparations, differing in physicochemical parameters. Risk and exposure assessment in both aquatic and freshwater model systems, expanding upon International Organisation for Standardisation (ISO), Clinical and Laboratory Standards Institute (CLSI) and Organisation for Economic Co-operation and Development (OECD) test protocols, have been conducted. In parallel to biological assessment, AgNPs have undergone physicochemical characterisation.

3.1 Physicochemical Properties of ENMs and AgNPs

Physicochemical properties must be considered and taken into account when evaluating the beneficial and potential toxicity of ENMs. These properties include specifically; shape, surface area, surface chemistry, dimension, and agglomeration in solution, see Figure 1 (Aillon et al., 2009; Gatoo et al., 2014; Stone et al., 2010). Although size can be the most distinguishing property, other unique physical and chemical properties begin to emerge as particles approach the nanometre size range (Auffan et al., 2009; Sayes & Warheit, 2009). Although the physicochemical properties differ across studies, some researchers have found that the effects of AgNPs can be similar to those produced by conventional ionic silver (Pal et al., 2007a), while other studies have demonstrated contrary findings showing that this is not the case (Griffitt et al., 2009). On the basis of such reports, it has become accepted that physicochemical characterisation is a necessary step in ENM characterisation and for furthering understanding of potential interactions. Physicochemical characterisation further permits comparison of ENM exposure studies, ensuring that like-for-like ENMs are compared and contrasted.

The identification and ranking of importance of individual physiochemical properties of ENMs, including AgNPs, for the prediction of their behaviour and interactions in the environment remains elusive. Indeed, the potential to associate behaviour and

interactions with single physiochemical properties may not be feasible due to the complex dependent nature these properties share. Recommendations for physicochemical characterisation are widespread in literature (Oberdörster et al., 2005a; Sayes & Warheit, 2009; Tiede et al., 2009a). A model proposed by Hassellov and Kaegi demonstrated in Figure 1, proposed criteria for reporting and characterisation of physicochemical properties (Hassellöv & Kaegi, 2009).

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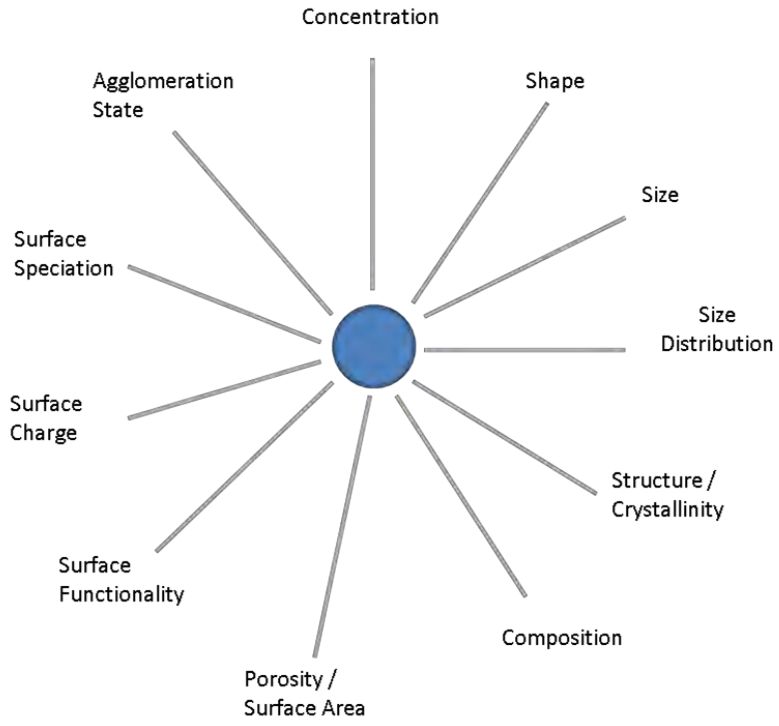


Figure 1: Physicochemical properties which are known to be factors in biological interactions and reactivity of a ENM, (adapted from Hassellov & Kaegi, 2009)

Standard physicochemical properties, briefly described as it relates to AgNPs, recommended to be characterised include:

(i) Size, including agglomeration and aggregation tendencies;
(ii) Morphology, including shape and crystal structure;
(iii) Surface area;
(iv) Chemical composition;
(v) Surface chemistry and reactivity;
(vi) Solubility;
(vii) Conductive, magnetic, and optical properties.

3.1.1 Size

Synthesis methods have been developed to produce monodisperse AgNPs of various shape and size distributions (Bar-Ilan et al., 2009). The size distribution of AgNPs, however, does not necessarily remain

constant and depends on the chemical and physical environment. AgNPs have been widely demonstrated to agglomerate or aggregate to form larger-sized clusters, which has consequences on the likelihood of release of silver ions, referred to as Ag^+ ions, from the particle and the particle's behaviour in the environment (O'Brien & Cummins, 2009). The dispersion state describes the extent to which particles become clustered by inter-particle attractive forces. Surface coatings and stabilising agents can enhance the stability of the dispersion state and maintain the original or intended size distribution. These phenomena can affect the fate and transport of AgNPs in the environment and in humans and biota. The release of AgNPs used in some products into the environment may occur either as individual AgNPs or as small clusters. It is also possible for AgNPs to be released from consumer products as composites or mixtures into the environment in an encapsulated form (Lowry & Casman, 2009). Persistence, accumulation and the translocation of AgNPs depend in part on their size, and if they have undergone agglomeration or aggregation (Ma-Hock et al., 2007; O'Brien & Cummins, 2009). Translocation of AgNPs may be more dependent on size alone, determining AgNP mobility in the environment and within the body (Fent, 2010; Liu et al., 2013), and enable cell penetration *in vitro* (Bar-Ilan et al., 2009; Morones et al., 2005).

3.1.2 Morphology, Shape

AgNPs can be synthesised into various forms, including particles, spheres, rods, cubes, truncated triangles, wires, films, and coatings (Pal et al., 2007b; Wijnhoven et al., 2009). AgNP shape can affect the kinetics of its deposition and transport in the environment. Depending on its surface structure and shape, different reactivity can be exhibited, as its shape could make it difficult for particles to approach each other (Oberdörster et al., 2005a). Such shape-related interactions can be controlled in some situations, by adding detergents or surface coatings to the particles to change their shape or surface charge (Handy et al., 2008b).

3.1.3 Surface Area

ENMs, due to their small size, have a greater specific surface area (SSA) when compared to the same mass of their bulk material, see Figure 2. This larger SSA of ENMs, relative to their mass or volume, increases their reactivity and sorption behaviour (Auffan et al., 2009; Tiede et al., 2009a,b). Increased SAA enhances chemical reactivity. Therefore, using AgNPs as an example, the smaller AgNPs have more reaction sites on their surfaces and are more sensitive to oxygen, a natural electron donor, than larger-sized particles (Auffan et al., 2009). Therefore, smaller AgNPs exhibit greater efficacy as biological agents or stressors in ecosystems or human health. Surface area also affects the ratio of silver ions (Ag^+) on the surface. This ratio may increase as particle size decreases. Therefore, it can be hypothesised that, for larger AgNPs with a smaller surface area-to-volume ratio, most of the Ag^+ might be unable to interact with the surrounding environment or on biological surfaces (Oberdörster et al., 2005a,b).

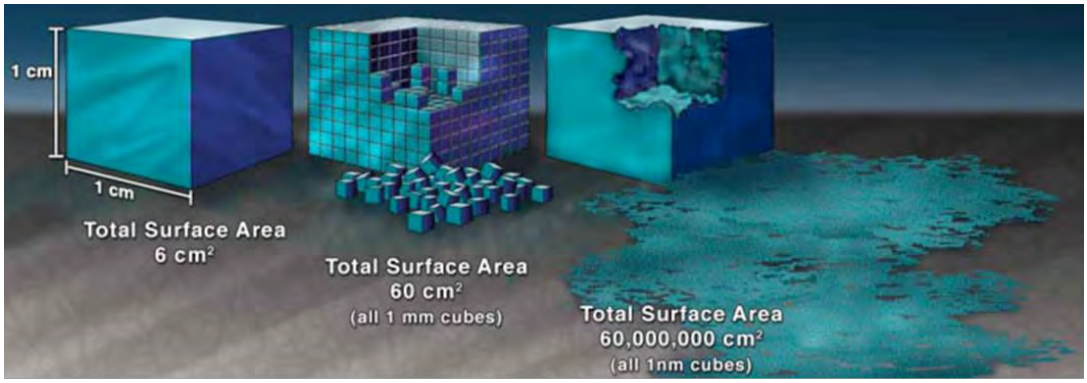


Figure 2: Illustration demonstrating size and specific surface area (SSA) from 'bulk' to nano size. Reproduced from http://www.nano.gov/nanotech-101/special with permission

3.1.4 Chemical Composition

Silver can exist in four oxidation states: Ag^0, Ag^+, $Ag^{2+,}$ and Ag^{3+}; free silver ion under natural conditions is Ag^+. Of these, Ag^0 and Ag^+ are the most commonly-occurring oxidation states in the environment. Existing literature demonstrates that forms of silver that release free Ag^+ ions are more toxic in environmental and human studies than other forms of silver that do not (Wijnhoven et al., 2009). Speciation would therefore strongly influence the quantity of silver available to affect living organisms. Positively-charged Ag^+ ions will associate with negatively-charged ligands, typically sulphide in freshwater and chloride in saltwater (Luoma, 2008). Surface coating used to stabilise the AgNPs in solution, to prevent agglomeration, or to add functionality to the AgNPs, is an important factor in chemical composition (Cumberland & Lead, 2009; Loza et al., 2014; Sayes & Warheit, 2009).

3.1.5 Surface Chemistry and Reactivity

The larger surface area and surface charge of AgNPs correlate with the availability of more possible reactive sites and greater chemical reaction potential, adsorption potential, and potential to aggregate or agglomerate. These properties can, in turn, affect the transport properties, behaviour, interactions, distribution, bioavailability, and effects of AgNPs in the environment (Loza et al., 2014; O'Brien & Cummins, 2009; Wiesner et al., 2009). Surface charge also influences particle stability in dispersions and overall solubility (Hassellöv et al., 2008; Sayes & Warheit, 2009). The coatings applied on AgNPs' surfaces consist of various chemical compositions, often a surfactant, polymer, or polyelectrolyte, which influence particle behaviour and persistence (Handy et al., 2008b; Lowry & Casman, 2009; Tiede et al., 2009a). Coatings can impart charge, either positive or negative, and can stabilise ENMs against clustering and deposition (Nowack & Bucheli, 2007; Wiesner et al., 2006).

3.1.6 Solubility, Dissolution

Solubility influences the fate and behaviour of ENMs in the environment, and also the dissolution rate and release of ions (Auffan et al., 2009; Wijnhoven et al., 2009). Metallic conventional silver is insoluble in water, while silver salts, including silver nitrate ($AgNO_3$) and silver chloride ($AgCl_2$), have

varying degrees of solubility, depending upon temperature. Cumberland and Lead (2009) reported approximately 1% dissolved silver when AgNPs, averaging ~13 nm, were added to a solution containing humic substances and sodium or calcium to mimic environmentally-relevant conditions. Other reports have suggested that total AgNP concentrations are approximately 10 times greater than the soluble AgNP concentration at 25°C in 0.22 μM filtered water (Griffitt et al., 2009). A substance's solubility or degradability generally tends to be inversely related to persistence in environmental conditions. Persistent materials that are slow to dissolve might be more available to biological systems, depending on the fate and transport of the substance in the environment.

Therefore, determination of solubility is key in prioritising risk location, be it point of manufacturing and discharge or downstream exposure. Life-cycle degradation is also associated with solubility. Rate of dissolution can be considered proportional to particle surface area; therefore, EMNs should dissolve faster than larger conventional materials, if considering surface area alone (O'Brien & Cummins, 2009). Particle concentration, surface morphology, surface energy, clustering, and other properties, however, are also relevant when considering dissolution.

3.1.7 Conductive, Magnetic, and Optical Properties

AgNPs have been studied, characterised and utilised for their optical properties extensively over the past two decades. AgNPs, like other noble metals, interact strongly with electromagnetic radiation, resulting in unique conductive, magnetic, and optical properties. Silver exhibits the highest surface plasmon resonance (SPR) band among all metals (Evanoff & Chumanov, 2005; Wijnhoven et al., 2009). A high SPR means that the electrons on the surface of a particle are highly interactive with electromagnetic fields, and as the surface plasmons resonate, the energy can be detected, quantified, and, in the case of silver, seen in the visible spectrum. These properties enhance the application and use of AgNPs in biomolecular labelling and in sensor technologies. Literature has demonstrated that these properties strongly depend on AgNP size, shape, spatial ordering, composition, and surface properties (Evanoff & Chumanov, 2005; Henglein, 1998).

3.1.8 Analytical Methods for Physicochemical Characterisation

Accurate analytical methods can aid in the understanding of the behaviour and properties of AgNPs. Point of characterisation is important for cross-study comparison and to understand AgNP behaviour. During exposure assessments, characterisation in environmental media, under exposure conditions, can allow for improved characterisation of exposure and their resulting impacts. The ability to monitor AgNPs in various media, however, is not straightforward. Although only the measurement of physicochemical properties, that are relevant from a risk assessment or risk management perspective, might be desired, the mechanistic understanding of biological effects of ENMs is still evolving, and the list of associated physicochemical

properties is still not completely understood or mapped. Furthermore, because physicochemical properties of AgNPs are dynamic and highly dependent on the surrounding media, instrumentation for characterisation in various environmental conditions could be useful (Tiede et al., 2009b). Techniques available to perform physicochemical characterisation have been reviewed elsewhere (Hassellöv et al., 2008; Maynard et al., 2006; Powers et al., 2006; Sayes & Warheit, 2009; Tiede et al., 2009a,b).

3.2 Physicochemical Methodologies for Laboratory Research

Approaches applied within this report at experimental level, used at onset and during assessment, are as follows:

a. UV-Vis spectrometry and Dark-field microscopy applied to indicate the distribution of size and shape, nanoparticle tracking.
b. Microscopy techniques such as Scanning Electron Microscopy (SEM), Atomic Force Microscopy (AFM) and Transmission Electron Microscopy (TEM) applied to study the measurement of size distribution, shape, surface area, sample heterogeneity and morphology, agglomeration/aggregation state, determination of concentration, and biological interaction upon exposure.
c. Dynamic Light Scattering (DLS) to study the size distribution and agglomeration/aggregation state.
d. Scanning Electron Microscopy with energy-dispersive X-ray spectroscopy (SEM-EDX) to determine chemical composition.
e. Surface charge, using a zetasiser to determine zeta potential.
f. Atomic Absorption Spectrometry (AAS) and Fourier Transform Infrared Spectroscopy (FTIR) for chemistry elemental analysis and determination of concentration.
g. Brunauer, Emmett and Teller (BET) analysis to determine surface area.

Detecting AgNPs (or any ENMs) in the environment, particularly the natural environment, is difficult due to current analytical limitations regarding detection at environmentally-relevant concentrations and the inability to distinguish natural materials in the nanoscale size range from ENMs (Domingos et al., 2009; Garza-Ocañas et al., 2010; Simonet & Valcárcel, 2008). Further to this, preparation for analytical analysis requires sample treatment and solvent stages, which consequently alter the samples by causing clustering and salt precipitation (Simonet & Valcárcel, 2008). Detecting nanoparticles in water or soil is further complicated by the heterogeneous nature of the sample matrix and the agglomeration and aggregation tendencies of the AgNPs.

3.3 Model Systems

3.3.1 Hydractinia echinata, Marine Invertebrate Model System

Hydractinia echinata (H. echinata), a colonial marine Cnidarian, is an established model organism for developmental biology and comparative immunology, and has also been widely used in toxicity testing and teratogenicity testing of pharmaceuticals. Hermit crab shells encrusted by colonies of *H. echinata* were collected from Kilkerin Bay, Carna, Co. Galway. Cultures are established in the laboratory of Dr. U. Frank (Martin Ryan Institute, NUI Galway).

H. echinata is maintained in seawater and fed daily with brine shrimp nauplii. Eggs can be collected daily after light-induced spawning. Embryonic development lasts three days, terminated by reaching the stage of a planula larva. The planula represents an already complex pattern of different cell types. The larva enters metamorphosis upon a well characterised exogenous stimulus. Within 24 hours, a primary polyp forms that will bud new polyps asexually. Sexual maturation is reached within two-three months. The morphology of the adult polyp can be deduced from the polar organisation of the larva. Exogenous compounds, as well as factors isolated from other cnidarians' tissue, have been demonstrated to affect patterning during metamorphosis. Thus, part of the larval tissue may be capable of regulation by external signals. They may be exposed to NPs through uptake of dissolved compounds, ingestion of food, or contact with suspended solids and sediments. In addition to AgNPs effects, bio-accumulation could also affect predators. The development and metamorphosis of *H. echinata* has been well studied. *H. echinata* tissues can be very lipid-rich, which allows for rapid uptake and storage of lipophilic contaminants. NP charge may therefore be a factor in their internal distribution.

3.3.2 Bacterial Systems

A model organism can be described as a non-human species that is studied to understand biological workings, with the prospect of discoveries providing knowledge into the functioning of other species (Fields & Johnston, 2005). The use of bacterial test systems permits the use of fast, relatively inexpensive and simple systems for ecotoxicity testing, permitting extrapolation for wider exposure scenario. Testing under environmentally-relevant and associated conditions presents a distinct advantage over using *in vitro* mammalian cell testing systems, which are used under a false condition, leading to results which may not be transferable to outside laboratory conditions. As the number of ENMs and ENPs continues to increase at a rapid rate, with little exposure data for a large proportion, such rapid testing may be utilised as initial screening tools.

This work utilises both (i) standardised test bacteria for susceptibility testing (CLSI), *Escherichia coli* and *Staphylococcus aureus,* and (ii) two members of the marine *Vibrio* species, *fischeri* and *harveyi,* from which ISO 11384 bioluminescence cytotoxicity assays are based. The broad nature of the bacterial systems applied here permits a number of assessment endpoints to be examined and also permits inter species response variation to be noted, while permitting Gram-positive and Gram-negative to be taken into account.

3.3.2.1 Escherichia coli

Escherichia coli, E. coli, is a gram-negative, rod-shaped bacterium, facultative anaerobe, which has the ability to grow in the presence or absence of oxygen, and has an optimal growth temperature of 37°C. *E. coli* is the most widely-studied organism for a number of reasons. It is easily cultured, fast growing, and has a comparatively large genome, which has since been sequenced. It is commonly found in the gastrointestinal tract of humans and many other animals. Most strains of the bacterial are harmless and are beneficial to the host organism, by preventing colonisation of unfavourable pathogens (Hudault et al., 2001). However, although a lot of strains are harmless, there are others that are pathogenic, which have been implicated in diarrhoea in infants and other infections in older patients, both demographics who suffer from weaker immune systems. This, in conjunction with its ubiquitous presence in the environment, makes *E. coli* an important model organism for many studies, both medical and environmental. It was one of the first models used in molecular biology and is still used as a model organism for gram-negative species of bacteria. Gram-negative bacteria, such as *E. coli*, or Salmonella species, often display a greater innate tolerance to exposure to chemical agents (antimicrobial compounds) (Russell, 1999). *E. coli* DSMZ 1103 was obtained as a lyophilised culture (Deutsche Sammlung von Mikroorganismen und Zellkulturen, Germany). This strain, also recorded as ATCC 25922, NCIB 12210 or WDCM 00013, is a referenced control strain recommended by the CLSI for susceptibility testing of novel compounds.

3.3.2.2 Staphylococcus aureus

Staphylococcus aureus, S. aureus, is an aerobic or anaerobic, non-motile, non-spore-forming, catalase- and coagulase-positive, gram-positive coccoid bacterium. It is a member of the commensal flora of the skin, nose and mouth in approximately 20% of humans, and is widespread in the environment (Kluytmans et al., 1997). It is an extremely important causative agent in a huge number of human infections. As a leading cause in hospital-acquired infections, this makes the microorganism a medically and environmentally-important model species. Strains of methicillin resistant *S. aureus* (MRSA) are common in hospitals throughout the world. Its perceived prevalence may be due to increased numbers, or simply increased awareness as a notifiable organism. Penicillin was one of the first treatment routes for treating infections from *S. aureus* infections. However, resistance in *S. aureus* for penicillin is extremely high, so other drugs were employed to treat it, the first being methicillin. Resistant strains for methicillin were clinically found, approximately two years after its introduction (Jevons et al., 1963; Jevons & Parker, 1964). Today, many *S. aureus* strains are resistant to beta-lactamase antibiotics (currently the most widely-used drugs in hospitals) (Fung-Tomc et al., 1991). Agents, including antimicrobials, often have different effects on Gram-positive organisms, compared to effects upon Gram-negative organisms (Russell, 1999). *S. aureus* DSMZ 2569 was obtained as a lyophilised culture (Deutsche Sammlung von Mikroorganismen und Zellkulturen, Germany). This strain, also recorded as ATCC 29213 or WDCM 00131, is a referenced control strain recommended by

the CLSI for susceptibility testing of novel compounds.

3.3.2.3 Vibrio species

The suitability and appropriateness of using bioluminescent marine bacteria to examine the potential toxicity of ENMs and ENPs remains to be validated, although as these species exist within nature and may therefore experience direct or indeed first contact with environmental agents, this suggests that they should be considered as a bio indicator. *V. harveyi*, a free living bioluminescent marine bacterium, has shown to have high levels of bioluminescence comparable to *V. fischeri* (Thomulka & Peck, 1995). These micro-organisms have the added advantage in that they can be cultured quicker at more ambient temperatures between 25-30°C, due to their mesophilic nature, and the fact that they are less fastidious. The applicability of using *V. logei*, another marine bioluminescent bacterium, in the toxicity assay has also been investigated elsewhere (Girotti et al., 2002). In this study, as well as using *V. fischeri* as a test organism (an OECD- and ISO-established species for water), a concurrent evaluation of toxicity towards *V. harveyi* has been performed.

Luminescent marine bacteria have different requirements according to their environmental niche conditions in which they naturally reside. As bioluminescence is directly related to metabolic state, it is paramount that optimal growth conditions are maintained for optimal bioluminescence levels. Factors such as temperature, salt concentration, and pH have direct effects on both growth and bioluminescence (Waters & Lloyd, 1985). Therefore, conditions for growth and bioluminescence will be optimised for both *V. harveyi* and *V. fischeri*, comparisons made and findings interpreted. The half maximal effective concentration (EC_{50}) value will be used to assess toxicity levels. This value is seen as the universal measurement in toxicity studies. It represents the minimum concentration of test substance needed to affect 50% of the population. The Inhibitory effect (INH) is reported as the percentage inhibition of bioluminescence. Once a test system has been used to generate a concentration range over which NPs are toxic, the likelihood of exposure to these concentrations must be considered to evaluate the risks associated with toxicities.

3.3.3 Danio Rerio (Zebrafish), Freshwater System

A preferred species for aquatic studies, developmental biology and ecotoxicology, *Danio rerio* (Zebrafish), has been utilised in laboratory settings since the 1950s (Detrich et al. 2004). The use of zebrafish within this study was supported by a growing body of information including:

(i) Greater than 2,100 records for zebrafish in the ECOTOX database (www.epa.gov/ecotox/)

(ii) Their development is rapid; three days after fertilisation, the embryo is essentially complete, with a functioning heart, circulatory and nervous system. Further to this, they have a life cycle of three-four months and produce large numbers of eggs (up to 200 per female) all year round. Mating takes place at the onset of dawn and is therefore easy to time. The embryonic development of zebrafish is very

well documented. This rapid development is comparable to three months of human development.

(iii) Its genome is one of the first vertebrate genomes to have been fully sequenced and annotated (www.ensembl.org/Danio_rerio). This enables a transcriptomic approach, utilising validated commercially-available arrays encompassing the entire transcriptome.

The zebrafish is a well-established test organism with NUI Galway, which has permitted a large number of molecular techniques, expertise and information to be built up. Recognised as an optimal organism for assessing mechanistically-oriented ecotoxicology in higher organisms, as a teleost, it represents half of all vertebrate species known. Its use is recommended in the Organisation for Economic Co-operation and Development (OECD) guidelines (OECD 1992)[9].

[9]ENV/JM/MONO(2012)16; OECD. Environment, Health and Safety Publications. Series on Testing and Assessment No. 171

4. Exposure Assessment

4.1 Fate and Transport Prediction Models

Current models applied for environmental exposure scenarios are not appropriate for use in predicting AgNPs' (or ENMs in general) fate and transport throughout environmental compartments. There exists a need for combining and adapting models of dispersive and convective atmospheric models (particles and gases) with models of fate and transport of chemicals and particles in surface waters and soils. Such a combined model may, in the future, be adapted for predicting environmental fate and transport of AgNPs, Ag^+ and associated complexes (Mueller & Nowack, 2008). Recently, Blaser et al. (2008a and 2008b) modelled the fate and transport of Ag^+, in the Rhine River, to assess potential risks from European use and disposal of plastic and textile consumer products containing AgNPs to municipal wastewater supplies. The application of such modelling and prediction requires the ability to include additional forces, including aggregation states and rates of clustering, interaction and absorption with ligands and natural particles, and to extend to encompass physicochemical parameters. Evaluation and model outcome validation by comparing model outputs with measured (laboratory-determined) values is currently a challenging task, due to the questionable reliability and sensitivity of analytical methods in detecting ENMs at environmentally-relevant concentrations, and under exposure conditions (Petosa et al., 2010; Stone et al., 2010; Tiede et al., 2009b).

For the immediate term, the optimal assessment of ENM exposure to living systems, and extrapolation into environmental scenarios and risk determination remains to be a laboratory-based assessment. To ensure uniform assessment of exposure, the condition/state of the ENM preparation must be known, a controlled biological model type is to be utilised under fixed, standardised conditions, and a stringent study design and adherence to standardised experimental protocol are required to be established.

4.2 Physicochemical Analysis; NP Synthesis and Characterisation

All glassware used for synthesis was treated with aqua regia solution (3 parts HCl to 1 part HNO_3) and washed thoroughly in analytical grade water. All chemicals and reagents were purchased from Sigma-Aldrich (Poole, UK), unless otherwise indicated. The choice of stabilising agents was determined, by ensuring the representation of the most utilised methods of stabilised preparations commercially available. Initial exposure testing to the stabilising agents used within this study demonstrated no observational effect, with respect to the concentrations used. This assessment was undertaken to ensure any AgNP exposure effect reported could be attributed to the presence of ENMs.

Silver nanoparticle (AgNP) stock solutions were synthesised using silver seed-catalysed reduction of Ag^+ (Fournet et al.,

2009). Briefly, 1.25 mM trisodium citrate, 500 mg/L poly sodium-4-styrene sulphonate (PSSS) and 0.3 mM sodium borohydride were stirred at a rate of ~800 rpm, and 0.25 mM silver nitrate ($AgNO_3$) was added at a rate of 10 mL/min. The seeds were allowed to age for four hours prior to use in the growth step. Two sizes of AgNPs were synthesised from the growth step, identifiable optically as red (spectral peak wavelength (λ_{max}) ranging 504 to 555 nm) and blue (λ_{max} ranging 667 to 745 nm), made with 10 mL and 2.5 mL of seed respectively. Seeds were mixed with 225 μM ascorbic acid under vigorous mixing; 450 μM $AgNO_3$ was then added. A stabiliser was then added, as detailed in Table 1. In total, eight unique preparations were synthesised.

Table 1: Stabilisation of Synthesised AgNPs

Ag-NP preparation	*Stabiliser*	*Final Concentration*	*Method of Stabilisation*
TSC (TSC_1, TSC_2)	Trisodium citrate	1.25 mM	Electrostatic
PVP (PVP_1, PVP_2)	Poly(vinylpyrrolidone)	0.1 w t%	Polymeric/Steric
BIO (BIO_1, BIO_2)	Gelatin	0.1 w t%	Biopolymeric/Steric
Thiol (Thiol_1, Thiol_2)	16-Mercaptohexacanoic Acid	10 mM	Thiol/Electrostatic

Two preparations of different diameter of each preparation have been produced to permit sizing affects to be considered labelled 1 and 2 respectively.
(mM, millimolar; wt%, percentage weight)

Six commercial AgNP preparations were purchased (Sigma-Aldrich, Poole, UK; PlasmaChem GmbH, Germany and BBI Life Science, Cardiff, UK). Commercially-supplied materials in powder forms were made into aqueous solutions by sonication. All preparations were made up to a working concentration of 50 ppm, which was used for all initial characterisation. Concentrations greater than 50 ppm were prepared by ultracentrifugation, when required. AgNPs' Characterisation UV/Vis absorption spectra of the preparations were acquired by using an USB2000 (Ocean Optics, USA) and a Helios alpha spectrophotometer (Thermo Unicam, UK). Direct size measurement and morphology assessment was performed using a Hitachi H7500 Transmission Electron Microscope (TEM). Hydrodynamic radius, size distribution, polydispersity (defined as a relative width of the size distribution) and aggregation state were determined by dynamic light scattering (DLS) measurements. Zeta potential (ζ) measurements were taken using a Zetasiser Nano (Malvern, UK). Surface area measurements were also determined. Physicochemical properties, including changes observed when held in filter sterilised egg water (FSEW), are detailed in Table 2. Observed changes in physicochemical aspects are discussed during exposure scenarios for each of the model systems.

4.3 Hydractinia echinata (Marine Model) Exposure

4.3.1 Animal Collection and Maintenance

Hermit crab shells encrusted by colonies of *H. echinata* were collected from Kilkerin Bay, Carna, Galway. The crabs were removed

from the shell. A number of hermit crabs were not removed from their shells; this was to facilitate symbiotic interaction. The animals were cultured in both (i) filtered natural seawater and (ii) artificial seawater. Both water types were kept at a constant temperature of 18°C and changed each day to ensure a constant salinity of 34 parts per trillion (ppt) and pH 8.2 – 8.4. Light exposure was kept constant at a 14:10 hours light hours:dark regime. The animals were fed once daily with *Artemia salina*. The *Artemia* cysts (ZM Systems, UK) were cultured in seawater at 18°C for 48 hours in a hatchery flask (ZM systems, UK), with constant aeration prior to feeding. Animals were washed one hour after feeding, using an airline or pasteur pipette to clean away any residual or digested food. The feed was supplemented with the addition of a fish and prawn mixture twice weekly. Fish (salmon, haddock and cod) and prawn were blended with ice until they reached a slurry consistency, and stored at -20°C in 50 mL tubes until needed. 10 ml of this supplement was then added to the *Artemia* and mixed prior to feeding. This also acted as a food source for the hermit crabs.

4.3.2 Animal Husbandry

Spawning and egg collection: *H. echinata* spawning is light-induced. Each morning, when the lights turned on at 08:00hrs, the animals were removed from their tanks and separated into dishes to collect eggs. Eggs were collected, washed in seawater, and placed in groups of 50 per petri dish, to permit hourly microscopy examination for selection of fertilised and also deformed egg removal. Eggs were incubated in seawater at 18°C. Every day for three days, the water was changed in the dishes and the developing eggs were cleaned. The light:dark cycle was kept constant, as for mature animals.

Induction to metamorphose: 72 hours post fertilisation (hpf), the larvae are artificially induced to metamorphose, using 116 mM Cesium chloride (CsCl). A stock solution of 560 mM CsCl is prepared (0.9428 g of CsCl in 10 mL of distilled water) which is diluted one part CsCl stock solution to four parts seawater and incubated for three hours at 18°C. Post incubation, the CsCl solution is removed, and filtered seawater used to wash the newly-developed primary polyps - washing is repeated three times. The animals were then transferred from the petri dish onto a glass microscope slide in a fresh petri dish to permit colonisation.

Culturing: Healthy animals adhered to the glass slides and developed to form colonies. Incubating seawater was changed daily, and light conditions remained constant. After five days of growth, daily feeding of artemia commenced.

Table 2: Physicochemical properties of AgNPs under synthesis and under Zebrafish test exposure conditions

NP Preparation Identifier	Source	Form	Concentration	MDD	TEM	DLS	DLS under test condition Freshwater (FSEW)	Shape description	Zeta potential	Zeta potential under test condition Freshwater (FSEW)
				diameter	*diameter*	*diameter*	*diameter*			*(Temperature and pH)*
			ppm (mg/L)	(nm)	(nm)	(nm)	(nm)		(mV)	(mV)
TSC_1	synthesised	suspension	50	N/A	46 ± 5	56.84	94.2	colloid	-15.1	-33.2
TSC_2	synthesised	suspension	50	N/A	110 ± 15	148.6	187.4	colloid	-16.2	-38.6
PVP_1	synthesised	suspension	50	N/A	52 ± 12	74.1	92.3	colloid	-10.2	-3.62
PVP_2	synthesised	suspension	50	N/A	140 ± 12	182.4	248.3	colloid	-12.7	-6.7
BIO_1	synthesised	suspension	50	N/A	48 ± 6	64.1	108.2	colloid	-19.4	3.36
BIO_2	synthesised	suspension	50	N/A	155 ± 17	180.7	260.8	colloid	-23.4	3.69
Thiol_1	synthesised	suspension	50	N/A	53 ± 2	84.2	102.3	colloid	-27.7	-32.2
Thiol_2	synthesised	suspension	50	N/A	108 ± 9	122.2	144.7	colloid	-28.3	-39.6
Si_1	Sigma- Aldrich, *Cat No 576832*	powdered	N/A	<100	78 ± 24	91	297.2	colloid	-27	-21.3
Si_2	Sigma- Aldrich, *Cat No 484059*	powdered	N/A	<150	204± 38	225.3	312.1	colloid	-16.4	-14.7
PL_1	PlasmaChem, *PL-Ag150*	powdered	N/A	100-150	140 ± 23	151.2	212.7	colloid	-19.3	-12.8
PL_2	PlasmaChem, *PL-AgW200*	powdered	N/A	N/A	4± 4 (w); 181 ± 16 (l	N/A	N/A	rod	-17.6	-11.2
BBI_1	BBI Life Science, *Cat No. EM.SC40*	suspension	N/A	40	42± 2	58.2	124.1	colloid	-21	-18.5
BBI_2	BBI Life Science, *Cat No. EM.SC80*	suspension	N/A	80	77± 8	101.3	184.6	colloid	-25.1	-19.5

NP, Nanoparticle; MDD, Manufacturer Determined Diameter upon release; TEM, Transmission electron microscopy; DLS, Dynamic light scattering FSEW, filter sterilized egg water.

4.3.3 Exposure to AgNPs

H. echinata larvae were exposed to concentrations of AgNPs ranging from ppb to ppm. AgNPs used throughout the exposure were characterised for their physicochemical parameters, in this case also in relation to seawater conditions, with high Cl^- being a contributing factor to stability, persistence, aggregation and accumulation.

Exposure larvae groups consisted of 5-10 animals (this varied according to the number of animals available at each time point). The exposures took place prior to inducing the animals, so the effects on early development could be monitored, and a more dramatic effect should be observed at this developmental stage. The animals were kept in the exposure and control solutions for three hours. Initial exposure concentration range of 100 to 0.01 ppm was examined. After the three-hour exposure, the larvae were washed three times with 0.22 µM filtered seawater and induced to metamorphose, as described in Section 4.3.2. The animals were then allowed to develop under the standard conditions (temperature and light cycle). Development was monitored by stereomicroscopy, to note any deviation from normal development, using unexposed animals as a control. All exposures were performed in triplicate to permit statistical analysis. Abnormal development was observed for all exposure groups, however, this was also noted for the healthy, non-exposed groups. Unfortunately, upon repetition, this was observed routinely, though with lesser and greater differences across the animals. Neither exposed nor healthy animals survived until they were capable of spawning. This resulted in no data being directly associated with exposure, with regards to the development of the *H. echinata groups*.

4.3.4 Histochemistry

In parallel with initial exposure studies, the examination of *H. echinata* by microscope approaches was undertaken, to permit the observation of cellular uptake, dysregulation and potential effects of AgNP exposure. Initially, *H. echinata* were fixed, after seven days of growth, on glass cover slips. *H. echinata* were relaxed before fixation, by placing them in 4% $MgCl_2$ (2 g of $MgCl_2$ in 25 ml filtered seawater and 25 ml double-distilled fresh water) for 30 minutes. To ensure the animals were ready for fixation, a

pipette was used to disturb the water, and response rates were noted by visual observation. The *H. echinata* were ready to be fixed when their response was slow and they appeared relaxed. After 30 minutes of $MgCl_2$ incubation, a wash was performed in 50:50 filtered seawater:freshwater, after which 4% paraformaldehyde (PFA) solution was added to cover the *H. echinata.* After 60 minutes at room temperature, the PFA solution was removed and the *H. echinata* washed three times with phosphate buffered saline (PBS) before being stored in the PBS solution. The petri dishes were covered, sealed with parafilm, and stored in a refrigerator at 4°C for staining.

H. echinata were stained with both 4',6-diamidino-2-phenylindole dihydrochloride (DAPI) and Acridine Orange (AO) in parallel, and also with AO separately. DAPI is a blue fluorescent nucleic acid stain which binds to DNA. It is a popular nuclear stain, as its bright blue fluorescence stands out when used with other red, yellow, or green fluorescent dyes that stain other cell components.

AO stains al nucleic acids (both DNA and RNA), and permits the comparison of images for effects of exposures or stresses for all types of DNA damage, including cellular. AO-stained DNA fluoresces at 525 nm and stained RNA fluoresces at >630 nm. It can also be a useful stain for measuring apoptosis. Some apoptotic cells can have increased plasma membrane permeability to fluorescent dyes. AO can cross through the plasma membrane of apoptotic cells, and when viewed under a fluorescent microscope, early-stage apoptotic cell nuclei fluorescence green and later-stage apoptotic cell nuclei fluoresce red.

AO staining protocol: 10 mg/mL of AO in distilled water stock solution was made and stored in the dark at 4°C. To make the stain, 4.5 mL of 0.1 M citric acid and 0.5 mL of sodium phosphate were added to 30 µL of the stock solution of AO. The staining protocol was modified to optimise staining from a number of progressive rounds, based upon fluorescent microscope results. The *H. echinata* were then washed three times with TBS-T, and gently shaken for five minutes. The AO stain was added for two minutes, washed with TBS four times and shaken for five minutes. The cover slip was then attached to the glass slide using glycerol. The glass slides were then stored in darkness to avoid any bleaching of the stain.

DAPI and AO double-staining protocol: The fixed *H. echinata* on cover slips were washed with Tris Buffered Saline Tween-20 (TBS-T) three times for five minutes. They were then treated with ImageiT block for 30 minutes to reduce background signals, by stopping any non-specific binding of the staining agents. This was then washed with TBS-T four times and shaken for five minutes. 1% Bovine Serum Albumin (BSA) in TBS was then added for 30 minutes, as a further non-specific binding blocking agent. The *H. echinata* were again washed twice with TBS-T as before, followed by two washes with TBS to remove any unbound BSA. The DAPI stain was then added, and incubated for 40 minutes, followed by AO staining for two minutes. The animals were washed with TBS as before, and the cover slip was then coated with ProLong Gold anti-fade and attached to the glass slide using

glycerol. The glass slides were then stored in darkness to avoid any bleaching of the stain.

Analysis and visualisation was then performed under standard fluorescent and confocal microscopes, as demonstrated in Figure 3 showing the *H. echinata* tentacle (a, b), stolon (c, d), a budding polyp (e, f), a stolon tip in greater detail (g) and a full animal (h). With confocal microscopy, it is possible to view individual layers of the sample by using spatial filtering to exclude any unfocused light, an advantage over standard approaches. The instruments used were an Olympux IX71 Inverted Fluorescent Microscope and a Zeiss LSM 510 Axiovert Inverted Confocal Microscope equipped with Millenia V Tsunami Multiphoton Laser.

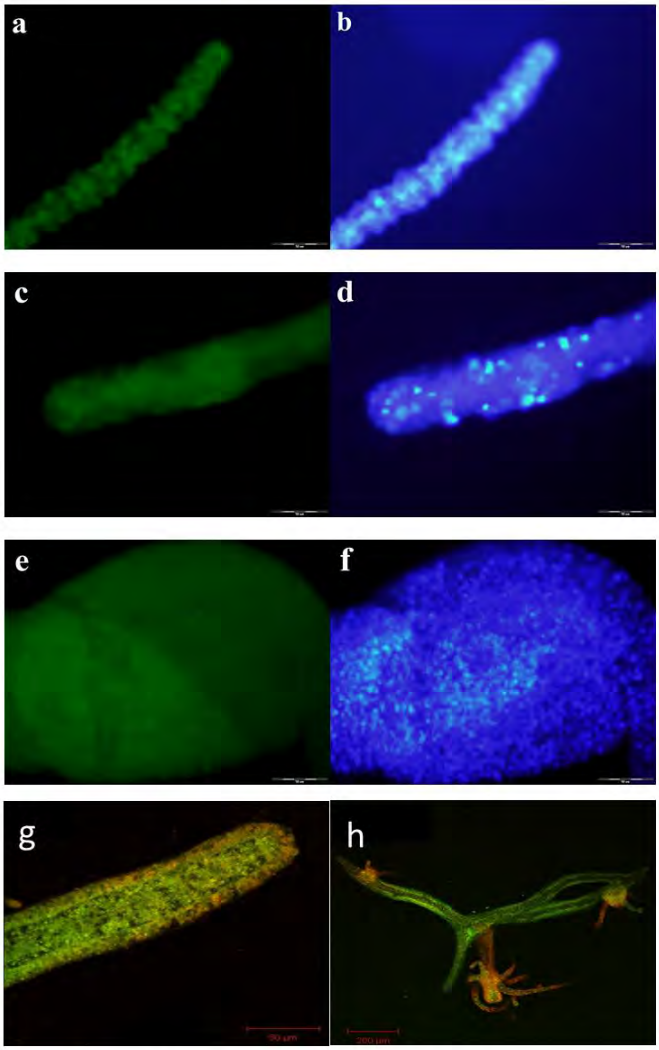


Figure 3: Double staining DAPI and Acridine Orange.
a, c, and e show AO stained nuclei, b, d, and f show DAPI stained nuclei . a) and b) tentacle; c) and d) stolon; e) and f) budding polyp.

AO stained H. echinata under confocal microscope. g) Stolon tip stained with AO; h) H. echinata with budding polyps stained with AO.

4.3.5 H. echinata - A Model System for assessment of Environmental and Biological Impact

Within this work, the application and potential use of the invertebrate model system has been assessed and demonstrated to be of future potential use. The requirement for complete understanding, behaviour and management of the system remains to be fully elucidated as a control model. It is anticipated that a model system, such as the *H. Echinata,* could provide a valuable tool in model environmental exposure scenario. Extensive development of this model system is ongoing within the research group of Dr. Uri Frank, NUI Galway[10]. Further assessments using this system are planned to be performed, again testing AgNPs, and also a range of ENMs which have been highlighted across the literature.

4.4 Bacterial Systems

A range of studies have established the bactericidal effect of silver in the nano range in Gram-negative and Gram-positive bacteria, but the mechanism of this effect has not been elucidated. A number of research groups have monitored the antibacterial activity of AgNPs in Gram-negative bacteria (*E. coli*, *V. cholera*, *P. aeruginosa* and *S. tiphy)* and Gram-positive bacteria (*B. subtilis, S. aureus* and *E. faecalis*), with some agreement across studies; i.e., the attachment of AgNPs to the surface of the cell membrane, leading to the disturb function, penetration of the bacteria and the release of Ag^{+}. A number of variables and comparisons could not be directly compared with across these studies, due to differences in experimental design and setup, conditions, assay type and the characterisation and profiling of the AgNPs themselves - areas which are addressed within the experimental design carried out within this work.

4.4.1 E. coli and S. aureus AgNP Exposure

4.4.1.1 Bacterial Strains, Media, Silver

Lyophilised cultures were resuscitated as per manufacturers' guidelines. Strains were prepared for long-term storage, using the Protect™ Beads Bacterial Preservation System (Technical Service Consultant Ltd., Heywood, UK). For retrieval of strains, Protect™ stocks were initially thawed at 4°C and then at room temperature for 10 minutes. A single latex bead was removed from the plastic cryovial using a sterile syringe needle (BD Microlance™, BD, Spain), and transferred to a 30 mL glass universal containing 20 mL of Mueller Hinton Broth, MHB (Oxoid, Basingstoke, UK) for each bacteria. The culture was incubated at 37°C, shaking at 120 rpm for 18 hours. Cultures were centrifuged (5000 x g, 10 min) and the pellet washed three times in a sodium phosphate buffer, pH 7.0. Cultures were standardised to an optical density (OD) of 0.1 (625 nm: Thermo Spectronic Heλios Є spectrophotometer, Madison, WI, USA) using sterile water. This suspension is referred to as "0.1 McFarland" standard. A second standard culture was prepared at an OD_{625nm} of 0.5, producing a '0.5 McFarland' standard.

MHB was prepared and sterilised in accordance with the manufacturers' guidelines (MH: Oxoid, UK). Prepared MHB had cation levels of 4 mg/L Ca^{2+} and 5.36

[10] http://www.nuigalway.ie/frank/

mg/L Mg^{2+}. A modified Muller Hinton Broth (MHB) (National Committee for Clinical Laboratory Standards, 2000) was also prepared, supplemented with Mg^{2+} and Ca^{2+} divalent cations, with the aseptic addition of $CaCl_2$ and $MgCl_2$ subsequent to autoclaving. All salts were purchased from BDH (Poole, UK). Cation levels were brought to the NCCLS recommendation; $Ca^{2+,}$ 10-12.5 mg/L; Mg^{2+}, 20-25 mg/L. Nutrient agar (NA) was prepared and sterilised in accordance with the manufacturers' guidelines (NA: Oxoid, UK).

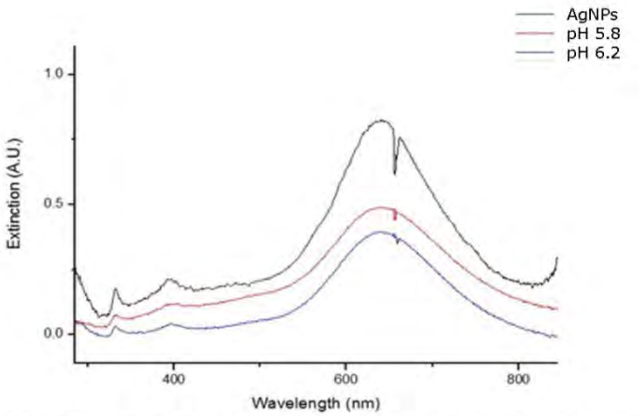


Figure 4: AgNP preparation UV-Vis spectral changes with pH variation from 5.8 to 6.2

Before following MIC protocols, as detailed by the CLSI and OECD, AgNPs were examined for their stability and the potential alternations which they may undergo during bacterial studies.

Initially, effects such as media pH were examined and found to be minimal on the effect of stability, as determined by UV-Vis spectral analysis (Figure 4). From this, the standardised media for CLSI testing, MHB was assessed. As demonstrated in Figure 5, CLSI standard routine testing protocol is not suited to the testing and determination of ENMs, with special reference to silver. A quick rapid analysis utilising UV-Vis indicates that the AgNPs undergo significant change upon exposure to the MHB test media. This is subject to the physicochemical properties of the ENMs and is associated with the surface coating used in the synthesis of the particles. Indicated in Table 1 are the stabilisers which were used throughout this work, also referred to in literature as 'capping agents'. Utilising an ion selective electrode, dissolution was seen to be dramatic, with an immediate release of Ag^{+} upon the introduction of AgNPs into the MHB. A gradual decrease in free Ag^{+} was observed, suggesting the formation of complexes within the media – interacting with ligands present. These assumed complexes could be reverted to free Ag^{+} upon acid treatment. Upon dilution of media, thereby reducing concentration of components, dissolution was observed to decrease, and when a 1:32 dilution was reached, no dissolution and a relative stability of the AgNPs was observed (Figure 5). It is noteworthy that differences in stability were related to both surface coating/stabiliser and also the ζ-potential. Aggregation within MHB media was not observed.

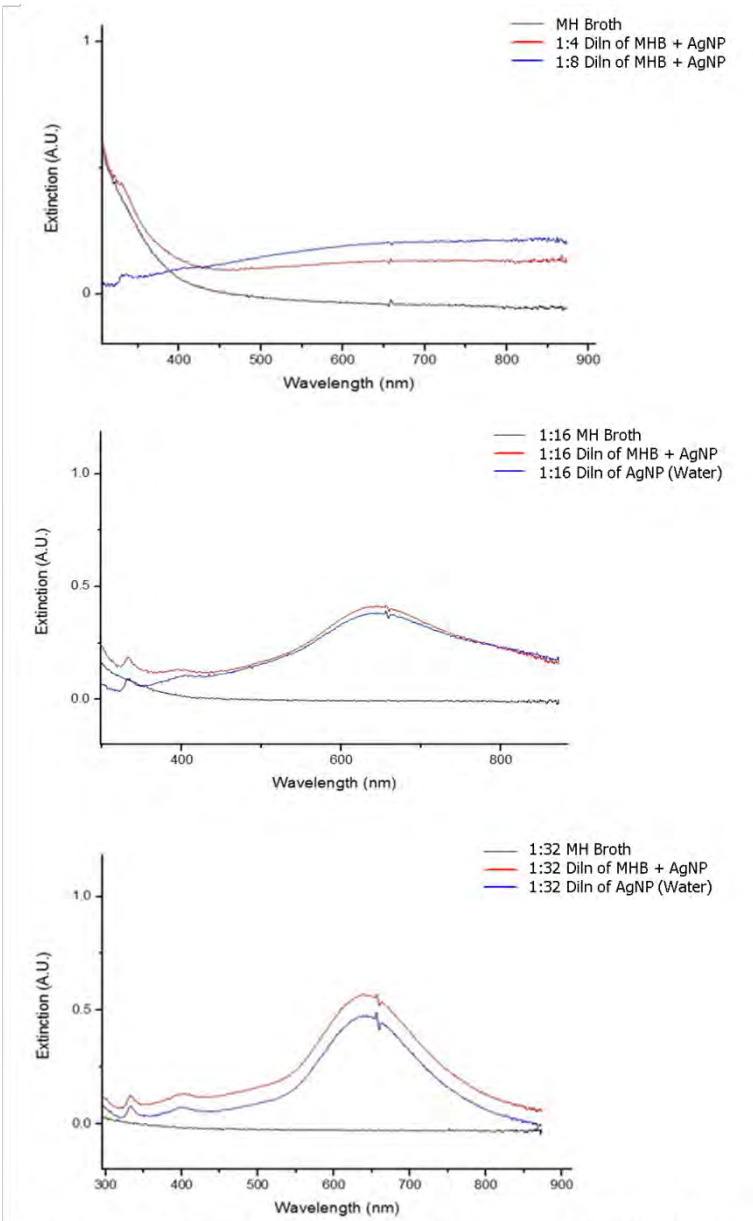
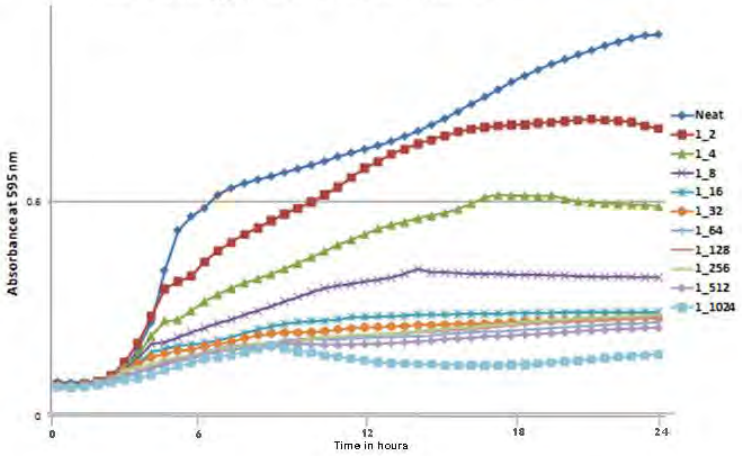


Figure 5: Effect and role of media on AgNP stability while undergoing exposure testing, rapid assessment by UV-Vis spectrum analysis. Identification of potential risk of applying existing testing protocols to novel materials

Dilution assessment indicated dilutions of 1:16 and greater were compatible with AgNPs not causing a dissolution or reaction with the media itself prior to bacterial load assessment. From this, an assessment and check for suitable bacterial growth were performed to see if a modified, diluted media analysis could be undertaken to determine minimum inhibitory concentrations (MICs) and bacteriostatic properties of AgNPs. The results, provided in Figure 6, show the potential of both test bacteria, *E. coli* and *S. aureus,* to grow satisfactorily, permitting OD_{595} readings, and for growth curves to be plotted. However, this was not observed at the required dilutions for testing to be performed. An alternative approach using M9 Minimal Medium was then investigated (Figure 7).

(A) E. coli growth profile in MHB with dilutions up to 1:1024. Growth recorded at OD_{595nm} over a 24 hour period



(B) S.aureus growth profile in MHB with dilutions up to 1:1024. Growth recorded at OD_{595nm} over a 24 hour period

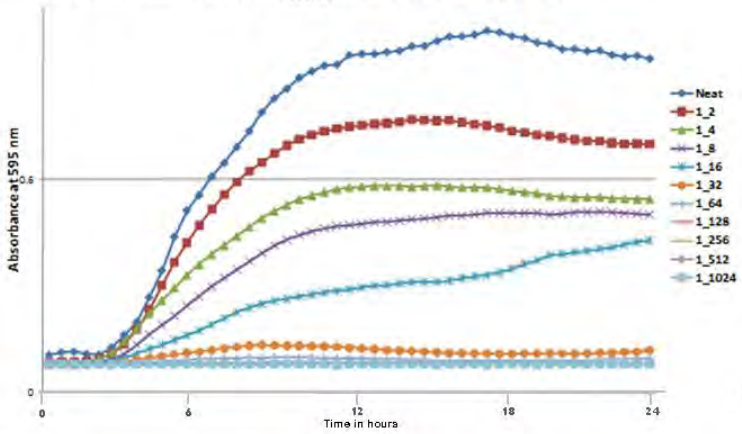


Figure 6: Monitored bacterial growth for (A) *E.coli and (B) S.aureus* in diluted preparations of Muller Hinton Broth (MHB), *as* required for the stability and integrity of the AgNP. Optical Density, OD, monitoring of growth profile was performed in a dilution range up to 1:1024, and measured at OD_{595nm} over a 24 hour period.

M9 Minimal Medium consists of M9 salts and supplements, each of which were prepared individually and combined aseptically after autoclaving (Maniatis et al., 1982). All salts were purchased from BDH (Poole, UK). Chemicals were of AnalaR grade. A five-fold concentration of M9 salts was prepared, with the addition of 6g sodium hydrogen-orthophosphate ($Na_2H_2PO_4$), 3g potassium di-hydrogen orthophosphate (KH_2PO_4), 0.5g sodium chloride (NaCl) and 1g ammonium chloride (NH_4Cl) to distilled water to give a volume of 1 L. Supplements added to 1 L final volume; (i) 2 ml of 1M Magnesium sulphate ($MgSO_4$), (ii) 10 ml of 20% (w/v) glucose or (iii) 0.1 ml 1M Calcium Chloride ($CaCl_2$). A further preparation of M9 with casamino acids M9 media was prepared as before, with the exception of the addition of 2 g/L of casamino acids (Difco, France), data shown in Figure 7.

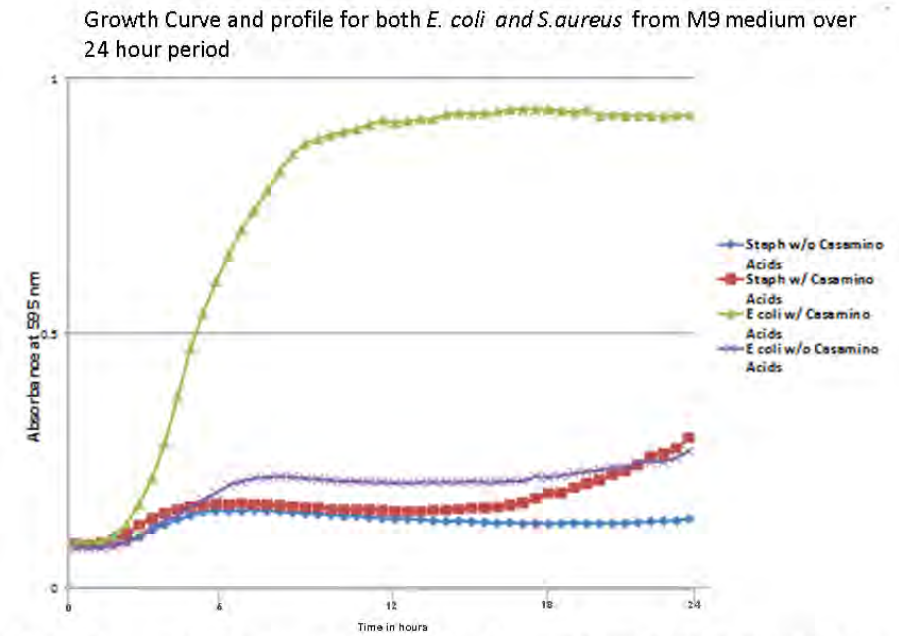


Figure 7: Bacterial growth for *E.coli and S.aureus* in M9 medium, measured at OD_{595nm} over a 24 hour period.
S. aureus did not grow to a significant turbidity (OD) in the presence of casamino acids (with, w/), and failed to grow in their absence (without, w/o), potentially auxotrophic. *E. coli* grew poorly in the low nutrient media (in comparison with MHB, demonstrated in Figure 5) in the absence of the supplemented Casamino acids., though sufficient growth was observed in the supplemented M9 medium.

It was determined through the UV-Vis analysis, dissolution measurement and growth experiments described above, that a classical MIC susceptibility testing was not a suitable method for determining the antimicrobial properties of the test nanoparticles.

A modified Minimum Inhibitory Concentration (mMIC) was designed and undertaken in the following section.

4.4.1.2 Bacterial culturing, susceptibility testing and analysis

Exposure testing: AgNPs, ion donor, and bulk metals are used, as characterised in Section 4.2, for initial physicochemical parameters. Subsequent analysis in media and under exposure conditions were performed in parallel.

Growth curves were determined using a 96-well format (GENios, Tecan, UK). Briefly, 5 µl of 0.1 McFarland standard of *E. coli* or *S. aureus* were used to inoculate 300 µl of medium. Media used for growth curve determinations included MHB, cation-adjusted MHB, M9, and M9 with supplemented casamino acids. The medium was previously diluted with the addition of sterile water to give doubling dilutions of the full-strength medium. Growth curves were carried out in the presence of a negative control, whereby the sterile medium was left un-inoculated. Cultures were incubated at 37°C. OD_{595} measurements were taken over the course of 24 hours, shaken for one minute, and then allowed to settle for five seconds before each reading.

A total viable count (TVC) was performed to determine the viability of bacteria, in both before and after exposure scenarios, as well as standardising and validating McFarland cultures. For the determination of a TVC, a solution containing bacteria was serial diluted and aliquots plated out and incubated overnight at 37°C. Total colony forming units (CFUs) were determined after 24 hours. Bacterial susceptibility to the silver-based materials (AgNP, ion donor, or bulk, as discussed in Section 4.2) was determined for *E. coli* and *S. aureus*.

A modified Minimum Inhibitory Concentration (mMIC) assay was performed in one of two ways:

(i) Susceptibility testing on a multi-well plate format. Here, exposure concentrations of 0.1, 1.0, 10, 25 and 50 ppm of each silver sample were prepared in 10 mM PBS (pH 7.6). 50 µl of 0.5 McFarland of the test bacterial strain was added to all wells, with the exception of the negative control, which contained no bacteria. A positive growth control, which contained no silver, was positioned on every plate. The plate was incubated at 37°C overnight and TVCs were determined after 24 hours, with changes in bacterial numbers in response to silver observed. All tests were performed in triplicate, with data presented in Table 3.

(ii) Susceptibility and exposure duration. Here, exposure concentrations of 0.1, 1.0, 10, 25 and 50 ppm of each silver sample were prepared in 10 mM PBS (pH 7.6) and 1.2 mL aliquots placed into 2 mL micro-centrifuge tubes (Sarstedt, Ireland). 0.3 mL of 0.5 McFarland of the test bacterial strain was then added to each silver preparation. A positive control, which contained no silver, and a negative control containing no bacterium, were used throughout. The tubes were incubated at 37°C, with TVCs carried out at two, four, six, and 24 hours, to calculate any drop and potential recovery in bacterial numbers in response to silver, with data presented in Table 4.

Table 3: Representative Total Viable Counts (TVC) of two AgNP preparations, ionic and bulk forms

	Total Viable Count (TVC) of Bacterial spp at 24 hours				
		E. coli		*S. aureus*	
	ppm	***T= 0***	***T= 24***	***T= 0***	***T= 24***
TSC_1	0.1	4×10^7	1.2×10^7	4.1×10^7	1.1×10^6
	1	4×10^7	3.2×10^6	4.1×10^7	2.2×10^3
	10	4×10^7	*N.G.*	4.1×10^7	*N.G.*
	25	4×10^7	*N.G.*	4.1×10^7	*N.G.*
	50	4×10^7	*N.G.*	4.1×10^7	*N.G.*
PVP_1	0.1	4×10^7	1.1×10^6	4.2×10^7	2.1×10^6
	1	4×10^7	2.4×10^5	4.2×10^7	2.3×10^4
	10	4×10^7	*N.G.*	4.2×10^7	*N.G.*
	25	4×10^7	*N.G.*	4.2×10^7	*N.G.*
	50	4×10^7	*N.G.*	4.2×10^7	*N.G.*
Ionic Ag^+	*0.1*	4.0×10^8	2.4×10^8	4.1×10^7	2.9×10^7
	1	4.0×10^8	1.4×10^7	4.1×10^7	2.5×10^5
	10	4.0×10^8	1.7×10^2	4.1×10^7	*N.G.*
	25	4.0×10^8	*N.G.*	4.1×10^7	*N.G.*
	50	4.0×10^8	*N.G.*	4.1×10^7	*N.G.*
Bulk Ag	*0.1*	4.1×10^8	1.4×10^8	4.2×10^8	4.0×10^8
	1	4.1×10^8	2.0×10^7	4.2×10^8	2.4×10^8
	10	4.1×10^8	1.7×10^6	4.2×10^8	1.7×10^7
	25	4.1×10^8	5.4×10^5	4.2×10^8	2.2×10^6
	50	4.1×10^8	2.0×10^5	4.2×10^8	1.2×10^6
N.G.= No Growth Observed					
T = Time in hours					

Table 4: Susceptibility and exposure duration effects of two AgNP preparations, ionic Ag^+ and commercial AgNP preparation

		E.coli					*S.aureus*				
	ppm	***T= 0***	***T= 2***	***T=4***	***T=6***	***T= 24***	***T= 0***	***T= 2***	***T=4***	***T=6***	***T= 24***
TSC_1	0.1	-	1.1×10^7	2.1×10^5	7.1×10^4	3.1×10^3	-	1.8×10^7	8.9×10^6	1.6×10^6	1.4×10^4
	1	-	*N.G.*	*N.G.*	*N.G.*	*N.G.*	-	1.9×10^7	5.1×10^6	3.2×10^5	2.6×10^2
	10	-	*N.G.*	*N.G.*	*N.G.*	*N.G.*	-	*N.G.*	*N.G.*	*N.G.*	*N.G.*
	25	-	*N.G.*	*N.G.*	*N.G.*	*N.G.*	-	*N.G.*	*N.G.*	*N.G.*	*N.G.*
	50	-	*N.G.*	*N.G.*	*N.G.*	*N.G.*	-	*N.G.*	*N.G.*	*N.G.*	*N.G.*
	Inoculum	2.2×10^7	-	-	-	4.3×10^7	2.1×10^7	-	-	-	3.9×10^7
PVP_1	0.1	-	1.5×10^7	5.2×10^6	2.1×10^4	2.4×10^3	-	1.9×10^7	6.5×10^6	4.4×10^6	5.2×10^4
	1	-	*N.G.*	*N.G.*	*N.G.*	*N.G.*	-	1.3×10^7	5.1×10^6	1.8×10^5	1.2×10^3
	10	-	*N.G.*	*N.G.*	*N.G.*	*N.G.*	-	6.3×10^5	4.2×10^4	3.2×10^4	*N.G.*
	25	-	*N.G.*	*N.G.*	*N.G.*	*N.G.*	-	8.2×10^3	1.2×10^3	8.2×10^2	*N.G.*
	50	-	*N.G.*	*N.G.*	*N.G.*	*N.G.*	-	1.8×10^2	1.2×10^1	347	*N.G.*
	Inoculum	2.2×10^7	-	-	-	5.2×10^7	2.1×10^7	-	-	-	4.2×10^7
Ionic Ag+	0.1	-	1.2×10^8	4.2×10^8	4.0×10^8	1.1×10^7	-	1.2×10^8	4.0×10^7	6.2×10^7	6.0×10^6
	1	-	1.2×10^4	1.2×10^4	1.6×10^2	2.8×10^6	-	3.6×10^6	1.2×10^5	8.0×10^3	1.3×10^1
	10	-	*N.G.*	*N.G.*	*N.G.*	*N.G.*	-	*N.G.*	*N.G.*	*N.G.*	*N.G.*
	25	-	*N.G.*	*N.G.*	*N.G.*	*N.G.*	-	*N.G.*	*N.G.*	*N.G.*	*N.G.*
	50	-	*N.G.*	*N.G.*	*N.G.*	*N.G.*	-	*N.G.*	*N.G.*	*N.G.*	*N.G.*
	Inoculum	1.6×10^7	-	-	-	1.7×10^8	1.4×10^7	-	-	-	1.6×10^8
Co. AgNP	0.1	-	1.4×10^8	1.8×10^8	4.1×10^7	1.2×10^5	-	1.0×10^7	1.0×10^7	1.2×10^7	6.0×10^7
	1	-	4.3×10^7	1.1×10^8	1.8×10^7	1.1×10^6	-	1.0×10^7	1.0×10^7	1.0×10^7	8.0×10^6
	10	-	*N.G.*	*N.G.*	*N.G.*	*N.G.*	-	6.0×10^3	4.0×10^1	6.0×10^3	1.1×10^1
	25	-	*N.G.*	*N.G.*	*N.G.*	*N.G.*	-	6.0×10^1	*N.G.*	*N.G.*	*N.G.*
	50		*N.G.*	*N.G.*	*N.G.*	*N.G.*	-	*N.G.*	*N.G.*	*N.G.*	*N.G.*
	Inoculum	1.8×10^8	-	-	-	4.0×10^8	1.3×10^7	-	-	-	4.0×10^7
N.G.= no growth observed											
T = Time in hours											

4.4.2 Marine Bioluminescent Test Organisms (V. fischeri and V. harveyi)

V. fischeri (DSM 7151) (previously named *Aliivibrio fischeri*) and *V. harveyi* (DSM 2165) were obtained as vacuum-dried culture from DSMZ. Bacterial strains were rehydrated and cultured under sterile conditions, according to manufacturers' instructions initially.

V. fischeri was cultured in marine broth containing 5 g/l peptone, 1 g/l yeast extract, 0.406 mM Fe ($C_6H_5O_7$), 332.8 mM NaCl, 61.96 mM $MgCl_2$, 21.24 mM $MgSO_4$, 16.22 mM $CaCl_2$, 7.38 KCl, 1.9 mM $NaHC0_3$, 672 nM KBr, 214 nM $SrCl_2$, 356 nM H_3BO_3, 32.8 nM Na_2SiO_3, 57.2 nM NaF, 20 nM NH_4NO_3, 56.4 nM Na_2HPO_4, pH 7.6 ± 0.2 at 16°C, with shaking at 90 rpm on a platform shaker. *V. harveyi* was cultured in LB 2% NaCl at 30°C, with shaking at 120 rpm, overnight. Growth was monitored at OD_{600nm} using a spectrophotometer. Bacterial cultures were harvested when they had reached late-exponential phase at OD_{600nm} within the range 0.8-1.0. For continued use, glycerol stocks (20% w/v) were prepared following two centrifugation (2 min, 16,110 x g) and two washing steps with fresh medium,

followed by re-suspension in 20% w/v glycerol and held at -80°C.

All industrial and commercially-produced ENMs are coated with polymeric stabilising agents, such as those listed in Table 1. ENMs' surfaces can be passivated to prevent degradation by agents, such as Mercaptohexadecanoic acid (MHA). Gelatin has been presented as a biocompatible coating which can be biodegraded, and is under investigation as a potential coating in drug delivery systems (Gaihre et al., 2009; Tseng et al., 2013). In order to see effects of stabilisers on AgNPs, while assessing their contribution to exposure design, the effects of NaCl and stability were determined for AgNPs, as detailed in Table 1 and Table 2. As indicated in Figure 8, the role of stabilisers is significant in terms of presentation of the AgNPs. The test AgNPs were exposed to a range of NaCl from 0.05% to 2.5%. Across the four different stabilising agents shown, a dramatic decrease in the spectral reading of the TSC-coated AgNPs can be observed for concentrations above 0.5 mM. A red shift can be observed for both TSC- and PVP-coated AgNPs, upon the introduction of any salt. This is demonstrative of the changes which require greater profiling and understanding with regards to environmental release.

4.4.2.1 Toxicity assay optimisation

Single colonies of each strain were inoculated into 25 mL volumes of 1% NaCl LB, 2% NaCl LB, marine broth and ISO standard media (0.5 g/L yeast extract, 5 g/L Caso-peptone, 32.57 mM glycerol, 513 mM NaCl, 6.5 mM NaH_2PO_4 H_2O, 12 mM $K_2H_2PO_4$ $3H_20$, 7.32 M $MgSO_4 \cdot 7H_2O$, 4.42 mM NH_4PO_4) and incubated at respective incubation conditions (30°C, 120 rpm for *V. harveyi* and 16°C, 90 rpm for *V. fischeri*). After 24 hours of culturing, OD_{595nm} readings were taken and appropriate volumes were sub-cultured into fresh medium to give a starting OD_{595nm} of 0.01. These fresh cultures were incubated for 20 minutes to achieve a homogenous culture.

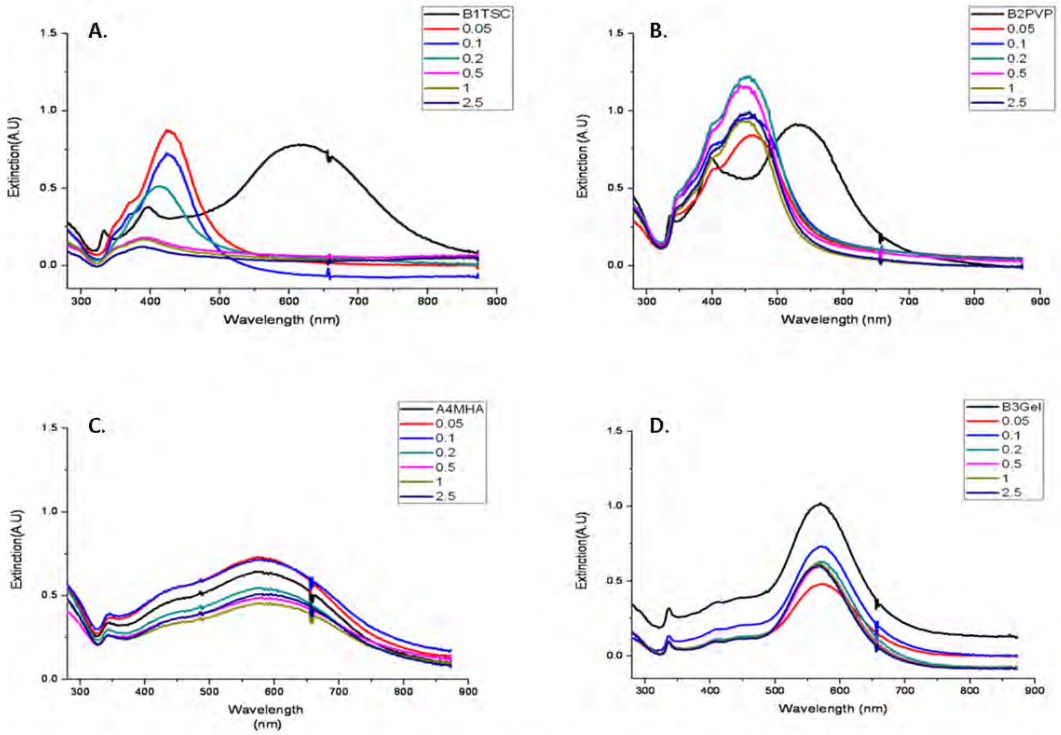


Figure 8: UV-Vis spectra of TSC (A), PVP (B), MHA (C) and Gelation (D) coated AgNPs in increasing concentrations of NaCl.

Flat-bottom, transparent cell culture (Sarstedt) and white, luminometer (Corning) 96-well plates were prepared, with 200 µl aliquots of the cultures in each well, in triplicate on each plate. OD_{595nm} readings and luminescence measurements were taken every 30 minutes, with shake duration of 15 seconds, and allowing 30 seconds settling time prior to each reading. Kinetic cycles were carried out over an 18-hour time period. Kinetic parameters were found to be identical for both OD_{595nm} readings and bioluminescence measurements, with the exception of temperature control. The 25 ml cultures from which the 200 µl aliquots were taken were further incubated at respective temperatures and under constant shaking. OD_{595nm} readings and bioluminescence measurements were taken every 30 minutes for the initial 330 minutes of growth, and then subsequent readings were taken after overnight growth, until a stationary phase was reached for all cultures. This allowed a more representative profile of the growth kinetics and corresponding bioluminescence levels in culture, which are often difficult to determine when in 96-well plate format. The optimum media used to culture the bacteria was selected, based on highest achievable levels of bioluminescence over a sustained period corresponding to a particular growth phase.

4.4.2.2 Bioluminescent-based toxicity assay

As a starting point, standard test protocols (ISO 11348-1, 2, 3) [11] were used as guidelines to evaluate the use of a luminescent-based toxicity assay for determining NP toxicities. The bioluminescence-based toxicity assay was

[11] http://www.iso.org/iso/home/search.htm?qt=11348&sort=rel&type=simple&published=on

performed in a flat-bottomed, 96-well luminometer microplate (Corning) at room temperature, using a Spectrafluor+ plate reader (Tecan). Luminescence readings were taken after five-minute exposures and every 2.66 minutes thereafter, for a period of 60 minutes. Luminescence intensity was expressed in 'relative light units (RLUs)'. All samples in the assays were in triplicate and each assay was carried out in triplicate.

Standard solutions of zinc sulphate heptahydrate ($ZnS0_4.7H_20$) (Fischer Scientific) and potassium dichromate ($K_2Cr_2O_7$) (Sigma Aldrich) were prepared in a 2% w/v NaCl solution, at concentrations of 439.6 mg/L and 9.84 mg/L respectively. Serial dilutions were preformed to generate a concentration range (439.6 mg/L, 219.8 mg/L, 109.9 mg/L, 54.95 mg/L, 27.475 mg/L, 13.7375 mg/L, 6.86875 mg/L) for $K_2Cr_2O_7$ and (9.84 mg/L, 4.92 mg/L, 2.46 mg/L, 1.23 mg/L, 0.615 mg/L, 0.3075 mg/L, 0.15375 mg/L) for $ZnSO_4.7H_2O$. These referenced standards acted as assay controls, and were included in each exposure, along with unexposed bacterial strain controls.

Bacterial culture exposure stocks were prepared from overnight cultures with an OD_{600nm} of 1. The 50 mL culture was centrifuged in a Beckman Coultier™ refrigerated centrifuge (Avanti J-20XP) for 15 minutes at 6,000 x *g*. Following centrifugation and decanting of supernatant, the bacterial pellet was re-suspended in 10 mL 2% w/v NaCl solution, which was pre-cooled on ice. The culture was held on ice and 8 mL of protective medium (333 mM $C_6H_{12}0_6{\cdot}H_2O$, 68.44 mM NaCl, 12.89 mM L-Histidine and 0.5 g/L BSA) was slowly added, while mixing over a period of 15 minutes. 1 mL aliquots of the suspension were dispensed into microtubes and stored at -20°C. These stock cultures were inoculated into 11.5 mL re-suspension media (ISO standard 5.5 buffer) (40.37 mM $C_6H_{12}0_6{\cdot}H_20$, 342.2 mM NaCl, 10 mM $MgCl_2{\cdot}6H_20$, 4.02 mM KCl 49.93 mM HEPES) prior to exposure and allowed to culture for 15 minutes.

100 µL of each nanoparticle test sample and its dilution series were added to a 96-well luminometer plate in triplicate. 100 µL of either chemical standard ($ZnS0_47H_20$ or $K_2Cr_2\ O_7$) and its dilution series were also added to designated wells. Control wells with 100 µL of high grade HPLC gradient H_2O were also prepared. 100 µL of bacterial culture in re-suspension media was then introduced into each well. Bioluminescence measurements were taken five minutes after exposure of the first test samples, using the Spectrafluor plate reader (Tecan), and up to 60 minutes after exposure (see Figure 9). The percentage inhibitory effect (INH%) of the AgNP suspensions were calculated from the differences in relative light intensity levels between the control and exposed bacteria at each time-point, as described in the ISO 11348 Standards (Table 5).

Table 5: Calculating the percentage inhibitory effect (INH%)

$$INH\%\ (\text{at time-point}) = \frac{[Ic\text{-}It\ ] \times 100}{Ic}$$

Ic, luminescence intensity (RLU) of the control bacterial suspension unexposed.

It, luminescence intensity (RLU) of the bacterial suspension after exposure with test sample.

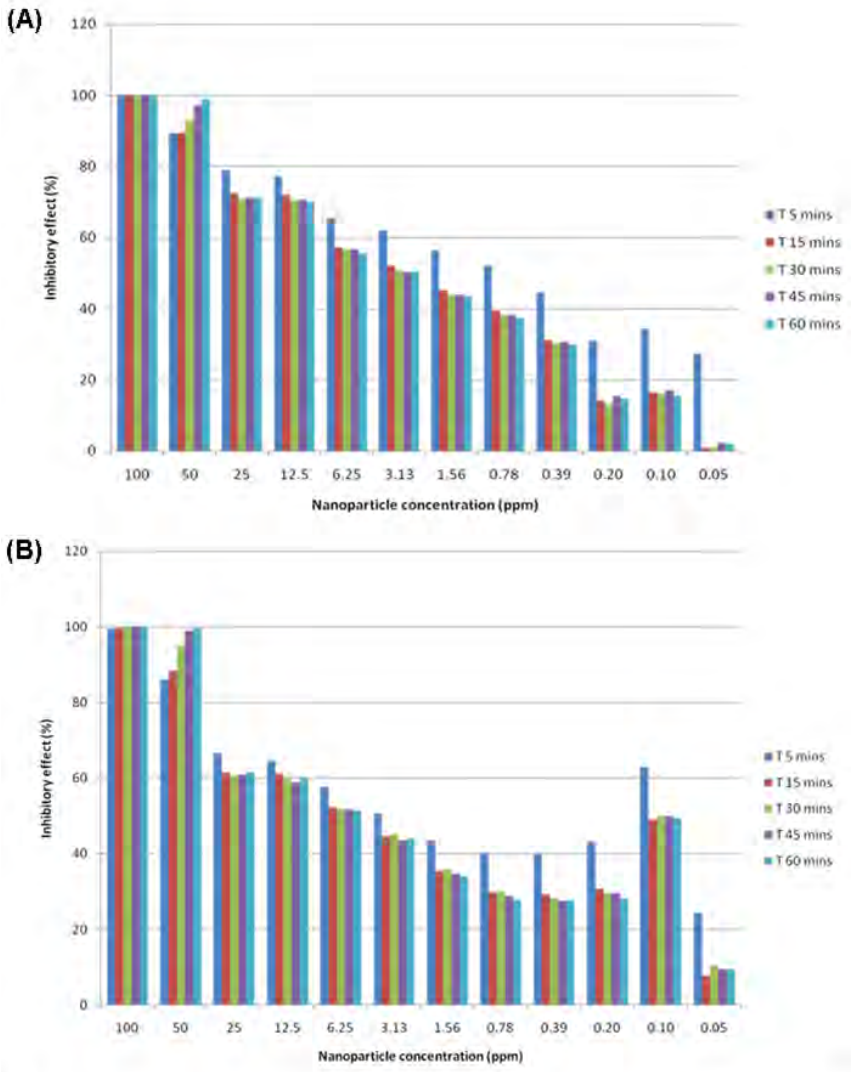


Figure 9: Inhibitory effect on *V. fischeri* as measured by bioluminescence over a 60 minute period, TSC stabilised (A) and PVP stabilized (B) shown above.
Concentration range of tested AgNP went from a maximum 100 ppm decreasing to 50 ppb.
Exposure assessment Performed under ISO 11348 protocols for toxicity reporting

A time-dependent increase in toxicity is seen at 50 ppm TSC_1. Inhibitory levels increase from 89% after five minutes' exposure to 99% after 60 minutes. A similar time-dependent increase in toxicity is seen at 50 ppm PVP_1. Inhibitory levels increase from 86% after five minutes' exposure to 100% after 60 minutes. At concentrations < 50 ppm, toxicity is less time-dependent, with the highest levels of toxicity seen after five minutes' exposure, and at all other time points, toxicity levels are relatively stable and showing less inhibition. This is seen most dramatically when *V. fischeri* is exposed to 0.05 ppm TSC_1 after five minutes, with approximately 30% inhibition, and then the levels drop to <5% inhibition after 15 minutes and remain <5% inhibition after 60 minutes. On average, inhibition levels drop by 12% between five and 15 minutes' exposure. This INH% trend may be as a result of toxicities changing from being a particulate effect to an ionic effect (see Figure 10).

The similarities in toxicities observed for TSC and PVP AgNPs are not unexpected, with both demonstrating a number of similar physiochemical properties when analysed by UV-Vis and viewed under a TEM analysis. When comparing toxicities, TSC AgNP appears to be slightly more toxic-showing, with higher inhibition levels. The fact that TSC AgNPs show slightly higher toxicities might be reflected in the smaller size range of these particles and the bare surface properties.

The effective concentration to inhibit luminescence by 50% (IC_{50}) ranged from 2.2 to 5.8 ppm for all AgNPs tested. The IC_{50} determined for silver ionic exposure was 0.48-0.52 ppm. It should be noted though that luminescence corresponds to the inhibitory effect of the test substance, in this case the AgNP preparations, and should not be considered solely as an assessment of viability, which requires further research to determine.

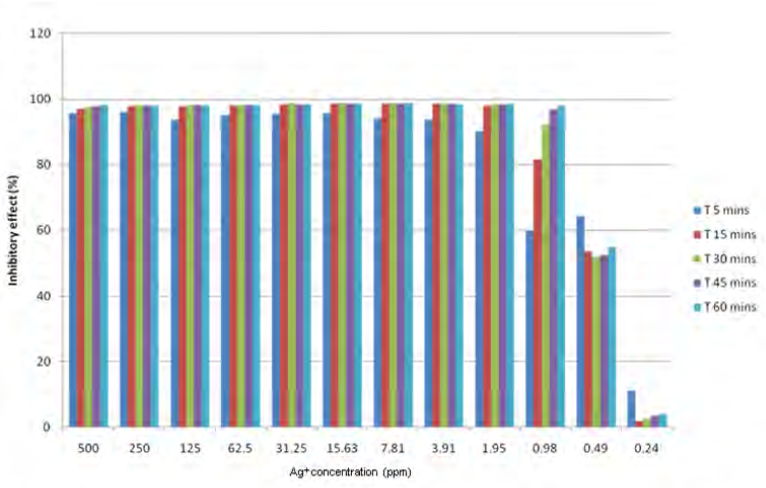


Figure 10: Inhibitory effect of silver ion on *V. fischeri* as measured by bioluminescence over a 60 minute period. Performed under ISO 11348 protocols.

4.4.2.3 Viability assessment

A 10-fold serial dilution in water was performed on the samples following a 60-minute exposure period, to allow a countable spread plate inoculation of 10^{-2} and 10^{-3} bacteria. 100 µL of each triplicate of the same dilution and all other triplicate dilutions were inoculated onto LBS agar plates (1% w/v Tryptone, 0.5% w/v Yeast extract, 2% w/v NaCl, 0.3% w/v Glycerol, 1.5% agar) and incubated at 30°C for *V. harveyi* and room temperature (16-23°C) for *V. fischeri* growth overnight. LBS agar was chosen, as it proved to be the fastest for observation of visible colonies. Plates were incubated (in triplicate) for 48 hours, permitting visible colony forming units (CFUs) to be counted. CFU counts were performed for each plate. During this work the assessment and comparison of utilising *V. harveyi* as an exposure model was explored (see Figure 11). These studies demonstrated (i) the suitability and (ii) the different sensitivities of bacterial strains to silver exposure in nano range forms.

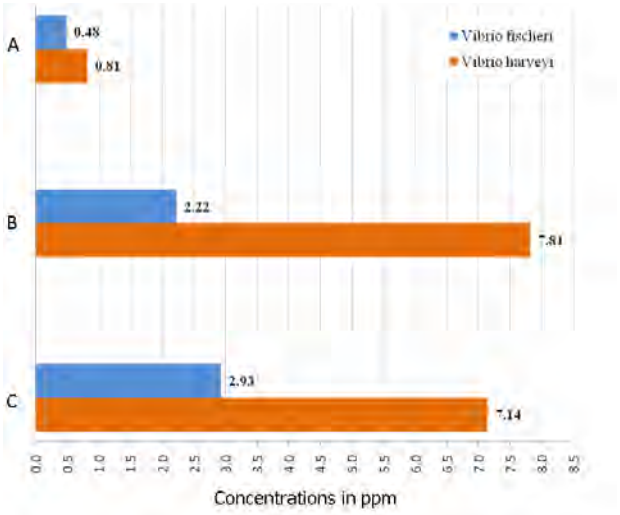


Figure 11 Using a 30 minute exposure time point, concentrations of silver ion (A), TSC AgNPs (B) and PVP AgNPs (B) which resulted in 50% inhibition of bioluminescence (IC_{50}) are demonstrated.

4.5 Zebrafish, Freshwater Model

4.5.1 Animal Husbandry and Exposure Study

Zebrafish (AB strain) were maintained in 10 L tanks with a day-night cycle of 14 hours light:10 hours dark, under standard conditions (Westerfield, 1995). They were fed twice daily with dried food and once daily with live brine shrimps (Artemia salina) (ZM Systems, UK). On the evening before spawning, zebrafish were separated into spawning trays at a gender ration of 2:1 male to female. Spawning was triggered upon exposure to light (Westerfield, 1995). Embryos were washed three times in 0.22 µm filter sterilised egg water (FSEW [60 µg/ml ocean sea salts]) and transferred to petri dishes. Healthy embryos were selected for normal development at cleavage-stage

(8–64 cell stage; 0.75–2.25 hours post fertilisation (hpf)), and exposures commenced immediately up to 72 hpf. Water conditions were kept within freshwater parameters throughout (0.2-1.0‰ salinity; pH 6.8-7.2; dissolved oxygen 7.7- 7.8mg/L; temperature 28.5°C).

4.5.2 Exposure of Embryos to AgNPs

An embryo toxicity assay was adopted following the OECD guidelines and research reports for the short-term toxicity test on fish embryo and sac-fry stages (Fraysse et al. 2006; OECD 1998 guidelines[13]). Initial range finding concentration determination and exposure assessment of AgNPs were performed in six-well plates and 96-well plates, with (i) single embryo and (ii) five embryos per well, with five replicates for each condition. Fish embryos were exposed to the test substance AgNPs, $AgNO_3$ or bulk silver at concentrations up to 1,000 ppm for a period up to 72 hpf, during which mortalities and concentration effects were scored at 24, 48 and 72 hpf. Concentration range cut-offs, at which survival was 95% or greater (lower limit) and where mortality exceeds 50% of the population at 24 hpf (upper limit), were identified.

Embryos were then treated with seven concentrations, ranging from 0.001 up to 100 ppm, for each AgNP dispersed in FSEW. Similarly, toxicity of bulk silver and ionic form Ag+ ($AgNO_3$) were evaluated at the same range. In order to avoid excessive adsorption, all plastics were pre-incubated with test material for up to 24 hours prior to commencement of testing, followed by rinsing twice with FSEW. Temperature (28.5°C) and pH (~6.8 to 7.1) levels were kept constant under static exposure conditions throughout. Observations were made up to 72 hpf under exposure conditions, at which point the exposure was stopped and embryos were held in FSEW. At 96 and 120 hpf, under standard, non-exposed culture conditions, embryos were further assessed. Time points are based on known developmental stages for criteria mortality, rate and time of hatching, development, touch responses, edema and heart rate (Kimmel et al., 1995). All observations up to 72 hpf were performed directly from the test condition, without removing developing embryos from the medium. Where embryos showed a failure to develop and coagulate, they were removed and cultured separately (Asharani et al., 2008). This step is essential in differentiating between malformed silver-treated embryos and dead embryos, both of which gave a white opaque appearance at 24 hpf (OECD 1998 guidelines [12]). Dead embryos were removed immediately from the medium during the observation period and counted.

The criteria for determination of death applied were (i) a cease in development, (ii) opaque discoloration and (iii) absence of body movement and/or absence of heartbeat. Experiments where controls showed more than 5% mortality were rejected. An untreated control group was used in all experiments and all conditions were tested using 100 embryos per treatment and

[12] www.oecd.org/chemicalsafety/testing/36817070.pdf; http://www.oecd-ilibrary.org/environment/test-no-212-fish-short-term-toxicity-test-on-embryo-and-sac-fry-stages_9789264070141-en

conducted in triplicate. Toxicity values (LC_{50}) were determined for each AgNP preparation at standard time point. Heart rates of a random selection of anesthetised embryos were directly observed for 15 seconds, rate per minute deduced from this, and the mean value was taken as the heart rate at that concentration of AgNP. Embryos were kept at room temperature for 30 minutes to allow the heartbeat to resume a steady rate prior to manual counting. Embryos were anesthetised with 168 mg/L tricaine methane sulfonate, MS-222 (Sigma-Aldrich, Poole, UK). The hatching rate for individual experiments was calculated as a percentage of surviving larvae hatched out at 72 hpf. The touch response of the larvae was tested by gently touching the sides of the trunk with a smooth pipette tip, and manual determination of rate/strength of movement recorded.

4.5.3 Dechorionation of Zebrafish Embryos

The chorion of 2.5 hpf embryos was softened by incubation in a 2 mg/ml pronase (Sigma–Aldrich, Poole, UK) for 1 ± 0.25 min at 28.5°C, followed by mechanical removal. Control experiments were carried out to ensure that pronase treatment did not have any effect on embryonic development. Dechorionated embryos were incubated in six well-plates and exposed to the determined concentrations range [0.001 to 100 ppm (100 mg/L)]. Reduced sample sizes of 20 embryos per treatment were performed in triplicate for this analysis.

4.5.4 Quantification of Ionic and Total Ag within Zebrafish Testing

Concentrations of ionic, dissolved and total silver forms were measured directly from FSEW. Briefly, from each exposure group (with embryos removed), 10 mL was taken and centrifuged at 15,000 rpm for one hour, followed by 0.22 µm membrane disc filtration (Whatman International Ltd, Maidstone England). Measurement of ionic silver was then performed using an ion selective electrode (ISE) (Nico2000, Middlesex, UK). Samples were then reduced using 2% nitric acid to determine silver content of this fraction (ionic and dissolved complexes). Concentrations of total silver were determined from test samples directly with nitric acid reduction, without centrifugation or filtering. Freshly prepared $AgNO_3$, ranging from 0.01 to 100 ppm, served as a standard for concentration estimation from a seven-point standard curve. The operational range of ISE probes was stated as 9×10^{-8} to 1 Molar (0.010 to 100000 ppm) by Nico2000, UK. ISE measurements also served for the quantification of AgNPs in the test solution for accurate exposure assessment.

4.5.5 Data Analysis

Cumulative toxicity scores for each 24 and 48 hpf were averaged and used as a metric of overall toxicity, including mortality and sub-lethal toxicity. LC_{50} values were calculated by probit analysis (USEPA Probit Analysis Programme, Ver 1.5) from 24 and 48 hpf survival data. All other statistical analyses and data management were performed using SPSS® Version 18.0 and Minitab® 16.1. The data were tested for homogeneity and normality. Statistical differences were compared to controls using

ANOVA. Differences in the frequency distribution of developmental abnormalities were compared across treatments using Chi-Square. Level of significance for all analyses was $p < 0.05$. As comparisons among means were considered to be of substantive interest a priori, no adjustment for multiple comparisons has been incorporated into the analysis (Rothman, 1990).

4.5.6 AgNPs Physicochemical Properties in Test System

Size, shape and charge were determined for all AgNP preparations. All AgNPs used within the study demonstrated a high degree of monodispersity, ranging in a diameter size across the preparations from 40-180 nm by TEM analysis (mean values). Hydrodynamic diameter (HDD) estimates under preparation conditions ranged from 56-225 nm; under exposure conditions in FSEW, the range was measured from 92-297 nm (Table 2). DLS HDD measurements increased by factors of 1.2 to 2.0 across the samples, with Si_01 increasing by a factor >3.0 under exposure conditions.

The behaviour of the AgNPs under test conditions, with increasing HDDs, is suggestive of particle aggregation. Aggregation of AgNPs has been typically associated with the surface modification, capping agent and the ζ-potential. Here, all preparations were observed to be undergoing aggregation to some extent, though no precipitation was observed for any AgNPs throughout test conditions. Measurement of the ζ-potential under the exposure conditions resulted in an overall charge variance, with the most notable shifts observed for the TSC (TSC_1, TSC_2), PVP (PVP_1, PVP_2) and the biocompatible gelatine preparations (BIO_1, BIO_2). Thiol-capped preparations demonstrated the highest degree of stability under these conditions.

Shifts in ζ-potential can be attributed to altered reactivity, with contributing factors of temperature, pH and salinity. Change in ionic strength, decreased electrostatic shielding, and formation of aggregates are also factors. These expansive differences in physicochemical parameters, seen across AgNP preparations, permit the examination of NP-induced levels of toxicity and response, in relation to physicochemical parameters. Shifts in ζ-potential under these conditions indicate a potential relationship with the capping agent, appearing to be independent of the particle diameter. UV–Vis spectroscopy use was to primarily confirm the presence of an absorbance peak, consistent with that expected for the plasmon resonance of AgNPs (see Figure 12). However, according to the authors, the application of this spectrometry required particle concentration to be greater than 15 ppm.

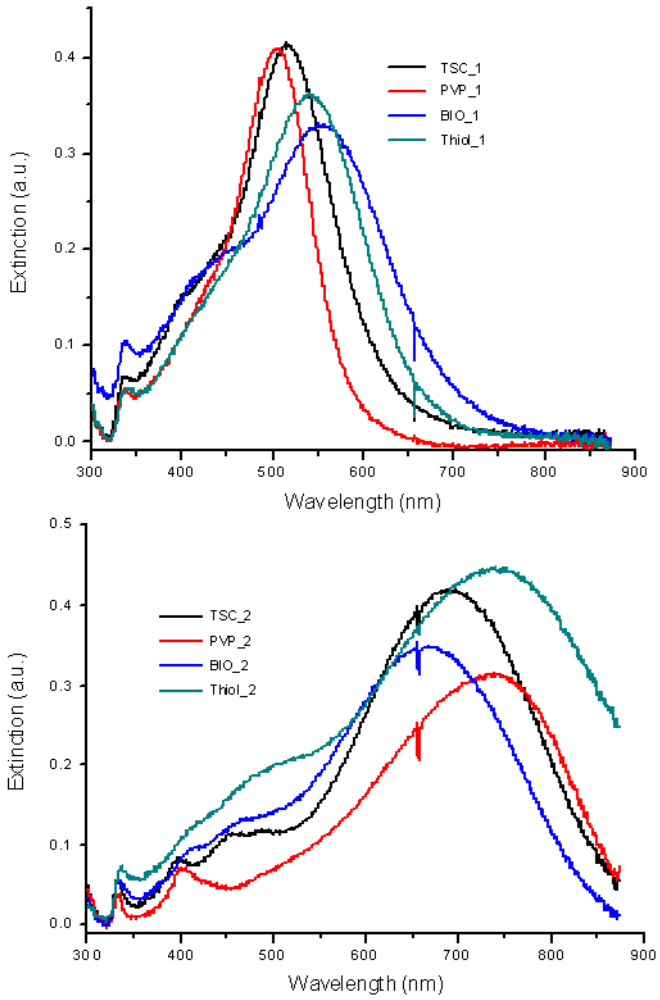


Figure 12: UV-Vis absorbance spectrum for synthesised Ag-NP preparations

Theoretical explanations on the toxicity of Ag and now AgNPs have been attributed to the release of Ag+ ions. The dissolution of ions from AgNPs is associated with physicochemical properties, such as size, surface areas and capping agent. AgNP preparations were incubated in FSEW over a five-day period at 28.5°C, mimicking exposure conditions to assess Ag+ dissolution. High-speed centrifugation and ultrafiltration were used to remove suspended metallic particles. The selected concentration (50 ppm) was based on initial range-finding experiments prior to the study. AgNPs, grouped on the basis of capping agents, follow a close pattern of dissolution with each other. PVP and thiol-stabilised AgNPs demonstrated the highest rate of dissolution within the test period (in FSEW), with greater than 40% silver in ionic form at 120 hours. In the case of PVP preparations, this may be linked with the observed shift in ζ-potential. However, thiol particles had demonstrated a level of stability of ζ-potential over the same period, reaching between -30 and -40 mV, which is typically accepted as moving towards a stable equilibrium. Over the course of 120 hours, both preparations

BIO_1, BIO_2 showed a reduced rate of dissolution when compared to other preparations, with less than 18% ionic form at 120 hours. A similar trend is observed for commercial preparations PI_1 and PI_2, during the initial 48-hour period. Rates of dissolution ranged from 6 - 12% up to 19 – 36% over the 48-hour period, from 24 to 72 hours (Figure 13). However, the release of ionic form and related toxicity require careful interpretation, as they will vary within test systems and environmental conditions widely.

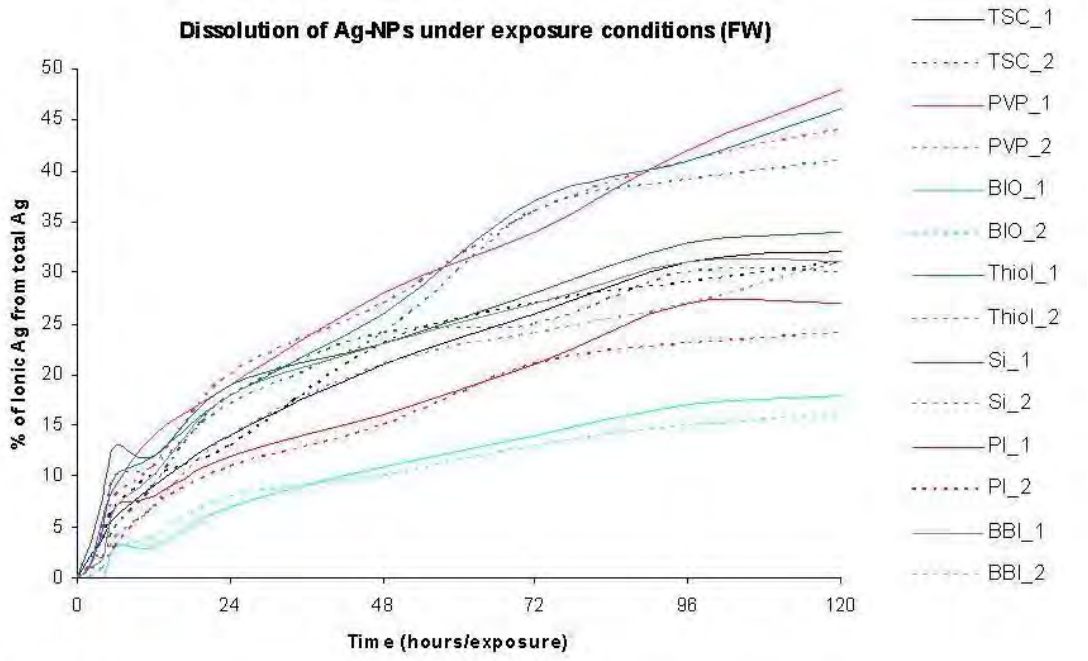


Figure 13: Dissolution of AgNPs in FSEW under exposure conditions

The formation of potential complexes is dependent upon both biologic and chemical loading of the test systems, as would be the case in environmental release scenarios. Indeed, mimicking complex formations that may occur within an environmental setting under natural conditions cannot be entirely modelled or assessed within a laboratory exposure setting. Characterisation indicated both ζ-potential and dissolution rate as key factors in the stability and presentation of the AgNPs to the test model system, and consequently their toxicities.

4.6 Exposure Outcomes

4.6.1 AgNPs on Mortality and Survival Rates

Control groups of embryos developed normally and showed an overall mortality of less than 4%. Exposure of embryos to the AgNP preparations resulted in a range of increased mortality, subject to both particle type and concentration (Figure 14, Figure 15 and Table 6). The range of LC_{50} values from 0.48 ppm up to 48 ppm at 24 hpf and 0.037 ppm up to 2.6 ppm at 48 hpf were determined by probit analysis on direct morality counts (Table 6). A range of toxicity,

demonstrating two orders of magnitude across AgNPs tested was observed. LC_{50} values, calculated directly from experimental observations, fall within the 95% confidence intervals. Determined probit values of the data set range from 0.003 to 57.41 ppm and 0.001 to 6.922 ppm for 24 and 48 hours respectively. The toxicity of synthesised AgNP preparations show a trend of increasing toxicity from biocompatible gelatine (BIO_1, BIO_2), TSC-capped (TSC_1, TSC_2), Thiol-capped (Thiol_1, Thiol_2) with PVP-capped (PVP_1, PVP_2) demonstrating the highest level of mortality of all the preparations tested (including commercial preparations). This trend is observed at both 24- and 48-hour time points. The commercially-assessed samples demonstrated LC_{50} values less than 15 ppm at 24 hours, with a number resulting in a LC_{50} of less than 7 ppm (Table 6). Exposure to all AgNP preparations showed significant toxicity compared to the control population over the range of concentrations used. Calculated LC_{50} values appear independent of diameter size.

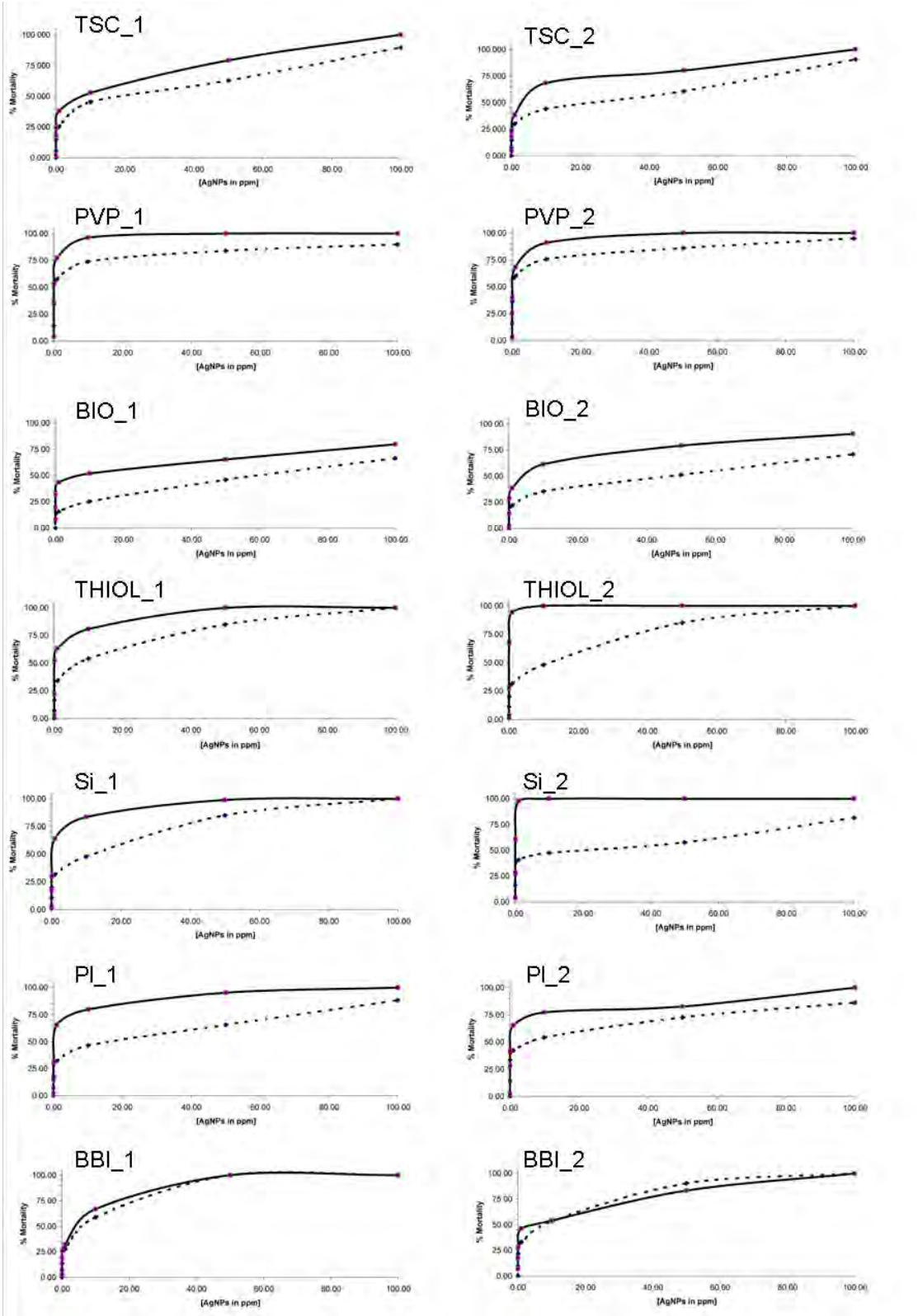


Figure 14: LC_{50} curves at 24 and 48 hours exposure
24 hpf mortality plotted in dash (·····); 48 hpf mortality (——)

Table 6: Probit analysis determined 24 and 48 hour LC_{10} and LC_{50} values

AgNP Sample	LC10 24hpf (95% CL) [PPM]	LC50 24hpf (95% CL) [PPM]	LC10 48hpf (95% CL) [PPM]	LC50 48hpf (95% CL) [PPM]
TSC_1	0.075 (0.006, 0.298)	7.39 (2.58, 25.29)	0.010 (0.001, 0.078)	1.584 (0.286, 9.618)
TSC_2	0.053 (0.002, 0.261)	6.902 (2.00, 31.08)	0.007 (0.002, 0.042)	1.039 (0.264, 4.069)
PVP_1	0.004 (0.001, 0.008)	0.642 (0.409, 0.999)	0.002 (0.001, 0.005)	0.063 (0.028, 0.134)
PVP_2	0.004 (0.002, 0.008)	0.487 (0.315, 0.744)	0.004 (0.001, 0.011)	0.154 (0.070, 0.323)
BIO_1	0.413 (0.053, 1.301)	47.89 (18.45, 203.49)	0.001 (0.001, 0.003)	2.634 (1.430, 5.082)
BIO_2	0.117 (0.048, 0.233)	24.87 (14.94, 45.08)	0.011 (0.001, 0.041)	1.712 (0.668, 4.474)
Thiol_1	0.053 (0.003, 0.222)	2.368 (0.709, 7.824)	0.003 (0.001, 0.013)	0.173 (0.049, 0.527)
Thiol_2	0.028 (0.001, 0.181)	2.24 (0.43, 12.67)	0.003 (0.002, 0.004)	0.037 (0.027, 0.051)
Si_1	0.071 (0.003, 0.344)	4.698 (1.268, 19.993)	0.007 (0.002, 0.019)	0.293 (0.137, 0.606)
Si_2	0.004 (0.001, 0.027)	4.868 (1.398, 23.201)	0.003 (0.002, 0.005)	0.038 (0.027, 0.052)
PI_1	0.040 (0.004, 0.157)	5.546 (2.048, 17.227)	0.008 (0.002, 0.019)	0.362 (0.186, 0.684)
PI_2	0.007 (0.001, 0.037)	2.174 (0.691, 7.422)	0.002 (0.001, 0.011)	0.269 (0.058, 1.010)
BBI_1	0.078 (0.002, 0.398)	1.890 (0.359, 9.079)	0.011 (0.001, 0.086)	0.755 (0.101, 5.257)
BBI_2	0.036 (0.001, 0.191)	1.838 (0.410, 8.151)	0.004 (0.001, 0.035)	0.955 (0.175, 5.236)

Probit analysis determined 24 and 48 hour LC_{10} and LC_{50} values.
Lethal Concentration values at LC_{50}, determined by probit analysis on direct morality/survival counts demonstrated a range of toxicity covering two orders of magnitude across AgNPs tested. Ranges determined for LC_{10} demonstrated a narrow distribution of morality/survival values . 95% confidence intervals are noted in brackets for each calculation.

Comparison between AgNPs, Ag^+, bulk Ag toxicities, and observed defects for all AgNPs had a lower mortality than equivalent Ag^+ ($AgNO_3$) concentrations, and higher toxicity values of the equivalent mass of bulk Ag control (Figure 16, Figure 17 and Table 6). LC_{50} values for both ionic and bulk Ag forms are within expected reported values. This may be suggestive that AgNP defects are due to the nanometre size range, and not directly the sole effect of the dissolved Ag^+ or from aggregate/complexes formed from these AgNPs. It is noteworthy to consider that AgNP test preparations would consist of AgNPs, aggregates and dissolved Ag^+, all of which may jointly exert influences on developing zebrafish embryos.

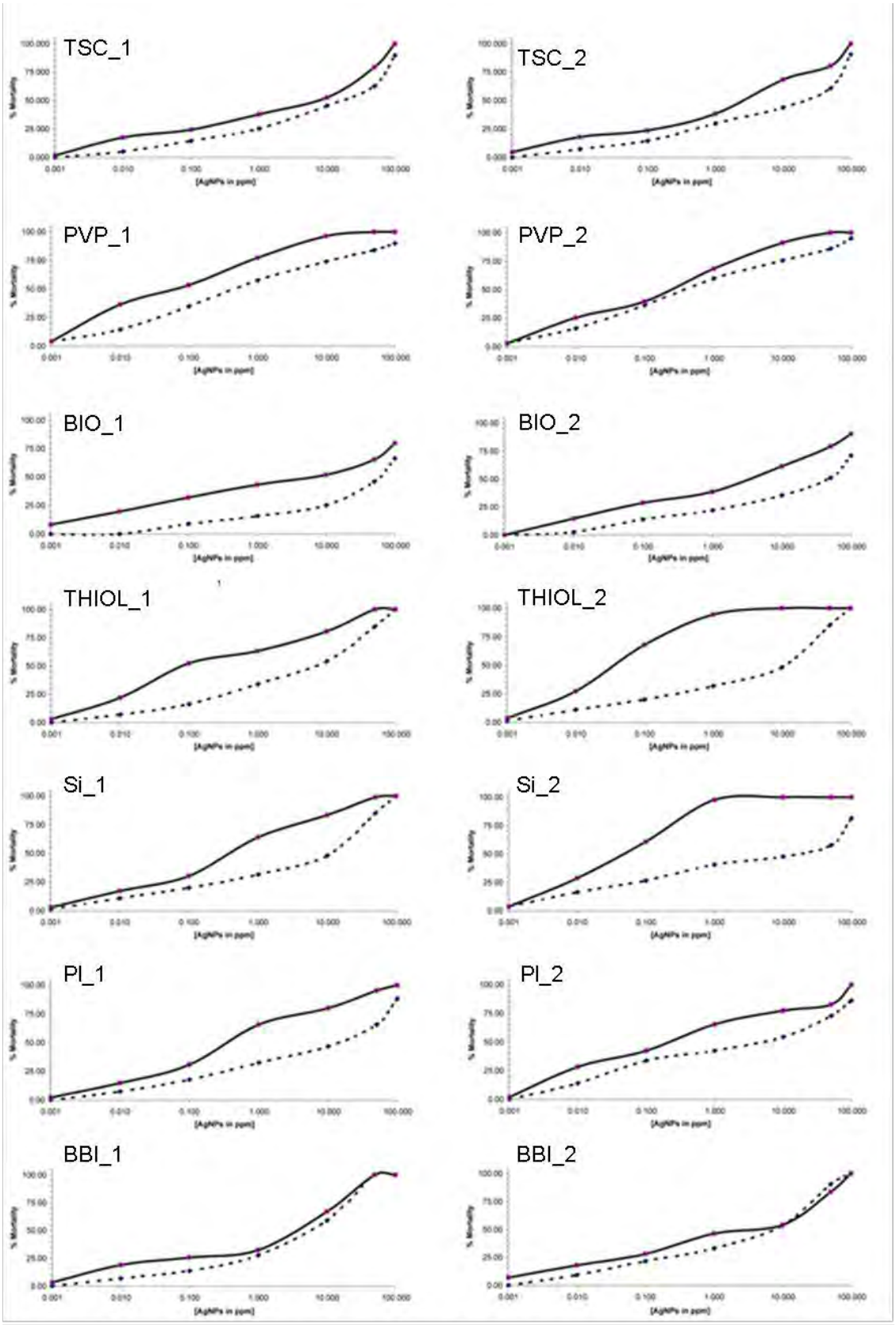


Figure 15: LC_{50} Log curves a 24 and 48 hpf exposure
24 hpf mortality plotted in dash (·····); 48 hpf mortality (——)

4.6.2 Heart Rate and Hatching

Heart rates of control and AgNP-exposed zebrafish embryos were recorded at 24, 48 and 72 hpf. A reduced heart rate was observed for a number of AgNP preparations, with decreasing rate proportional to increasing concentrations (Figure 16). Differences in rate are easily observed at 72 hpf at 10 ppm. The most dramatic rate change is seen for PVP preparations 1 and 2, compared to non-exposed control population (Figure 16). At 72 hpf, all exposures were stopped and zebrafish were maintained in FSEW, and permitted to continue to develop under normal conditions. Hatching rates were calculated from surviving embryos at 72 hpf, and are given as percentages hatched at 72 hpf (Figure 17).

Hatching rates can be influenced by the developmental state of the embryo, environment and the condition of the chorion itself. For developmental factors, interaction between the AgNPs can be assumed, be it directly via entering the embryo, or by secondary effects, e.g. dysregulation of ion channels. Figure 17 shows the percentage of hatched embryos from surviving populations at 72 hpf, along with the hatching rate of the Ag+ ($AgNO_3$) population. The reduced hatching effect can be observed, with a similar trend for all 14 AgNP preparations. Though the reduced hatching deviates from control (χ^2 = 21.9, 5 d.f., p > 0.001), no one AgNP preparation demonstrated a greater effect. Significantly, however, embryos exposed to $AgNO_3$ demonstrate a dramatic drop in hatching, compared to the most inhibitive AgNP tested, PVP_1; testing against the least inhibitive AgNP (Bio_1), this significance is increased (χ^2= 46.513, 5 d.f., p > 0.0001), indicating a potential difference in the toxicity effects of AgNPs compared to those of $AgNO_3$, in relation to hatching as a parameter.

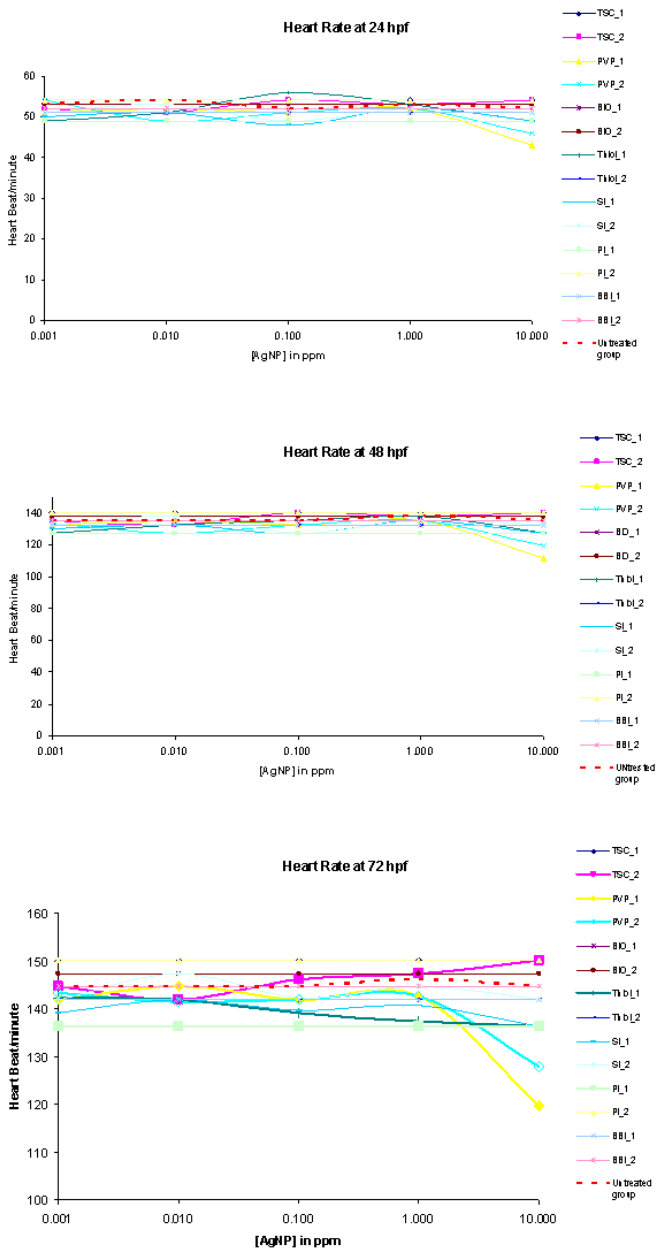


Figure 16: Heart Rate of AgNP exposure

Heart rates of 10 anaesthetised embryos were directly observed for 15 seconds, and rate per minute deduced from this and the mean value was taken as the normal heart rate for that exposure concentration of AgNP. Embryos were kept at room temperature for 30 minutes to allow the heartbeat to assume a steady rate prior to manual counting. Counts were taken at 24, 48 and 72 hpf. Highlighted at 72 hpf are PVP exposure groups.

4.6.3 Chorion Shielding and Larvae Mobility

Under laboratory conditions, the chorion can be enzymatically weakened. Using a reduced sample number, the chorion was removed to assess the role that it plays. However, in shielding and potential accumulation, findings suggested that the chorion did not provide a barrier to exposure, as mortality was similar for both groups. Post-exposure embryos at 72 hpf were treated with pronase to permit (i) the time duration of chorion breakdown and (ii) free swimming to be observed. Non-hatched 72 hpf control embryos were released after 93 (±8) seconds of pronase digestion. Release is not solely due to enzymatic breakdown of the chorion; other contributing factors include the stage of development, strength, overall health and range of mobility. In contrast, exposed embryos required in excess of 340 seconds to be able to swim free, if indeed any movement was noted at all. Effects of AgNP or Ag^+ cannot be excluded as causative factors in the hardening. Once hatched, a mobility assessment was performed. This assessment is a measure of the zebrafish moving away from a point contact, and is measured by visual monitoring. Normal larvae responded by swimming away, while exposed larvae demonstrated a range of reduced mobility including circling, rolling and delayed response. Larvae exposed to Ag^+ did not show any significant movements, and where present, had dramatically reduced heart rates. On this basis, these larvae were euthanised at this point.

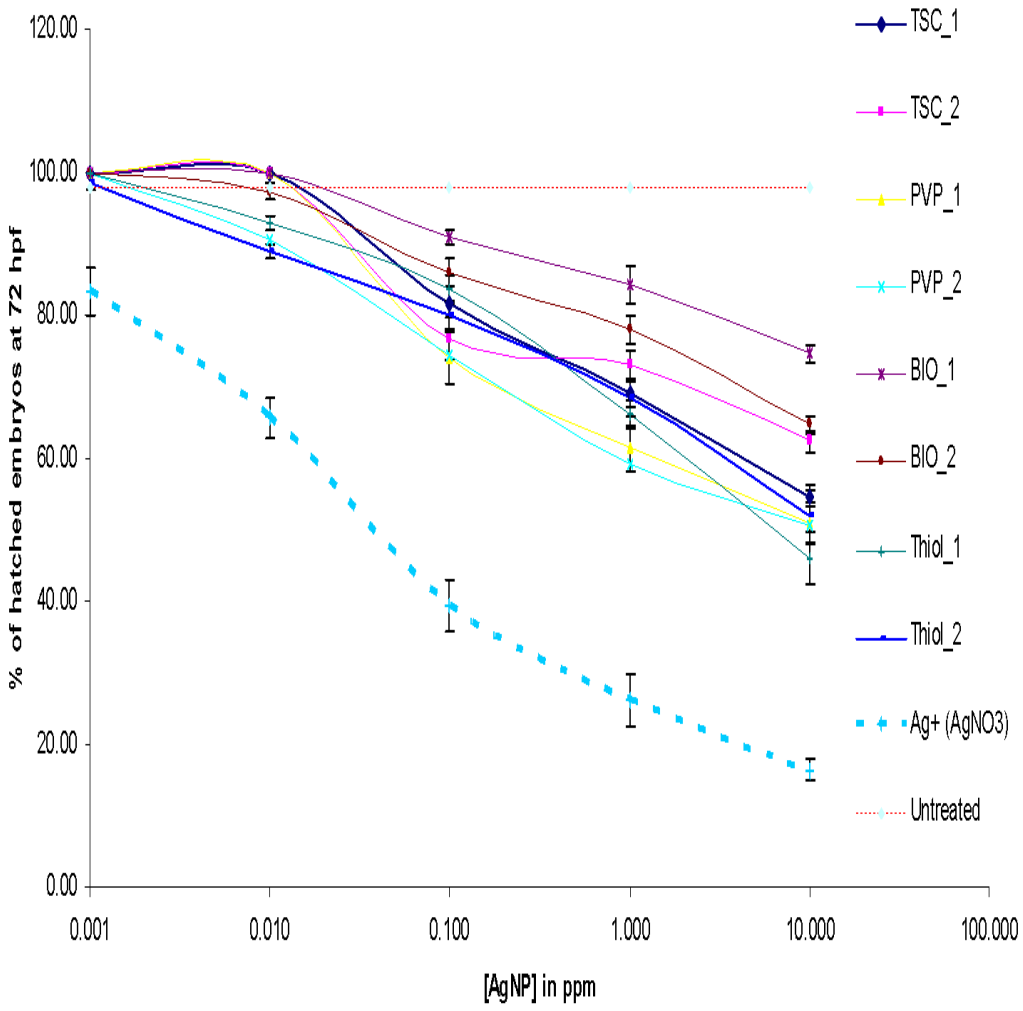


Figure 17: Hatched embryos from surviving populations at 72 hpf

The percentage of hatched embryos at 72 hpf from the surviving population. The eight in house synthesis AgNPs are shown which demonstrate the range of hatching rates, over concentration range. Control non-exposed embryos where 94-100% hatched at 72 hpf. A relationship between concentration and hatching can be seen. Across the AgNPs a hatching rate variation of 46% to 74% at 10 ppm was observed, for Thiol_1 and BIO_1 respectively.

5. Application of Transcriptomics

Over the last decade, environmental systems monitoring has seen the continuing growth and application of genomic technologies, to enable molecular biomarkers to measure and monitor environmental exposure scenarios. This has been greatly aided by whole transcriptomic profiling utilising microarrays, targeting the molecular responses of a selected organism before, during and post exposure, thus providing a whole organism response, and potentially indicating the compensatory mechanisms activated and mechanisms of toxicity of test agents.

With the continuing expansion of ENMs, and a growing need to attain and determine exposure risk, microarray transcriptomic analysis is one avenue of analysis which could rapidly be used for testing and prediction of exposures, while also permitting a standardised exposure protocol to be widely applied, and data from cross-studies to be easily merged for meta-analysis of ENMs, including AgNPs. Microarray-based methods may also provide a novel approach to detect AgNPs in the environment, through the identification of panels of novel biomarkers.

5.1 Exposure, Sample Collection and Preparation

Within this work, transcriptomic analysis was performed on 24 and 48 hpf zebrafish which had undergone exposure, as detailed in Section 4.5.2 (0.001 up to 100 ppm), and were used to examine the transcriptomic profile under exposure conditions. Exposure concentrations, which were utilised for transcriptomic analysis, were grouped from 0.001 to 0.100 ppm, which encompasses current proposed environmental exposure levels.

Briefly, once exposure time had been completed, embryos were homogenised in TriPure reagent and total RNA was extracted, according to the manufacturer's protocol (Roche), and DNAse I column treatment (Qiagen) was performed to remove traces of contaminating DNA (Qiagen). Analysis of concentration and integrity using a Nanodrop ND-1000 (Thermo Fisher Scientific) and a 2100 Bioanalyser (Agilent, Santa Clara, CA) were then performed on harvested RNA (Figure 18). The incidence of false positives, due to individual embryos variability samples, were pooled (n = 25 to 30) for those treated under the same exposure conditions (AgNP type, time and concentration). As low yields were detected, due to the small sample size, whole transcriptomic analysis, WTA2 (Sigma) was performed on all samples (Figure 19).

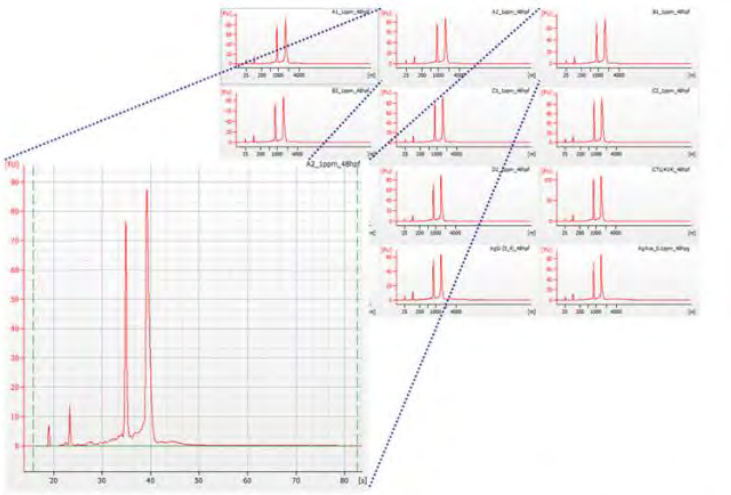


Figure 18: Bioanalyser 2100 analysis of RNA extraction samples demonstrating dominant peak profiles for S18 and S28

WTA amplification products (amplified cDNA) are routinely used as the sample for microarray target for expression analysis, using platforms such as the NimbleGen™ array platform, which has been utilised in this work[13].

[13] http://www.sigmaaldrich.com/life-science/molecular-biology/whole-genome-amplification/whole-transcriptome.html

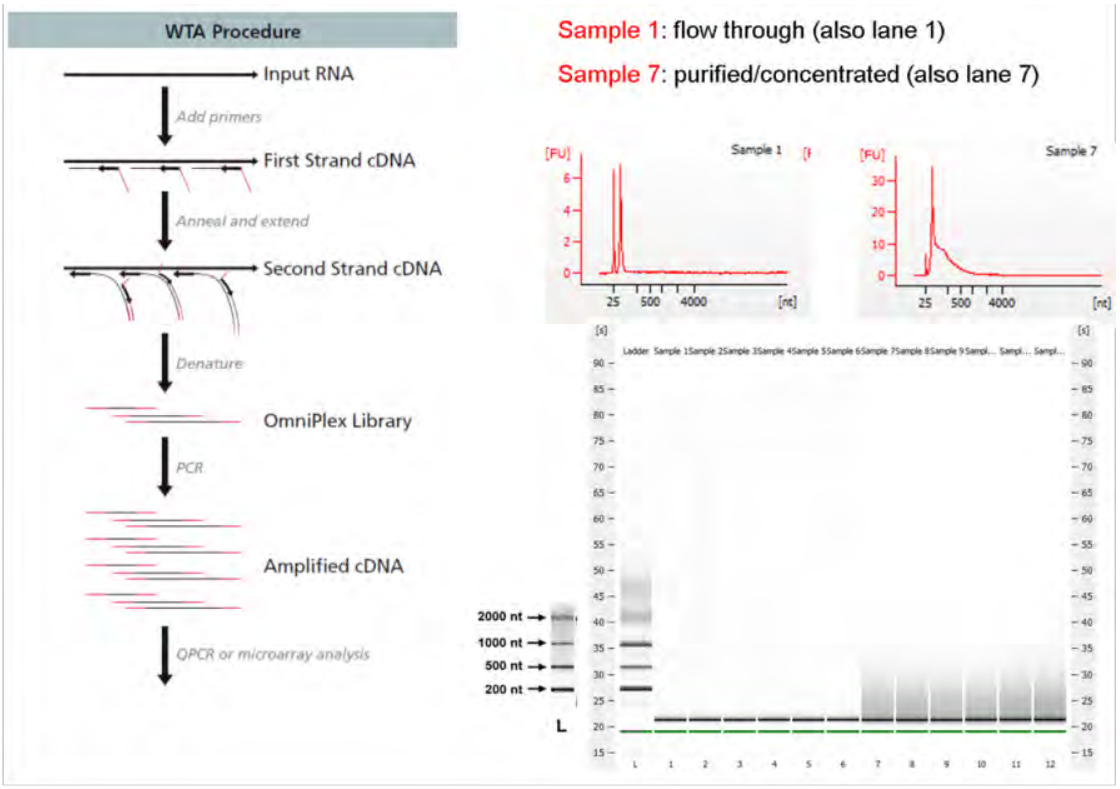


Figure 19: Whole Transcriptomic Amplification (WTA), and on the right, WTA samples visualized on Bioanalyser 2100. The resultant cDNA is ready for labelling and down stream array analysis

Equivalent masses of RNA from all samples were subjected to WTA (output demonstrated in Figure 19), which resulted in the generation of sample cDNA libraries which were presented for array analysis.

Microarray analysis was performed by Roche Nimblegen, a microarray and expression service provider. Equal amounts of amplified cDNA from controls and exposure treatments were used for fluorescent labelling (Cy3 and Cy5) for microarray analysis. Samples were then applied to the Nimblegen 12x135K zebrafish gene expression array, which covers 38,000 transcripts from the zebrafish, each targeted with a number of probes (genome build Zv7). Hybridisation and scanning procedures were conducted according to the fixed service protocols of Nimblegen. Raw fluorescent data was then returned for analysis, corrected for background noise applying the Robust Multi-Array Analysis (RMA) algorithm, and then normalised. Normalised datasets were then examined in ArrayStar (DNASTAR, Madison, USA).

5.2 Global Analysis of Transcriptomics

Analysis of non-exposed control zebrafish, with AgNPs' exposed populations performed by the students' t-tests, and applying correction by Benjamini-Hochberg's false discovery rate (FDR), indicated 455 genes differentially expressed with greater than 1.5 fold change in expression, of which 259 were retained with 95% confidence (see Figure 20).

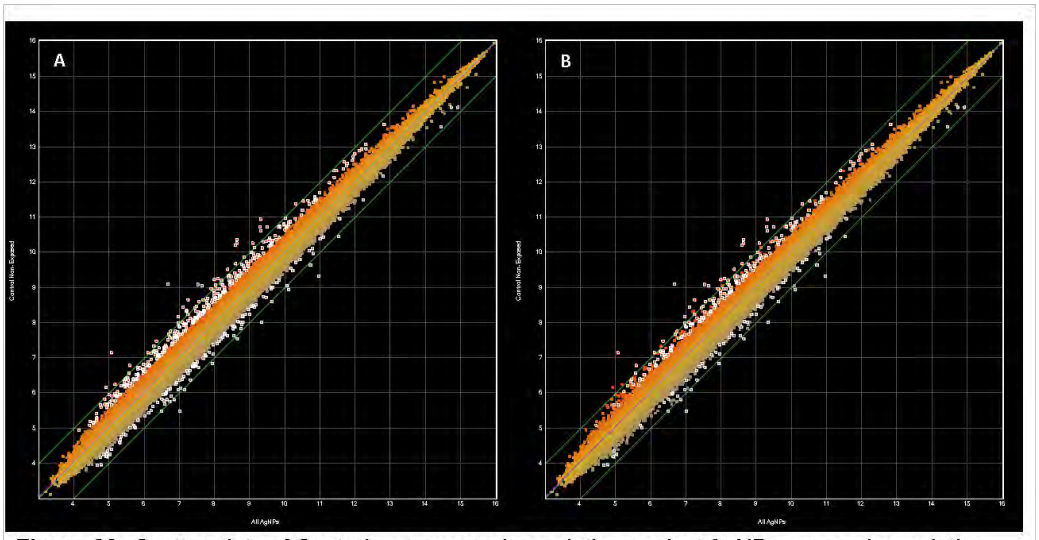


Figure 20: Scatter plots of Control non-exposed population against AgNPs exposed population. R2 = 0.9935 (Linear Correlation)
Cut-off of 1.5 fold change. Transcript expression differences reported for (A) 455 indicated transcripts; (B) 259 transcripts with 95% confidence indicated.

Increasing the stringency of analysis and examining transcripts with fold change greater than 2 with 95% confidence, reduced the number of transcripts of interest to 40. A heat map and details of these transcripts are provided in Figure 21 and Table 7 respectively.

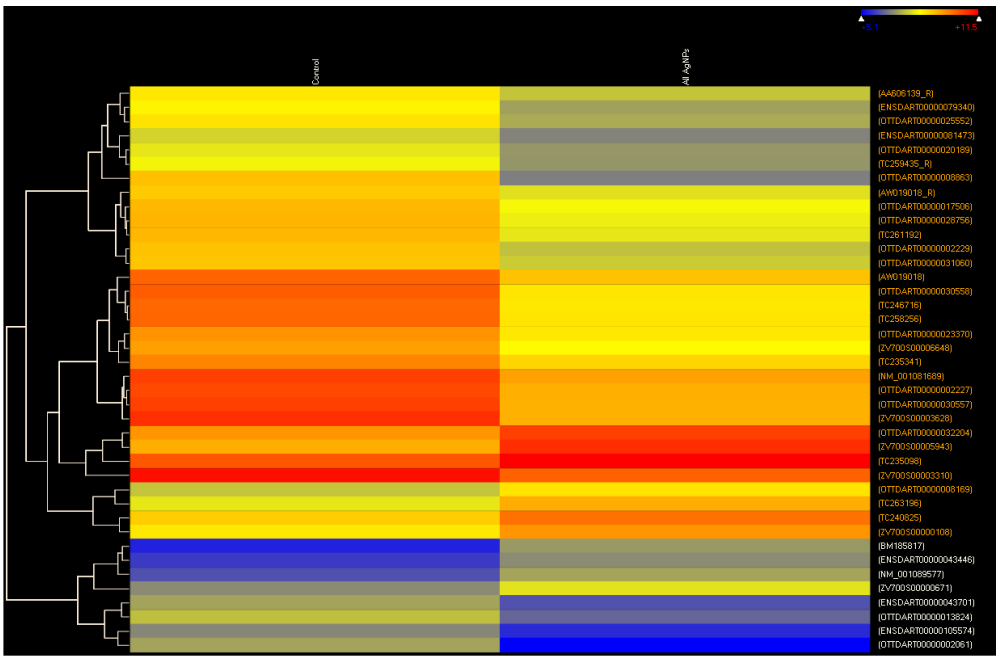


Figure 21: Clustering heat map of 40 transcripts demonstrating 2.0 fold change with 95% confidence.

Table 7: Genes clustered in figure 21, meeting >2 fold change in transcript expression, 95% confidence. Fold change and *p*-value listed. Associated URL with gene probe provided

Transcript_SEQ_ID	Fold change, <2	P value	URL
OTTDART00000008863	5.326 down	0.00223	http://vega.sanger.ac.uk/Danio_rerio/transview?transcript=OTTDART00000008863&db=core
OTTDART00000002061	4.215 down	0.00204	http://vega.sanger.ac.uk/Danio_rerio/transview?transcript=OTTDART00000002061&db=core
OTTDART00000030558	3.342 down	0.00924	http://vega.sanger.ac.uk/Danio_rerio/transview?transcript=OTTDART00000030558&db=core
TC258256	3.083 down	0.000544	http://compbio.dfci.harvard.edu/tgi/cgi-bin/tgi/tc_report.pl?gudb=zfish&tc=TC258256
ZV700S00003628	3.076 down	0.00338	http://www.ncbi.nlm.nih.gov/UniGene/clust.cgi?ORG=Dr&CID=121926
TC246716	3.068 down	0.0175	http://compbio.dfci.harvard.edu/tgi/cgi-bin/tgi/tc_report.pl?gudb=zfish&tc=TC246716
OTTDART00000002229	2.936 down	0.000794	http://vega.sanger.ac.uk/Danio_rerio/transview?transcript=OTTDART00000002229&db=core
OTTDART00000030557	2.691 down	0.00803	http://vega.sanger.ac.uk/Danio_rerio/transview?transcript=OTTDART00000030557&db=core
OTTDART00000025552	2.668 down	0.00214	http://vega.sanger.ac.uk/Danio_rerio/transview?transcript=OTTDART00000025552&db=core
OTTDART00000031060	2.624 down	0.000639	http://vega.sanger.ac.uk/Danio_rerio/transview?transcript=OTTDART00000031060&db=core
ENSDART00000079340	2.509 down	0.0107	http://www.ensembl.org/Danio_rerio/transview?db=core;transcript=ENSDART00000079340
OTTDART00000002227	2.449 down	0.00134	http://vega.sanger.ac.uk/Danio_rerio/transview?transcript=OTTDART00000002227&db=core
TC261192	2.348 down	0.00314	http://compbio.dfci.harvard.edu/tgi/cgi-bin/tgi/tc_report.pl?gudb=zfish&tc=TC261192
NM_001081689	2.329 down	0.0105	http://www.ncbi.nlm.nih.gov/entrez/viewer.fcgi?db=nuccore&id=126157454
TC259435_R	2.295 down	0.0208	http://compbio.dfci.harvard.edu/tgi/cgi-bin/tgi/tc_report.pl?gudb=zfish&tc=TC259435
ENSDART00000105574	2.277 down	0.0346	http://www.ensembl.org/Danio_rerio/transview?db=core;transcript=ENSDART00000105574
AW019018	2.268 down	0.00786	http://www.ncbi.nlm.nih.gov/sites/entrez?db=nucleotide&cmd=search&term=AW019018
OTTDART00000028756	2.237 down	0.00579	http://vega.sanger.ac.uk/Danio_rerio/transview?transcript=OTTDART00000028756&db=core
ZV700S00006648	2.214 down	0.00223	http://www.ncbi.nlm.nih.gov/UniGene/clust.cgi?ORG=Dr&CID=107751
OTTDART00000013824	2.184 down	0.0175	http://vega.sanger.ac.uk/Danio_rerio/transview?transcript=OTTDART00000013824&db=core
OTTDART00000023370	2.113 down	0.00742	http://vega.sanger.ac.uk/Danio_rerio/transview?transcript=OTTDART00000023370&db=core
ZV700S00003310	2.077 down	0.0148	http://www.ncbi.nlm.nih.gov/UniGene/clust.cgi?ORG=Dr&CID=114305
AW019018_R	2.070 down	0.0276	http://www.ncbi.nlm.nih.gov/sites/entrez?db=nucleotide&cmd=search&term=AW019018
ENSDART00000043701	2.053 down	0.00548	http://www.ensembl.org/Danio_rerio/transview?db=core;transcript=ENSDART00000043701
AA606139_R	2.048 down	0.00495	http://www.ncbi.nlm.nih.gov/sites/entrez?db=gene&cmd=search&term=AA606139
TC235341	2.033 down	0.0371	http://compbio.dfci.harvard.edu/tgi/cgi-bin/tgi/tc_report.pl?gudb=zfish&tc=TC235341
OTTDART00000020189	2.020 down	0.00756	http://vega.sanger.ac.uk/Danio_rerio/transview?transcript=OTTDART00000020189&db=core
ENSDART00000081473	2.015 down	0.0126	http://www.ensembl.org/Danio_rerio/transview?db=core;transcript=ENSDART00000081473
OTTDART00000017506	2.006 down	0.0183	http://vega.sanger.ac.uk/Danio_rerio/transview?transcript=OTTDART00000017506&db=core
ENSDART00000043446	2.049 up	0.0476	http://www.ensembl.org/Danio_rerio/transview?db=core;transcript=ENSDART00000043446
ZV700S00000108	2.050 up	0.0133	http://www.ncbi.nlm.nih.gov/sites/entrez?db=nucest&cmd=search&term=BC044204.1
NM_001089577	2.074 up	0.045	http://www.ncbi.nlm.nih.gov/entrez/viewer.fcgi?db=nuccore&id=147906215
TC235098	2.117 up	0.0228	http://compbio.dfci.harvard.edu/tgi/cgi-bin/tgi/tc_report.pl?gudb=zfish&tc=TC235098
OTTDART00000032204	2.143 up	0.0123	http://vega.sanger.ac.uk/Danio_rerio/transview?transcript=OTTDART00000032204&db=core
ZV700S00000671	2.160 up	0.0122	http://www.ncbi.nlm.nih.gov/UniGene/clust.cgi?ORG=Dr&CID=132802
OTTDART00000008169	2.162 up	0.0477	http://vega.sanger.ac.uk/Danio_rerio/transview?transcript=OTTDART00000008169&db=core
TC240825	2.231 up	0.0035	http://compbio.dfci.harvard.edu/tgi/cgi-bin/tgi/tc_report.pl?gudb=zfish&tc=TC240825
TC263196	2.531 up	0.015	http://compbio.dfci.harvard.edu/tgi/cgi-bin/tgi/tc_report.pl?gudb=zfish&tc=TC263196
BM185817	2.871 up	0.0346	http://www.ncbi.nlm.nih.gov/sites/entrez?db=nucest&cmd=search&term=BM185817
ZV700S00005943	3.120 up	0.0033	http://www.ncbi.nlm.nih.gov/UniGene/clust.cgi?ORG=Dr&CID=81057

AgNPs were separated and analysis subjected to the type of AgNP used during exposure (see Table 8), which indicated closely matched numbers of fold change greater than 4. These transcripts are detailed in Table 9, and compared expression changes against ionic form exposure.

Table 8: Gene number group on basis on fold change (>1.5, 2.0, and 4.0) for each AgNP stabiliser/capping agent, against control non-exposed population

	No. of genes with fold change greater than		
AgNP	>1.5	>2	>4
TSC	1442	164	5
PVP	710	84	5
Thiol (MHA)	1752	184	6
Bio	698	70	2

It is noteworthy that, when examined individually, and examined at >4 fold change, each of the four stabilising agents have two transcripts in common. This appears to be unique to AgNPs, as these genes, though they altered expression when exposed to Ag^{+} conditions, are not among the most prominent (Table 9). Though the true association and significance of these findings remain to be substantiated, the data obtained from this study suggests that a transcriptomic profiling may be suitable as an impact assessment approach and also as a potential monitoring tool, perhaps through the utilisation of a reduced number of transcripts for analysis.

Table 9: Transcript information for greater than 4 fold change from each AgNP stabiliser/capping agent used during exposure studies

AgNP type	Transcript information for those identified to be greater then 4 fold change			
TSC	Transcript_SEQ_ID	Fold change	P value	URL
	OTTDART00000008863	5.815 down	0.00193	http://vega.sanger.ac.uk/Danio_rerio/transview?transcript=OTTDART00000008863&db=core
	OTTDART00000002061	4.215 down	0.00204	http://vega.sanger.ac.uk/Danio_rerio/transview?transcript=OTTDART00000002061&db=core
	ZV700S00003628	3.076 down	0.00338	http://www.ncbi.nlm.nih.gov/UniGene/clust.cgi?ORG=Dr&CID=121926
	NM_001081689	2.329 down	0.0105	http://www.ncbi.nlm.nih.gov/entrez/viewer.fcgi?db=nuccore&id=126157454
	ZV700S00005943	3.120 up	0.0033	http://www.ncbi.nlm.nih.gov/UniGene/clust.cgi?ORG=Dr&CID=81057
PVP	Transcript_SEQ_ID	Fold change	P value	URL
	OTTDART00000008863	6.154 down	0.00184	http://vega.sanger.ac.uk/Danio_rerio/transview?transcript=OTTDART00000008863&db=core
	OTTDART00000002061	3.815 down	0.00314	http://vega.sanger.ac.uk/Danio_rerio/transview?transcript=OTTDART00000002061&db=core
	ZV700S00006123	2.373 down	0.0526	http://www.ncbi.nlm.nih.gov/UniGene/clust.cgi?ORG=Dr&CID=80239
	AI353629	1.584 up	0.264	http://www.ncbi.nlm.nih.gov/sites/entrez?db=nucleotide&cmd=search&term=AI353629
	OTTDART00000008169	2.162 up	0.0477	http://vega.sanger.ac.uk/Danio_rerio/transview?transcript=OTTDART00000008169&db=core
Thiol	Transcript_SEQ_ID	Fold change	P value	URL
	OTTDART00000008863	5.246 down	0.00243	http://vega.sanger.ac.uk/Danio_rerio/transview?transcript=OTTDART00000008863&db=core
	OTTDART00000002061	3.495 down	0.00334	http://vega.sanger.ac.uk/Danio_rerio/transview?transcript=OTTDART00000002061&db=core
	OTTDART00000030558	3.342 down	0.00924	http://vega.sanger.ac.uk/Danio_rerio/transview?transcript=OTTDART00000030558&db=core
	TC258256	3.083 down	0.000544	http://compbio.dfci.harvard.edu/tgi/cgi-bin/tgi/tc_report.pl?gudb=zfish&tc=TC258256
	ZV700S00003628	3.076 down	0.00338	http://www.ncbi.nlm.nih.gov/UniGene/clust.cgi?ORG=Dr&CID=121926
	BM185817	2.871 up	0.0346	http://www.ncbi.nlm.nih.gov/sites/entrez?db=nucest&cmd=search&term=BM185817
Bio	Transcript_SEQ_ID	Fold change	P value	URL
	OTTDART00000008863	5.944 down	0.00191	http://vega.sanger.ac.uk/Danio_rerio/transview?transcript=OTTDART00000008863&db=core
	OTTDART00000002061	4.112 down	0.00217	http://vega.sanger.ac.uk/Danio_rerio/transview?transcript=OTTDART00000002061&db=core
Ionic Exposure	Transcript_SEQ_ID	Fold change	P value	URL
	TC246716	3.068 down	0.0175	http://compbio.dfci.harvard.edu/tgi/cgi-bin/tgi/tc_report.pl?gudb=zfish&tc=TC246716
	ENSDART00000105574	2.277 down	0.0346	http://www.ensembl.org/Danio_rerio/transview?db=core;transcript=ENSDART00000105574
	OTTDART00000017506	2.006 down	0.0183	http://vega.sanger.ac.uk/Danio_rerio/transview?transcript=OTTDART00000017506&db=core
	OTTDART00000028253	1.809 down	0.0255	http://vega.sanger.ac.uk/Danio_rerio/transview?transcript=OTTDART00000028253&db=core
	NM_001033093	1.426 down	0.27	http://www.ncbi.nlm.nih.gov/entrez/viewer.fcgi?db=nuccore&id=74315903
	ENSDART00000052235	1.396 down	0.264	http://www.ensembl.org/Danio_rerio/transview?db=core;transcript=ENSDART00000052235
	ENSDART00000063754	1.213 down	0.495	http://www.ensembl.org/Danio_rerio/transview?db=core;transcript=ENSDART00000063754
	TC240885	1.083 down	0.777	http://compbio.dfci.harvard.edu/tgi/cgi-bin/tgi/tc_report.pl?gudb=zfish&tc=TC240885

Further analysis and validation is required before individual transcripts or grouped transcripts can be proposed as AgNPs' monitoring tools.

Access to these data sets will be made available from NUI Galway upon request.[14]

[14] Please contact authors for dataset access, available through the Glycoscience group, NUI Galway, Ireland webpage, under the EPA link.

5.3 Further Applications of Transcriptomics in ENM and ENP Exposure

The potential application of transcriptomics has been explored within this study. As the need for an increasing number of ENMs to be assessed for exposure risk grows, a need for a highly informative, standardised, and rapid analysis is required. Unlike a number of existing approaches, transcriptomics presents the potential for a complete biological effect to be monitored and assessed.

Altered gene expression within a model system can be contributed as altered regulation of RNA expression, due to expose stimuli. However, limitations in the interpretation of altered expression profiles require functional confirmation studies to establish valid biomarkers. Initial transcriptomic datasets can aid in the identification of future proteomic targets to be assessed as functional biomarkers. The transcriptomic approaches applied within this study are still early-stage tools, in both environmental management and NP risk assessment. In keeping with observations from within human clinical transcriptomic analysis, a number of integrated genes are typically only marginally regulated, on an up-down basis. Therefore, all genes indicating a fold change of 1.5 or greater require further analysis and laboratory investigation to propose or exclude potential biomarkers of AgNP exposure effect.

6. Conclusions and Recommendations

6.1 Conclusions

There continues to be a need for reliability, accuracy and consistency in the testing and reporting of nanoparticle toxicity for environmental monitoring and surveillance, industrial safety and human health risk. Classical toxicology approaches may be utilised, albeit modified from existing protocols, to suit these novel materials, with adherence and adaption to the 3 Rs' philosophy; reduction, refinement and replacement (Scholz et al., 2008; Warheit & Donner, 2010). With the unique attributes and properties of these ENMs and ENPs, they represent challenges beyond standard testing procedures, with requirements for greater chemical and physical material characterisation. At the outset of this work, the authors hypothesised that AgNP preparations with unique physicochemical parameters would produce different exposure effects and toxicity levels (LC_{50}), therefore presenting unique environmental risks including persistence, based upon altered stabilisation and physicochemical parameters (El Badawy et al., 2010). Using dose-response experiments, the authors determined the toxic potency of the tested AgNPs, taking into account bulk silver and ionic silver ($AgNO_3$), thereby permitting the determination of the potential 'nano' effects, removing observations attributable to bulk and ionic forms.

Metal ENMs/ENPs are anticipated to aggregate and in turn precipitate, thus forming metal clumps and complexes, depending on the environment (Petosa et al., 2010; Stebounova et al., 2010). These changes directly alter any risk assessment interpretation of initial material testing. Roles associated or attributed to a singular physicochemical parameter remain to be further validated, with a need to look both at (i) a broad set of ENPs of a single composition and (ii) applied more generally to a number of particle types and compositions. Reports have indicated the roles that particle size, surface chemistry, shape, surface area and stabilisation agents play in contributing directly with interaction, aggregation states, particle uptake, bioaccumulation and translocation (El Badawy et al., 2011; Fubini et al., 2010; Jiang et al., 2008).

Regarding physicochemical characterisation of the AgNPs within this reported work, relative stability and behaviour was observed throughout, in stock preparations. The greatest modification during initial characterisation was across the determined ζ-potential (Table 2). Minimal ζ-potential values may be associated with shielding effects in the case of AgNPs capped with agents, such as PVP or gelatine, both neutral polymers (Charles et al., 2010). AgNPs used within this study have also undergone characterisation throughout exposure conditions (Section 4). AgNPs have been exposed and presented in a range of aquatic and media environments for exposure assessment. Across this range of test environments, from seawater (*H. echinata*), sterile egg water (zebrafish) and numerous bacterial media types, the AgNPs displayed change from aggregation to

complete dissolution into Ag^+, and complex formation to stability in 'nano' form. AgNP stability and behaviour are of major concern within nanotechnology, are key parameters in risk assessment (Petosa et al., 2010), and are key issues in the completion of life-cycle assessment of ENPs (Mueller & Nowack, 2008; Petosa et al., 2010; Som et al., 2010).

Indeed, it is noteworthy that within bacterial testing, the immediate effect of culture media was observed to have dramatic effects on the AgNP preparations when alterations to the form and concentration of silver were presented to the bacteria. Immediate outcomes indicated that all such future studies should assess and consider (1) the test bacteria species, (2) suitability of media and (3) a suitable endpoint of measurement. Without the monitoring and assessment of the test system, dissolution and ionic effects could easily be reported as 'nano' attributed effects, when it is a function of ionic concentration.

Dissolution profiles, demonstrating the release of Ag ions from the AgNPs (with reference to FSEW), do not support the zeta potential shifts alone throughout. Surface area, morphology, energy, adsorbing species, and aggregation are all relevant parameters in dissolution properties (Borm et al., 2006). These rates may be, in this test system, attributed to a combination of capping agents, energy and aggregation.

6.1.1 H. echinata

Initial testing and exposure work was performed using the marine invertebrate, *H. echinata* (Section 4.3). The use and development of *H. echinata* as a model system for laboratory application is in compliance with the 3Rs' directive. Further to this, the adult stage of *H. echinata* and other cnidarians also contains a population of i-cells which are capable of giving rise to both somatic tissues and gametes (Kunzel et al., 2010), and based upon building literature, it has been proposed as an excellent model for research on stem cells, metamorphosis, and allorecognition (Kraus et al., 2014). However, within this study, the authors were unable to utilise this system to its full potential. CsCl-induced metamorphoses developmental changes were noted for both exposed and non-exposed larvae. The authors believe that this may be due to a population-associated infection or pre-existing environmental stress, as these larvae failed to develop to adult stage. This being the case, the authors have not presented exposure data from this system. Within NUI Galway, under the guidance of Dr. Uri Frank [15], this model is under development and becoming established both for biomedical research and environmental monitoring.

6.1.2 Bacterial Systems

The utilisation of bacteria under ISO11384 and CLSI testing guidelines are now standard assessment protocols for the determination of exposure risk, often as preliminary testing to predict human exposure risk. However, within this report, the authors have observed a number of factors which should be considered when undertaking this assessment.

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There are widspread reports of the profiling of ENMs utilisng bacterial systems across literature. Within this work, the authors chose to use recognised CLSI-established protocols, which would permit the determination of bacteriocidal and bacteriostatic effects of the AgNPs using two reference standard test bacteria, *E. coli* and *S. aureus*. As referred to previously, recommended standard media was found to have detrimental effects on the presentation and the stability of the AgNPs. Though observed here, the authors are unaware of such reporting or consideration elsewhere in literature. This is also supported by their results, when using modified test approaches (Section 4.4.1), where observations indicate that Ag^+ has a greater toxicity than AgNP exposure. Under standard testing, without consideration or adaption for particle dissolution and aggregation, AgNPs appear to have greater toxicity – though the authors now expect this to be attributed to the media affect. When modified protocol, as proposed herein, is utilised, initial effects are greatest with Ag^+ exposure.

At the concentration ranges tested for bacteriostatic/bacteriocidal effects, both AgNPs and Ag^+ demonstrated similar level effects, though for viability AgNPs produced complete bacteriocidal effects at concentrations <10 ppm, while Ag+ required concentrations from 10 – 100 ppm for a similar outcome. Further protocol refinement is required for the standardisation of such bacteriocidal/bacteriostatic assessment, allowing for media influences on outcomes.

ISO 11384 testing utilising *V. fischeri* was expanded to permit the inclusion of *V. harveyi* (Section 4.4.2), following international accepted protocols for exposure. Though a short exposure assessment (60 minutes), the authors found the determination of bioluminescence to be a rather simplistic measurement. Therefore, the authors added a viability assessment of culture after 60 minutes' exposure. Bioluminescence-based assays, such as those contained within ISO 11384, are relatively rapid and permit application in the field, and are therefore valuable tools for environmental monitoring. AgNPs here demonstrated an IC_{50} range of 2.2 to 5.8 ppm (Figure 9), which is a tight range containing all preparations. Ag^+ was recorded as having an IC_{50} range of 0.48 ± 0.04 ppm (Figure 10), indicating that Ag^+ has a greater effect on the natural bioluminescence of the bacteria. However, similar enumeration of viable cells was observed for match concentrations of AgNPs compared directly to Ag^+. Therefore, the effects of 'ion' or 'nano' forms on true bacterial populations may not be as dramatically different as first anticipated. This was further highlighted by direct comparison of a 30-minute incubation (Figure 11), where Ag^+ demonstrated a 5 – 10 fold increase in toxicity based upon bioluminescence. Indeed, a 2.5 fold difference can be observed between the two vibrio species. With suitable control and incorporating a viability step, the ISO 11384 may serve as a suitable bacterial assay for ENMs' assessment.

6.1.3 Danio rerio (Zebrafish)

The early-developing zebrafish embryo is generally considered to be the most sensitive stage in the life-cycle of a teleost, being particularly sensitive to low level,

environmentally-relevant exposure concentrations (OECD guidelines 203, 210, 212). A number of citations have reported ENP, ENM lethal concentrations and developmental effects, including with zebrafish, while numerous cell-based profiling exposures have been performed and the mechanisms of this toxicity proposed (Asharani et al., 2011; Bar-Ilan et al., 2009; Lewinski et al., 2008; Yen et al., 2009). Environmental exposure scenarios require a consideration of a potential or an estimated exposure concentration, to permit initial range-finding toxicity evaluation (Joner et al., 2007; Stone et al., 2010). Levels and concentrations utilised in studies need to focus on potential exposure scenarios and not affect leading exposure studies.

The authors determined that the range of testing should be from 0.001 to 100 ppm, with 100 ppm extending beyond the concentration required to observe and report exposure effects. The risk of environmental exposure ever reaching or going beyond 100 ppm is considered highly improbable, without accident industrial release. Exposures performed using zebrafish, and followed for up to 72 hours under exposure conditions, determined a range of LC_{50} values from 0.4 ppm up to 47.89 ppm at 24 hpf. A range of toxicity of two orders of magnitude across all AgNPs was tested. This range reduced to 0.04 to 2.6 ppm after 48 hpf.

The toxicity range is indicative of multiple parameters contributing to exposure outcome, immediately suggesting a single AgNP preparation cannot be used to infer toxicity levels on other preparations. Reports in literature to date have reported toxicity values ranging from 25 to 50 ppm (Asharani et al., 2008), 100 μg/ml (equivalent to100 ppm) resulting in 43 ± 7 percent mortality (Asharani et al., 2011), ≥3μM Ag^+ (Powers et al., 2010) and ranging from 93.31 to 137.26 μM (equivalent to ~ 0.9 to 1.2 ppm) at 120 hpf, depending upon the diameter (Bar-Ilan et al., 2009). The most toxic AgNPs identified here were the PVP preparations, in relation to zebrafish testing, and TSC and PVP for bacterial systems tested. A similar nanoparticle preparation, stabilised with PVP, has previously been tested and reported (Asharani et al., 2011). Physicochemical variation, along with concentration and molecular weight of PVP used, may be contributory factors in exposure outcome across studies (Chung et al., 2008). Deviations in the reported exposure toxicity values, due to inter-laboratory differences in handling, zebrafish phenotype, end points used, or statistical approach to data interpretation, exist.

6.1.4 Summary

Overall, these results suggest the toxicity of AgNPs in aquatic species and bacterial systems depends on a number of factors, with the key factors being; capping agents, zeta potential and Ag+ dissolution rate.

The utilisation of capping agents in the synthesis of AgNPs is to prevent aggregation through electrostatic and/or steric repulsion and for functionalisation. The most prevalent capping agents currently in use are citrate, sodium borohydride and polyvinylpyrrolidone (Tang et al., 2007). The dissolution of AgNPs and the rapid increase in Ag+ within test solution (FSEW, averaging ~25 % of total silver in 120 hours and media for bacteria ranging from 12-70% after 24 hours)

indicates an important relationship with observed toxicity. It should be noted that the dissolution rate is related to size and surface area, therefore the overall shape of the particle is critical.

Key to assessment of exposure effects is the selection of suitable model systems and reference control materials. Here, $AgNO_3$ has been used as the precursor for AgNP synthesis, the silver source used to produce the tested AgNPs, and as the ion donor control (Ag^+), which permitted nano – bulk – ionic effects to be observed and attributed.

The level at which AgNPs, and indeed ENPs/ENMs in general, may be present in the environment remains unknown and therefore an assumption of little or indeed no environmental impact is not possible (Tiede et al., 2009b). Ag may exist in different oxidation states, i.e. Ag^0 (metallic silver), Ag^{+1}, Ag^{+2}, and $Ag+^3$, of which the latter two are unstable in aquatic environments (Wijnhoven et al., 2009). The reactivity of Ag^0 is low. Ag^{+1} possesses a strong binding affinity for thiol and disulfide groups. The toxicity of AgNPs may be explained by several mechanisms, such as (i) the excessive binding and interaction of AgNPs directly, or in the form of Ag^{+1} preventing the uptake or exchange of media components, (ii) translocation into the organism in AgNP form, where dissolution occurs on entry in an oxidised state or (iii) translocation and irreversible accumulation. There has been a strong indication that the majority of ENPs in waters are captured by clearing sludge in wastewater treatment plants (Limbach et al., 2008; Nowack, 2009).

In this study. wide-ranging adverse effects of AgNPs in aquatic species currently supported in literature have been demonstrated, highlighting the need to assess properties, such as the dissolution rate as a measurement and the contributing factor of potential toxicity. Applications of AgNPs require carefully approach and assessment prior to large-scale commercialisation. A toxicological concern, outside of the current scope of this report, is the possible uptake of AgNPs by aquatic and marine populations. A number of studies have demonstrated the occurrence of translocation, highlighting a number of potentially-associated physicochemical properties (Krug et al., 2011; Luo, 2007) Building upon the authors' findings, further ongoing studies are directed towards the translocation, morphological and bioaccumulation of AgNP-treated embryos, at levels below the demonstrated LC_{50} values.

Transcriptomic data generated within this work (Section 5) is being further explored for the identification of potential early biomarkers in the recognition of exposure of zebrafish to AgNP. Though this is an early-stage investigation, it holds promise, as it may lead to the application of molecular markers using greater sensitivity, and has the potential to be used in additional exposure ranges beyond current exposure scenario modelling. This, in turn, may lead to or support further proteomic-based (functional marker) approaches for the environmental monitoring of ENMs.

The authors can conclude that AgNPs have the potential to cause health and associated ecotoxicological issues due to being within

the nano size range and also due to the dissolution of Ag^+ states, which are subject to the immediate environment that the test material is released into. This factor needs to be considered and addressed in the selection of suitable test parameters. The dissolution and fate of ionic Ag will be key in determining if a risk will be continuous or a single point risk, i.e. only at point of exposure, or if complex formation should be an ongoing concern. The role and contribution of physicochemical parameters, such as surface chemistry, remain to be fully understood. Questions regarding AgNP accumulation, bio persistence and translocation, in relation to physicochemical properties, need to be addressed.

6.2 Recommendations

A need for the implementation of standards as follows:

(i) Guidelines for physicochemical characterisation are required, as minimal characterisation should be required on all manufactured ENMs.

A minimal requirement of physicochemical profiling should be required for all ENMs, both at point-of-synthesis/fabrication, and throughout the life-cycle of the nanomaterial. Life-cycle assessment should also anticipate environmental release and model accordingly.

(ii) Reference standard tests for ENMs should be encouraged to be used and incorporated within exposure scenario testing and modelling.

(iii) Existing biological assays do not appear to be suitable for the determination and measurement of risk – a database of modified protocols and guidelines should be established and eventually standardised. This extends the need for studies to include more than a single system for observation.

(iv) Continued development of model systems for environmental impact assessment, such as the *H. echinata*.

(v) Further exploration into the application of high capacity, highly informative screening approaches is required, to permit regulatory decisions on both national and international levels.

This includes transcriptomics and proteomic approaches. Such technologies would permit numerous studies in parallel, monitoring numerous biological events and providing informative results in a quick manner.

All imports and exports should be required to list if they either directly contain ENMs or have been produced with the application of ENMs. ENMs could form a new listing (similar to the E-number system used for foods), where consumers and end-users can be informed with regards to what ENMs may be contained. Such a system could be linked to highlighting the potential risks or effects of these ENMs.

Within Ireland, a national database should be established, to record and monitor the industrial use and growth of the ENM field nationally. This would permit the establishment of environmental monitoring and an ENM watch list.

Project Outputs

Published Papers (Accepted at time of report preparation)

1. Cunningham, S., Brennan-Fournet, M.E., Ledwith, D., Byrnes, L., Joshi, L. Effect of nanoparticle stabilisation and physicochemical properties on exposure outcome: acute toxicity of silver nanoparticle preparations in zebrafish (Danio rerio). Environ Sci Technol. 2013 Apr 16;47(8):3883-92, doi: 10.1021/es303695f.

Oral Presentations

1. Cunningham, S., Brennan-Fournet, M., Ledwith, D., Voisin, M., Byrnes, L. and Joshi. L. Assessment of exposure to silver nanoparticles on Zebrafish development in relation to their physicochemical properties. Nanotoxicology 2010. International meeting, June 2010. Edinburgh Napier University, Edinburgh, Scotland.
2. Cunningham, S., Brennan-Fournet, M., Ledwith, D., Voisin, M., Byrnes, L., Boyd, A., Fleming, G. and Joshi. L. The assessment of exposure risk, persistence and accumulation of nanoparticle silver in the aquatic and marine environment. 3rd NanoImpactNet Conference; For a healthy environment in a future with nanotechnology. Lausanne, Switzerland; February 2011.

Poster Presentations

1. Cunningham, S., Brennan-Fournet, M., Ledwith, D., Voisin, M., Byrnes, L. and Joshi, L. Assessment of exposure to silver nanoparticles on Zebrafish development in relation to their physicochemical properties. Nanotoxicology 2010. International meeting, June 2010. Edinburgh Napier University, Edinburgh, Scotland. Poster and Selected for platform presentation.
2. Cunningham, S., Brennan-Fournet, M., Ledwith, D., Voisin, M., Byrnes, L. and Joshi, L. Developmental and morphological assessment of Zebrafish up to 72 hours post fertilisation under exposure to nanoparticle silver.
3. Voisin, M., Connolly, M., Ledwith, D., Boyd, A., Joshi, L., Fleming, G., Brennan-Fournet, M. and Cunningham, S. Toxicity screening of Ag and Zn nanoparticles using ISO and CLSI bacterial model systems.
4. Cunningham, S., Brennan-Fournet, M., Ledwith, D., Voisin, M., Byrnes, L., Boyd, A., Fleming, G. and Joshi, L. 3rd NanoImpactNet Conference. For a healthy environment in a future with nanotechnology. Lausanne, Switzerland; February 2011. Poster and Invited platform presentation.

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Acronyms and Annotations

AAS	Atomic Absorption Spectrometry
Ag	Silver
Ag^{+}, Ag^{2+}, and Ag^{3+}	Ionic Silver
Ag^{0}	Zero-Valent Silver
AgCl	Silver Chloride
AgS	Silver Sulphate complexes
Ag_2S	Silver Sulphide or Argentite
$AgNO_3$	Silver Nitrate
AgNPs	Silver Nanoparticles
AFM	Atomic Force Microscopy
AO	Acridine Orange
BET	Brunauer, Emmett and Teller
BSA	Bovine serum albumin
Ca	Calcium
$CaCl_2$	Calcium Chloride
CFU	Colony Forming Unit
Cl^{-}	Chloride Ion
CLSI	Clinical and Laboratory Standards Institute
CsCl	Cesium Chloride
°C	Degrees Celsius
DAPI	4',6-diamidino-2-phenylindole
d.f.	degrees of freedom
DLS	Dynamic Light Scattering
DNA	Deoxyribonucleic Acid
DO	Dissolved Oxygen
EC_{50}	Concentration resulting in a Non-Lethal Toxic Effect in 50% of test population
ECETOC	European Centre for Ecotoxicology and Toxicology of Chemicals
EDS	Energy-Dispersive X-Ray Spectroscopy
ENM	Engineered nanomaterials
ENP	Engineered nanoparticles
EPA	Environmental Protection Agency
EU	European Union
FDR	False Discovery Rate
FSEW	Filter Sterilised Egg Water
FT-IR	Fourier Transform Infrared Spectroscopy
HCl	Hydrochloric Acid
HDD	Hydrodynamic Diameter
hpf	hours post fertilisation

HPLC	High Performance Liquid Chromatography
H_2O	Water
HNO_3	Nitric Acid
HR-TEM	High-Resolution Transmission Electron Microscopy
IC_X	Concentration Causing an Inhibitory Response in X% of the Test Population
ICP-MS	Inductively Coupled Plasma – Mass Spectroscopy
IDL	Instrumental Detection Limit
ISE	Ion-Selective Electrode
ISO	International Organisation for Standardisation
INH	Inhibitory effect (reported in %)
λ_{max}	Lambda max, the wavelength at which the maximum fraction of light is absorbed by a solution
LB	Luria Broth
LC_{10}	Concentration Resulting in Mortality of 10% of the Population
LC_{50}	Concentration Resulting in Mortality of 50% of the Population
LSPR	Localised Surface Plasmon Resonance
MATC	Maximum Acceptable Toxic Concentration
MDD	Manufacturer Determined Diameter
MDL	Method Detection Limit
MHA	Mercaptohexadecanoic Acid
MHB	Muller Hinton Broth
mg	milligramme
MIC	Minimum Inhibitory Concentrations
MINChar	Minimum Information for Nanomaterial Characterisation
mL	millilitre
mM	millimole
MRSA	Methicillin Resistant S.aureus
mV	milli-Volts
µg	microgramme
µl	microlitre
µM	micromole
NA	Nutrient Agar
Na^+	Sodium Ion
NaCl	Sodium Chloride
NCCLS	National Committee for Clinical Laboratory Standards
N.G.	No Growth observed (used with reference to bacterial testing)
NH^{4+}	Ammonium ion
nm	nanometre
NM	Nanomaterials
NPs	Nanoparticles
NO^{3-}	Nitrate ion

OECD	Organisation for Economic Co-operation and Development
OD	Optical Density
PBS	Phosphate Buffered Saline
PFA	Paraformaldehyde
PCA	Principal Component Analysis
PEC	Predicted Environmental Concentration
PEN	Woodrow Wilson Centre's Project on Emerging Nanotechnologies
PFA	Paraformaldehyde
ppb	parts per billion
ppm	parts per million
ppt	parts per trillion
PSSS	Poly Sodium-4-Styrene Sulphonate
PVP	Polyvinylpyrrolidone
REACH	Registration, Evaluation, Authorisation and Restriction of Chemicals
RMA	Robust Multi-Array Analysis
RNA	Ribonucleic Acid
rpm	revolutions per minute
RLU	Relative Light Units
SCENIHR	Scientific Committee on Emerging and Newly Identified Health Risks
SEM	Scanning Electron Microscopy
SEM-EDX	Scanning Electron Microscopy with Energy-Dispersive X-Ray Spectroscopy
SPR	Surface Plasmon Resonance
SSA	Specific Surface Area
TBS	Tris Buffered Saline
TBST	Tris Buffered Saline, with Tween 20 added
TEM	Transmission Electron Microscopy
TSC	Tri Sodium Citrate
TVC	Total Viable Count
µg/g	Microgrammes per gramme
µg/L	Microgrammes per litre
USA	United States of America
wt%	percentage weight
ζ	zeta potential

EPA Research Report 150

Assessment of exposure of marine and freshwater model organisms to metallic nanoparticles

Authors: Dr. Stephen Cunningham and Professor Lokesh Joshi

epa Research
Climate - Water - Sustainability
Identifying pressures • Informing policy • Developing solutions

The applications and use of nanoparticles continues to extend across technology sectors, requiring suitable assessment and monitoring of human and environmental impacts. In this report, a study was undertaken to determine the potential exposure limits of silver nanoparticles, and to identify attributes which may alter their persistence and accumulation within marine and fresh water systems.

Identifying Pressures

This report demonstrates the number of applications and uses for which nanotechnologies have currently been explored, indicating the extent of both human and environmental exposure to various nanoparticles that require careful monitoring and assessment.

Informing Policy

From this work it is concluded that silver nanoparticles have the potential to cause health and ecotoxicological effects associated both with their 'nano' physicochemical properties and from the dissolution of silver (Ag+) states. Both factors need to be considered and addressed in the selection of suitable test parameters. The dissolution and fate of ionic silver will be key in determining if a risk will be, single point (i.e. only at point of exposure), continuous or if complex formation should be a monitoring concern.
The role and contribution of physicochemical parameters such as surface chemistry remain to be fully understood. Questions regarding silver nanoparticle accumulation, bio-persistence and translocation with relation to physicochemical properties remain to be addressed.

Developing Solutions

This report highlights the need for the implementation of guidelines for: (a) the physicochemical characterisation of manufactured engineered nanoparticles; (b) Life-cycle assessment to anticipate environmental release and model accordingly; (c) biological impact assessment, as existing assays do not appear suited to the determination and measurement of risk – a database of modified protocols and guidelines should be established and eventually standardised. Proposed alternatives and suggestions are made throughout the report.

Within Ireland, a national database should be established, to record and monitor the industrial use and growth of the engineered nanoparticle field nationally. This would permit the establishment of environmental monitoring and an engineered nanoparticle watch list.

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