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How to cite this thesis
THE AQUATIC TOXICOLOGY OF GOLD NANOPARTICLES

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Thesis
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 Philosophiae of Doctor
In
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In the
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University of Johannesburg

Supervisor: Prof. V. Wepener

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Summary

Toxicity laboratory tests are used world-wide to manage environmental resources such as water quality and are considered to be the first step in a tiered approach in establishing guidelines for setting maximum acceptable concentrations of specific pollutants. The aim of this study was to evaluate the aquatic toxicology of gold nanoparticles (nAu) using a combination of acute and chronic ecotoxicological bioassays. Particle distribution and agglomeration in the reconstituted water medium was evaluated using several microscopy techniques as well as characterization tools which included dynamic light scattering and Fourier transform infrared spectrometry. Range finding exposure tests were done to determine the LC50 of nAu in *Pseudokirchneriella subcapitata, Daphnia pulex, Daphnia magna* and 14 day old *Danio rerio* using standard OECD protocols where ionic gold and solvent controls were also included. The acute toxicity showed a bimodal response for nAu and an LC50 was only calculated for *D. pulex* at 75.314mg/l while ionic gold concentrations had an LC50 with a 95% confidence level at 0.01mg/l and 4.85mg/l daphnia and 14 day old zebrafish respectively. The *in vivo* nAu distribution and mechanical effect was observed using several microscopy techniques which showed the attachment of nAu to the surface of algae and daphnia, uptake via the gills and storage in cartilage and muscle tissue in zebrafish as well as nAu ingestion of agglomerates visible to the naked eye (40mg/l and 45mg/l). A reproductive study in *D. magna* to determine the effect of nAu on molting patterns related to reproduction showed that as exposure concentrations increased daphnia molting also increased and a lower correlation was seen between reproduction and molting as concentrations increased. Sub lethal testing was done by exposing adult zebrafish (*D. rerio*) to nAu for 96 hours at a concentration range of 5mg/l to 45mg/l with a 5mg/l interval between concentrations. Male fish livers were stored in RNA later and grouped samples were used for DNA microarray. Real time polymerase chain reactions (RT-PCR) were used to determine changes in gene expression in the liver comparing male fish to female fish. The results obtained from scanning the gene chip gave an indication of events in the cell based on 15 618 genes being regulated. The gene ontology was further investigated using ArrayStar® to show pathway interactions as well as the clustering of genes. The 20mg/l and 25mg/l nAu concentrations showed similar genetic clustering and these were related to 40mg/l; and 45mg/l respectively. By using a process of elimination genes were narrowed down to 75, genes that are not yet properly understood in the zebra fish genome and genes that had a fold change less than threefold were eliminated. Pathways were then looked at and the following genes were focused on and used
for primer design and RT-PCR: Cat, Sod, MT1, MT2, gpx1a1, Cyp1a, Cyp1c1, Cyp11a, Cyp17a1, Cyp19a1 and Bactin, which was used as endogenous control. The male fish had a higher fold change when compared to female fish and had up to a six times fold change when compared to control groups. The steroidogenesis pathway for both males and females was up regulated at the previously mentioned clustered concentrations (20mg/l, 25mg/l, 40mg/l and 45mg/l). The up or down regulation of genes was then further confirmed by looking at biomarker responses of acetylcholine esterase (AChE), metallothionein (MT), cytochrome P450 (CYP450 - organic compounds e.g. EDCs) and oxidative stress biomarkers explored were protein concentrations of catalase (CAT), superoxide-dimutase (SOD), lipid peroxidation (LP), protein carbonyls (PC) and non-enzymatic reduced glutathione (GSH) in whole body organisms to explore whether genetic changes were influential of circulating protein levels after a 96 hour period. The affected biomarkers were PC which showed protein damage, SOD showing slight oxidative stress and MDA which showed a decrease while an increase in concentration would be indicative of biomarker response, the GSH showed an increase in activity which could be related to up regulation of the GPX1 gene. The CYP450 showed a response in nAu concentrations but no response (comparable to the control) in ionic gold concentrations. With the use of different microscopy techniques particle distribution and bioaccumulation could be observed in muscle tissue and the intestine of zebrafish. It was also seen on the carapace and within the gut of daphnia altering molting patterns and reproduction. This study gave evidence that the mechanical effects of nAu with aquatic organisms was more hazardous than the chemical effects seen in other toxicant studies and a shift needs to ensue to focus more research on these mechanical toxicity effects in nanoparticle aquatic ecotoxicity studies.
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List of Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>% O$_2$</td>
<td>Percentage dissolved oxygen</td>
</tr>
<tr>
<td>°C</td>
<td>Degrees celsius</td>
</tr>
<tr>
<td>µl</td>
<td>Microgram per liter</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------------------------------------------</td>
</tr>
<tr>
<td>µm</td>
<td>Micro meter</td>
</tr>
<tr>
<td>AChE</td>
<td>Acetylcholine esterase</td>
</tr>
<tr>
<td>ACS</td>
<td>American Chemical Society</td>
</tr>
<tr>
<td>ADP</td>
<td>Adenosine diphosphate</td>
</tr>
<tr>
<td>Bactin</td>
<td>Beta actin</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>CAT</td>
<td>Catalase</td>
</tr>
<tr>
<td>CEA</td>
<td>Cellular energy allocation</td>
</tr>
<tr>
<td>CYP</td>
<td>Cytochrome</td>
</tr>
<tr>
<td>D.O.</td>
<td>Dissolved oxygen</td>
</tr>
<tr>
<td>DEESEP</td>
<td>Direct Estimation of Ecological Effect Potential</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribose nucleic acid</td>
</tr>
<tr>
<td>DTA</td>
<td>Direct Toxicity Assessment</td>
</tr>
<tr>
<td>DWA</td>
<td>Department of Water Affairs</td>
</tr>
<tr>
<td>$E_a$</td>
<td>Energy reserves/energy available</td>
</tr>
<tr>
<td>EC</td>
<td>Electrical conductivity</td>
</tr>
<tr>
<td>$E_c$</td>
<td>Energy consumption</td>
</tr>
<tr>
<td>EDS</td>
<td>Energy dispersant spectrum</td>
</tr>
<tr>
<td>EF1a</td>
<td>Elongation factor</td>
</tr>
<tr>
<td>ETS</td>
<td>Electron transport system</td>
</tr>
<tr>
<td>FFT</td>
<td>Fast fourier transform</td>
</tr>
<tr>
<td>FTIR</td>
<td>Fourier Transform Infrared Spectroscopy</td>
</tr>
<tr>
<td>g/l</td>
<td>Grams per liter</td>
</tr>
<tr>
<td>GO</td>
<td>Gene ontology</td>
</tr>
<tr>
<td>Gpx1a</td>
<td>Glutathione peroxidase</td>
</tr>
<tr>
<td>GSH</td>
<td>Non-enzymatic reduced glutathione</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>h</td>
<td>Hours</td>
</tr>
<tr>
<td>ICP-MS</td>
<td>Inductively Coupled Plasma Mass Spectrometry</td>
</tr>
<tr>
<td>ICP-OES</td>
<td>Inductively coupled plasma atom emission spectroscopy</td>
</tr>
<tr>
<td>IVT</td>
<td>In vitro transcription</td>
</tr>
<tr>
<td>LP</td>
<td>Lipid peroxidation</td>
</tr>
<tr>
<td>M</td>
<td>Molar</td>
</tr>
<tr>
<td>MANOVA</td>
<td>Multiple analysis of variance</td>
</tr>
<tr>
<td>MDA</td>
<td>Malondialdehyde</td>
</tr>
<tr>
<td>mg/l</td>
<td>Milligrams per liter</td>
</tr>
<tr>
<td>MT</td>
<td>Metallothionein</td>
</tr>
<tr>
<td>nAu</td>
<td>Gold nanoparticles</td>
</tr>
<tr>
<td>NBF</td>
<td>Neutral buffered formalin</td>
</tr>
<tr>
<td>NCBI</td>
<td>National Center for Biotechnology Information</td>
</tr>
<tr>
<td>NIOH</td>
<td>National Institute for Occupational Health</td>
</tr>
<tr>
<td>nm</td>
<td>Nano meter</td>
</tr>
<tr>
<td>OECD</td>
<td>Organization for Economic Co-operation and Development</td>
</tr>
<tr>
<td>PC</td>
<td>Protein carbonyls</td>
</tr>
<tr>
<td>RIN</td>
<td>Ribose nucleic acid integrity number</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribose nucleic acid</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>Real time polymerase chain reaction</td>
</tr>
<tr>
<td>s</td>
<td>Second</td>
</tr>
<tr>
<td>SEM</td>
<td>Scanning electron microscopy</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of the mean</td>
</tr>
<tr>
<td>SOD</td>
<td>Superoxide-dimutase</td>
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</table>
SPSS  Statistical Package for Social Science
TDS  Total dissolved solids
TEM  Transmission electron microscopy
UJ  University of Johannesburg
WET  Whole Effluent Toxicity
ΔΔCt  Quantitation comparative Ct

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Acknowledgments

Firstly none of this would have been possible without the financial assistance approved by the South African Department of Science and Technology. I would like to thank the University of Johannesburg for continued assistance throughout this journey to the PhD that has lasted a decade; this would include the Department of Zoology support staff and all members of the Faculty of Science. Funding for the project was made possible through the Department of Science and Technology (OECD Nanogold project) and the University of Johannesburg. I would like to say a big thank you to Frankline Keter (Mintek) for manufacturing and supplying the gold nanoparticles used in the study. I would like to send heartfelt appreciation to Prof Victor Wepener for always believing in me to “let’s just try” and going out of his way to be an unforgettable mentor. I have been fortunate to have a wonderful team and I would like to specifically thank Prof Johan Janse van Vuren for his experienced advice and laughs, Dr Richard Greenfield for always being willing to assist on all fronts, Prof Ina Wagenaar and Dr JC van Dyk for assisting me with histology and always supporting me as a person, R Gerber and C Edwards for understanding that biomarkers are tedious and sometimes sympathy helps, Dr Nel for her continued expert advice on daphnia cultures and feeding protocols, E Kroukamp (Spectrum Analytical Facility) for all metal analysis, L Luhkele (Doornfontein campus) for input on nanoparticle exposures, T. James and all other students and staff in the laboratory that assisted me in any way. The Departmental colloquiums, Nano Workshop and ISTA16 conference organizers for allowing me the continued opportunity to present preliminary work and for feedback on the project. I would like to thank Prof Mary Gullinam, Kailen Boodhia, Melissa Vetten (NIOH) for allowing me to use their facilities for microscopy and providing advice on new findings within cell line research wherever possible. Prof Kerstin Hund-Rinke (Fraunhofer Germany) for allowing me the opportunity to work in her laboratory and introducing me to techniques associated with nanoparticle toxicity testing. A sincere thank you to the following sensei: Prof Mayumi Ishizuka, Prof Yoshinori Ikenaka, Associate Prof Shouta Nakayama and all students at Hokkaido University School Veterinary Medicine (Japan) for assistance with all Danio rerio genetics. Thank you to Dr A Jordaan (North-West University) for all assistance with microscopy. I would like to thank my all friends and family, specifically my little brother, for keeping me sane and being extremely supportive. To my parents Barry and Christine Botha who have always believed in my dreams and showed me the ways to greet them. And to my partner, Elca Jacobs, for standing by me on days when I didn't think I could stand with anyone. It truly takes a village. My sincerest thank you.
Chapter 1: Study rationale

1.1. Brief introduction to gold nanoparticles (nAu)

In recent years gold nanoparticles (nAu) have been studied and developed within the biological and photothermal therapeutic contexts. The plasmon resonance on the surface of nAu has electrons that create a magnetic field when irradiated with a laser which is converted to heat (Durazo and Kompella, 2011; Akiyama et al., 2009; Boisselier and Astruc, 2009). The major clinical interest is within the application of novel drug delivery systems and targeted delivery into cells (Yah, 2013; Janowski et al., 2012; Alanazi et al., 2010; Fernandes et al., 2010) as well as microelectromechanical systems (Voskerician et al., 2003) and diagnostic systems (El-Sayed et al., 2005). Exposure of nanoparticles can occur in different stages of the lifecycle of the product, from their synthesis, application (which can include ingestion or intravenously), weathering to their waste disposal. Due to little research within this field there are currently no guidelines for their use, therefore the previously mentioned exposure routes could build up in the environment (Yah, 2013; Arora et al., 2012; Byrne et al., 2010; Delgado, 2010; Lewinski et al., 2008).

Cancer is the primary target of nanoparticle delivery agents, as the conditions are favourable to the particle. The high surface area to volume ratio is advantageous for loading drug molecules in the particle surface where recognition molecules, like antibodies, may also be attached and help achieve active targeting. For metallic nanoparticles the use of light energy and heat (Alkilany, et al., 2012; Yamashita et al., 2011) may be used to actively release drugs from the particle in addition to passive release within the organism. Nanogold has a strong absorbance in the near infrared light region (Akiyama et al., 2009). For these reasons nAu are of high interest within the therapeutical and drug delivery fields, conversely the fate of the actual particle once the drug has been released is not frequently studied (Alkilany, et al., 2012; Elsaesser and Howard, 2012).
Studies which have thus far been performed on mice (Akiyama et al., 2009) and blue mussel *Mytilus edulis* (Tedesco et al., 2010a; Tedesco et al., 2010b; Tedesco et al., 2008) have had conflicting results. These studies have been based on different surface modifications, site specificity and delivery mechanisms to cells (Wang et al., 2008; Pissuwan et al., 2011; Cui et al., 2012; Alkilany et al., 2012). Cui et al. (2011) showed that gold nanoparticles have the ability to undergo endocytosis, which forms the major part of drug delivery. However, it was found that the smaller the particle, the greater the uptake and thus, the higher the toxicity within the cell. Larger particles undergo aggregation and then adsorb to the surface of the cell, as is the case in human cervical carcinomas stimulated uncontrolled growth. Therefore depending on the coating used the particle uptake will most likely alter (Oh and Park, 2014; Klaine et al., 2008).

A study within the rabbit cornea showed that after seven days the gold nanoparticle diffused out of the area but the distribution was not followed (Sharma et al., 2011). Tedesco et al. (2010) found that gold nanoparticles in blue mussel accumulated in the digestive gland with low levels present in the gills. This showed an effect on proteins, specifically thiols, where moderate oxidative stress was found. The size of the particle would affect the accumulation and uptake of the particle into the cell as found by Hirn et al. (2011) and the larger the particle the higher the uptake concentration in the liver. In a review article by Mesbahi (2010) it was stated that more animal cell lines were needed to determine and clarify dose enhancement of the particle. Aillon et al. (2009) stated that due to the characteristics of gold nanoparticles it is easily understood why these are targeted to be used for drug delivery. However, the same reasons these are so attractive for use are those which could increase the toxicity of the particle to biological systems. Elsaesser and Howard (2012) revealed that nAu can cross the materno-foetal barrier and fullerenes were fatal to mouse embryos’. There have been limited in vitro studies performed, with even less in vivo studies, which show the effect on the organism as a whole (Roy et al., 2014; Yah, 2013; Johnston et al., 2012; Boisselier and Astruc, 2009; Linkov et al., 2008; Fischer and Chan, 2007).

The proposed study would help contribute data and statistical results to this breach of scientific knowledge in the aquatic environment and would do so across different aquatic trophic levels. This study will assess how the particle could change in an aquatic environment which is where these particles would very likely eventually end up as by products and waste.
1.2. Study hypothesis

The proposed hypothesis for this study is that since nAu are being developed for use within the drug and therapeutic fields they will have a low toxicity to aquatic biota.

1.3. Aim and objectives

The aim of the study is to evaluate the aquatic toxicology of nAu using a combination of acute and chronic ecotoxicological bioassays.

To meet the aim the following objectives were set:

- Determine the physico-chemical properties of nAu and ionic Au in the aquatic exposure media to determine what changes occur over time when a supplied stock concentration is used to make up nAu concentrations in standard aquatic medium.
- Use a suite of assessments specific to nanoparticle toxicity studies, which would include Inductively Coupled Plasma Mass Spectrometry (ICP-MS), transmission electron microscopy (TEM), dynamic light scattering, Fourier Transform Infrared Spectroscopy (FTIR), to determine the characterization of nAu in aquatic media.
- Determine the acute toxicity of nAu and ionic Au using the bacteria (Vibrio sp.) bioluminescence viability test; algal (Pseudokirchneriella subcapitata Korshikov) growth inhibition test (OECD-TG); a 48h freshwater water flea (Daphnia pulex) lethality test (OECD-TG202) and 96h freshwater zebrafish (Danio rerio) lethality test (OECD-TG203).
- Establish the nAu uptake, tissue distribution and effect on P. subcapitata (algae), Daphnia magna and D. rerio (zebrafish) using standard histological processing, cryosectioning, CytoViva® dark field hyperspectral imaging, ICP-MS, scanning electron microscopy (SEM) and TEM.
- Determine whether clustering exists between genes using D. rerio as a model aquatic organism by means of GeneChip® Microarray analysis after exposure to a range of chronic exposure concentrations.
• Use a standard suite of biomarkers namely acetylcholine esterase (AChE), metallothionein (MT- metal exposure) and cytochrome P450 (CYP450 - organic compounds e.g. EDCs), and the oxidative stress biomarkers catalase (CAT), superoxide-dimutase (SOD), lipid peroxidation (LP), protein carbonyls (PC) and non-enzymatic reduced glutathione (GSH) as well as the cellular energy allocation to determine changes in circulating protein levels.

1.4. Chapters outline

Chapter one provides a brief overview of the context of the study, the study hypothesis, aim and objectives. Chapter two will discuss physico-chemical properties and particle characterization and changes to these properties in the different environmental exposure media utilized in the study. Chapter three presents the acute toxicity of nAu to aquatic organisms at different trophic levels, i.e. bacteria, algae, daphnia and zebrafish using standardized OECD protocols. The toxicity of nAu is also compared to the bulk chemical, i.e. ionic gold. Chapter four will give insight into nAu uptake, distribution and effects in algae and daphnia after acute exposures and will also include a reproductive assessment of daphnia relating to variations in molting patterns. Chapter 5 will discuss nAu uptake, distribution and effects in the model organism D. rerio across a broad range of exposure concentrations as well as several sub cellular effects including genetic fold changes in the liver comparing male and female fish, genetic relations to gene ontology and whole body biomarker responses relating to characterization results. The final chapter will relate conclusions from each chapter and give recommendations for future studies on nanoparticle toxicity.
Chapter 2: Particle characterization

2.1. Introduction

The scientific discipline of ecotoxicology has been successful in studying effects of pollutants such as metals on aquatic systems (Wepener et al., 2011; Erickson et al., 2008; van Leeuwen et al., 2005; van Leeuwen and Galceran, 2004; Wilkinson and Buffle, 2004; Pyle et al., 2002; Wood, 2001; Bervoets and Blust, 2000; Campbell, 1995; Cusimano et al., 1986). When studying these aquatic systems many parameters are routinely monitored, these include temperature, pH, conductivity, dissolved solids and oxygen concentration. With an increase in the manufacture and potential release of engineered nanomaterials into the environment, research into the way these materials should be monitored needs to be carried out (Kühnel and Nickel, 2014). It is particularly pertinent as standard protocol is applicable for exposure techniques but the monitoring protocol will no longer suffice. The nanoparticle characterization needs to be standardized so that all toxicity testing is adequate and comparable (Wepener et al., 2013; Klaine et al., 2008).

Nanoparticles come in several sizes, varying in shape, charge and a variety of chemical compositions (Shaw and Handy, 2011; Samberg et al., 2009). Their behaviour and toxicity are dependent on these varying factors (Johnston et al., 2012). Figure 2.1 presents a summary of the different parameters that could influence nanoparticle toxicity.
Figure 2.1. Physico-chemical parameters which need to be assessed when classifying nanoparticle behaviour and ecotoxicity.

The concentration would refer to the amount of nanoparticles present in the given sample i.e. the milligrams of nanoparticle per liter sample; this is made up according to standard protocol (Kühnel and Nickel, 2014) or is received in the desired concentration from a manufacturer. As is the case for aquatic exposures, e.g. a standard Organization for Economic Co-operation and Development (OECD) fish medium would be made up to different concentrations (OECD 1992). Nanoparticles come in varying forms which include nanorods, nanometal ions, single-, double-, multiwalled carbon nanotubes and composites made from several metals such as quantum dots (Shaw and Handy, 2011). The behaviour and biological uptake would vary depending on these factors. Due to the nanoscale it is suggested that the mechanism is through endocytosis rather than ion channels along the plasma membrane (Cui et al. 2011; Shaw and Handy, 2011; Klaine et al., 2008). Oh and Park (2014) discussed endocytosis and exocytosis of several different nanoparticles according to their size and shape finding that at 50nm spherical nAu had the highest rate of cellular uptake while rod shaped gold nanoparticles had a lower rate of uptake with a higher aspect ratio. Smaller spherical gold nanoparticles had the fastest exocytosis while nanorods with smaller aspect ratios showed a decreased rate of exocytosis when compared to larger ones. Therefore size and shape are directly influential on cellular uptake (Oh and Park, 2014; Elsaesser and Howard, 2012; Albanese and Chan, 2011; Kunzmann et al., 2011; Cho et al., 2009a; Chithrani and Chan, 2007). The distribution of size is therefore important as it will be
directly related to concentration and nanoparticle size, the bioavailability is an important factor when related to effects and toxicity (Glenn and Klaine, 2013; Mühling et al., 2009). Nanoparticles are able to aggregate where various components are held together by strong forces and the external surface area is lower than the calculated surface areas of individual components. These aggregates are able to agglomerate where they are held together by weak forces which results in a similar surface area when comparing the sum and calculated components (Klaine et al., 2013; Wepener et al., 2013).

Nanoparticles tend to aggregate when ‘naked’ or pristine and are therefore capped to reduce toxicity and interaction with one another. Gold nanoparticles are usually capped with citrate, amino or thiol groups (Pal et al., 2013). As is the case with citrate capped nAu the citrate capping functionalizes once the nanomaterials come in contact with the fish medium thereby allowing the active sites to bind to constituents present in the water. The particles will therefore differ in various ways, they could be cationic or anionic, lipophillic or lipophobic, hydrophillic or hydrophobic and how extensively they will interact will change based on these properties (Xia et al., 2010; Lankveld et al., 2010).

Various literature show that due to particle functionalization the cationic nAu are able to interact with the biological organism due to the electrostatic forces of the negative membrane potential and therefore cause moderate toxicity while anionic nAu that form neutral or weak negatively charged groups do not exert any toxicity (García-Cambero et al., 2013; Schaeublin et al., 2011; Goodman et al., 2004). As the surface area of nanoparticles change so do the interactions between them (Wang et al., 2011). Agglomerations are loose bonds between nanoparticles and have recently been described to be van der Waals forces, this occurs when the electrostatic repulsive forces are lower than the van der Waals forces (Oh and Park, 2014). The forces that exist would be dependent on the ions that the nanoparticles have been bound to in the prepared water medium, while the biological uptake would be dependent of the interactions with the negatively charged lipid bilayer of the biological membrane (Goodman et al., 2004). Even in the solid state atoms are not static but vibrate at equilibrium, this frequency emitted is dependent on the size, length and strength of any bonds formed. The natural frequency of the bond is stimulated by radiation at the same frequency of the molecular vibrations and this is the infrared region of the electromagnetic spectrum which may be measured in wavenumbers (Coates,
It is therefore possible to determine the functional groups on the nAu surface. The ability for nAu to interact with other compounds within a medium as well as with one another will affect the zeta potential or surface charge and particles would therefore be cationic (positive) or anionic (negative) (García-Cambero et al., 2013; Goodman et al., 2004).

The aim of this chapter is to determine the changes that occur in citrate capped nAu characterization when stock concentrations of nAu are diluted within an aquatic medium used for standardized toxicity tests.

2.2. Materials and methods

For the purposes of this study the following characteristics were determined physico-chemical water parameters (pH, temperature, conductivity and oxygen concentration), concentration, size distribution, surface charge, surface functionality and agglomeration patterns based on the recommendations by Von der Kammer et al. (2012); Stone et al. (2010) and Klaine et al. (2008).

2.2.1. Gold nanoparticle stock solutions

MINTEK, a science council in South Africa, prepared and supplied the nAu stock solutions. The stock solution, which was made up of 14±2nm nAu with product code TMU14G, batch numbers (20130304FKP49b; 20130308FKP52; 20140905BM001) was prepared by standard citrate reduction techniques according to Murphy et al. (2008) and Fren (1973) and were sterilised using the filtration method.
2.2.2. Preparation of environmental media as diluent for nAu suspensions of varying concentrations

The reconstituted water for daphnia toxicity testing was performed by making up a stock solution which was prepared by dissolving 2.59g sodium bicarbonate (NaHCO$_3$), 1.2g calcium sulphate (CaSO$_4$.2H$_2$O), 0.08g potassium chloride (KCl) and 2.46g magnesium sulphate (MgSO$_4$.7H$_2$O) in 1L of distilled water in a volumetric flask and filling up to 20L with distilled water. The reconstituted water for fish toxicity testing was prepared by dissolving 11.76g CaCl$_2$.2H$_2$0, 4.93g MgSO$_4$.7H20, 2.59g NaHC0$_3$, and 0.23g KCl separately in distilled water in a 1L volumetric flask. According to OECD (1992) 500ml of each solution was added together and filled up to 20L using distilled water. The reconstituted water mediums were aerated for 24hrs (Truter, 1994) prior to use. The nAu stock solution was placed in an ultra-sonicator bath and sonicated for two hours prior to use. To make up the required exposure concentrations relevant volumes of the nAu stock was added to the environmental media water to make up the desired concentrations. Nine concentrations were prepared for the fish medium (5mg/l, 10mg/l, 15mg/l, 20mg/l, 25mg/l, 30mg/l, 35mg/l, 40mg/l and 45mg/l) and six concentrations were prepared for the daphnia medium (1mg/l, 5mg/l, 15mg/l, 25mg/l, 35mg/l and 45mg/l) respectively.

2.2.3. Measurement of physico-chemical chemical water properties

Physico-chemical water parameters were measured according to standard test protocols (OECD, 1992). Measurements were taken at the start of the test and at 24 hour (h) intervals until the tests were concluded. The pH, electrical conductivity (EC), total dissolved solids (TDS), oxygen saturation (% O$_2$) and dissolved oxygen (DO) concentration were measured using a handheld Eutech pH 110 RS232C meter, Eutech CON 110 RS232C conductivity and TDS meter, and Eutech DO6 DO and temperature meter. The oxygen saturation was maintained above 60% for the duration of the test by bubbling compressed air into the exposure beakers.
2.2.4. Nanoparticle exposure concentration

Total gold concentrations were determined at the start of the toxicity bioassay (0h) as well as at the conclusion of the assay (48h or 96h where relevant) to verify nominal concentrations in both ionic and nAu exposures. Samples (11ml) were acidified with 3ml suprapur® 30% hydrochloric acid (Merck) and 1ml 65% HNO₃ to ensure that all of the gold was in ionic form. Inductively coupled plasma atom emission spectroscopy (ICP-OES) (Spectro Arcos FSH12) techniques were used to analyse the water samples.

2.2.5. Characterization of nAu concentrations

Dynamic light scattering (Malvern Zetasizer Nano series, NanoZS) was used to measure the hydrodynamic size distribution and zeta potential of the nAu in solution and Fourier Transform Infrared Spectrometry (FTIR) (Perkin Elmer FTIR-spectrometer, Spectrum 100) was used to determine the nAu surface functionality and give insight into possible reasons for particle aggregation. These measurements were taken at 0h as well as at the conclusion of the test, for fish medium this was after 96h while for the daphnia medium this was 48 hrs. Transmission electron microscopy (TEM) (FEI Tecnai G2) was used to quantify nAu diameter and nAu surface morphology as well as show particle aggregation patterns. One drop of nAu medium was dropped onto a carbon coated copper grid and allowed to settle for a few minutes. The excess water was removed using a filter paper by touching only the edge of the droplet and the grid was allowed to dry before examination at high resolution (200 kV).
2.3. Results

2.3.1. Physico-chemical water parameters

The physico-chemical water parameters were maintained within the OECD guidelines (OECD, 1992) as shown in Table 2.1. The temperature ranged between 20-23.9°C across all exposures, the pH was maintained between 7 and 8 but had a minimum of 6.24 and a maximum of 8.95 for chloroauric acid exposures. The DO concentration ranged between 4.44mg/l and 9.78mg/l and as previously discussed the oxygen percentage (O\textsubscript{2} %) was well maintained above 60% according to standard protocol. The daphnia medium had a lower conductivity (EC) and total dissolved solids (TDS) when compared to the fish medium; this was expected as the reconstituted water is made up of different salts. The EC ranged between 198-420µS/cm for the daphnia medium and 621-809µS/cm for the fish medium. The TDS ranged between 104-267mg/l for the daphnia medium, while for the fish medium the range was between 309-485mg/l.
Table 2.1. Physico-chemical water quality characteristics presented as mean (minimum and maximum) values in different bioassays conducted on the citrate buffer, gold nanoparticles and chloroauric acid.

<table>
<thead>
<tr>
<th>Species</th>
<th>OECD guidelines</th>
<th>Temp. (˚C)</th>
<th>pH</th>
<th>D.O. (mg/l)</th>
<th>O₂ (%)</th>
<th>EC(µS/cm)</th>
<th>TDS (mg/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Citrate Buffer</td>
<td></td>
<td>20.5</td>
<td>7.85</td>
<td>5.43</td>
<td>64.6</td>
<td>339</td>
<td>171</td>
</tr>
<tr>
<td>D. pulex</td>
<td></td>
<td>(20 - 21.2)</td>
<td>(6.57 - 8.36)</td>
<td>(4.44 - 6.08)</td>
<td>(61.7 - 67.4)</td>
<td>(309 - 374)</td>
<td>(156 - 189)</td>
</tr>
<tr>
<td>D. magna</td>
<td></td>
<td>20.3</td>
<td>7.7</td>
<td>7.26</td>
<td>77</td>
<td>310</td>
<td>155</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(20 - 21.9)</td>
<td>(6.63 - 8.72)</td>
<td>(91.4)</td>
<td>(297 - 362)</td>
<td>(148 - 181)</td>
<td></td>
</tr>
<tr>
<td>D. rerio</td>
<td></td>
<td>23.3</td>
<td>8.26</td>
<td>4.77</td>
<td>69.6</td>
<td>708</td>
<td>350</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(22.7-23.9)</td>
<td>(7.10-8.65)</td>
<td>(6.00-8.72)</td>
<td>(62.6-71.5)</td>
<td>(309-398)</td>
<td></td>
</tr>
<tr>
<td>nAu</td>
<td></td>
<td>20.7</td>
<td>7.66</td>
<td>5.05</td>
<td>63.1</td>
<td>328</td>
<td>167</td>
</tr>
<tr>
<td>D. pulex</td>
<td></td>
<td>(20-21.9)</td>
<td>(7.05-8.7)</td>
<td>(4.4-6.1)</td>
<td>(60.7-72.6)</td>
<td>(208-420)</td>
<td>(104-220)</td>
</tr>
<tr>
<td>D. magna</td>
<td></td>
<td>20.2</td>
<td>7.78</td>
<td>7.23</td>
<td>78.4</td>
<td>309</td>
<td>155</td>
</tr>
<tr>
<td>D. rerio</td>
<td></td>
<td>22.6</td>
<td>7.88</td>
<td>4.49</td>
<td>68.1</td>
<td>674</td>
<td>336</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(22.4-23.9)</td>
<td>(7.13-8.42)</td>
<td>(4.04-6.26)</td>
<td>(60.1-98.7)</td>
<td>(642-765)</td>
<td>(321-412)</td>
</tr>
<tr>
<td>Chloroauric acid</td>
<td></td>
<td>20.6</td>
<td>7.9</td>
<td>5.35</td>
<td>66.3</td>
<td>344</td>
<td>176</td>
</tr>
<tr>
<td>D. pulex</td>
<td></td>
<td>(20-22)</td>
<td>(6.93-8.95)</td>
<td>(4.35-6.13)</td>
<td>(62.7-71.5)</td>
<td>(198-382)</td>
<td>(104-267)</td>
</tr>
<tr>
<td>D. magna</td>
<td></td>
<td>21.6</td>
<td>8.22</td>
<td>7.57</td>
<td>83.3</td>
<td>332</td>
<td>168</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(20.4-22.4)</td>
<td>(7.59-8.43)</td>
<td>(6.4-9.78)</td>
<td>(62.5-99.7)</td>
<td>(296-381)</td>
<td>(148-259)</td>
</tr>
<tr>
<td>D. rerio</td>
<td></td>
<td>23</td>
<td>7.92</td>
<td>4.98</td>
<td>72</td>
<td>677</td>
<td>350</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(21.8-23.7)</td>
<td>(6.24-8.67)</td>
<td>(4.2-6.044)</td>
<td>(62.8-85.3)</td>
<td>(621-739)</td>
<td>(311-485)</td>
</tr>
</tbody>
</table>
2.3.2. Nanoparticle exposure concentration

To verify the concentrations added to each exposure group, total gold concentrations were measured. The nominal ionic gold concentrations and measured concentrations were in close agreement (Figure 2.2). After a 48h and 96h period the concentrations were maintained within the start concentration limits. The total gold concentrations in the nAu exposures (Figure 2.3) showed up to a 20% difference between nominal and measured gold concentrations.

Figure 2.2. Mean measured gold concentrations (mg/l) ± standard deviation of the ionic gold (HAuCl₄) exposures at all exposure concentrations used in this study.
2.3.3. Particle size distribution

The largest proportion of nAu particles (i.e. >90%) in the daphnia medium across all exposure concentrations showed agglomerations greater than 566 nm (Table 2.2). As the exposure concentration increased so did the average sizes of the agglomeration, e.g. in the 45mg/l exposure the average sizes were 3175.5nm (Table 2.2.).

Figure 2.3. Mean gold concentration (mg/l) ± standard deviation measured in the nAu exposures used in this study.
Table 2.2. Size distribution of gold nanoparticles across all concentrations in daphnia medium.

<table>
<thead>
<tr>
<th>Nominal concentration</th>
<th>Size distribution (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 h</td>
</tr>
<tr>
<td>2 mg/l</td>
<td>76.4 ± 32.1</td>
</tr>
<tr>
<td>5 mg/l</td>
<td>181.2 ± 29.3</td>
</tr>
<tr>
<td>15 mg/l</td>
<td>148.3 ± 12.9</td>
</tr>
<tr>
<td>25 mg/l</td>
<td>799.5 ± 16.8</td>
</tr>
</tbody>
</table>

In contrast to the nAu particle aggregation/agglomeration in daphnia media, the dynamic light scattering indicated that the size distribution in fish media is represented by two peaks in terms of percentage intensity (Figure 2.4). The particle size distribution is therefore not presented as the average size as there were distinct differences and this would not give an accurate indication of the sizes available for biological uptake within the media. Therefore the size distribution is represented as two dominant size classes per concentration with the corresponding percentage intensities at 0h as well as at 96h.
Figure 2.4: Raw data sheet showing Z-Average size (nm) and peaks indicating size (nm), percentage intensity and standard deviation (nm).

The nanoparticle size distribution was determined at 0h and again at 96h to observe any changes in aggregation over time. Results are presented by indicating the main size distribution classes in terms of the percentage contribution after 0h and 96h. Percentage intensity of gold nanoparticles (red) at 0h, where the indicated size (blue) had an intensity of more than 60% followed by the value at less than 40% percentage intensity (Figure 2.5). The same was shown at 96h where more than 55% of nanoparticles had the indicated size. In four concentrations (10mg/l, 20mg/l, 25mg/l and 45mg/l) the particles had less than 45% percentage intensity of the indicated size.

At 0h in the 5mg/l concentration 89% of the sample had a size distribution of 150.7nm and 12% of nanoparticles were 12.64nm. After 96h more than 90% of particles were 133.7nm in size. At 10mg/l all particles measured in the sample were around 75.79nm while after 96h the particles (82.7%) had agglomerated to 986.4nm while 17.3% remained approximately 79.64nm in diameter. At 15mg/l 89% were in the size range of 89.49nm, while 11% were at 15.51nm. After 96h particles had all aggregated to 96.6nm. At the onset of the 20mg/l exposure 88% of the particles had aggregated to 127.9nm and 12.2% to 17.12nm in diameter. After 96h 64% of the sample had aggregated to 121.5nm in size. At the 25mg/l concentration just over half (59.6%) of the sample was 298.4nm in size while just under half (40.4%) of the sample was 71.48nm. After 96h the same ratio was maintained where 54.5% of the sample had a size of 455.6nm and 45.5% had a size of 96.89nm. At 30mg/l the prevalence was 72.3% distribution of 163.1nm particles and 27.7% at 40.27nm, while after 96h all particles had agglomerated to 201.5nm. The 35mg/l concentration revealed that 91% of the sample had aggregated to 96.89nm and 9% of the sample remained around 14nm in size. After 96h all particles had aggregated to 138.2nm. The 40mg/l concentration showed an immediate aggregation at 0h where 57.4% of the sample was 464.9nm and 42.6% of the sample was 57.55nm in size. After 96h all sample measured contained a unanimous size of 226.9nm. At the highest concentration, 45mg/l, 79.9% of the sample had particles 82.52nm in size and 15% remained around the original size of 14nm. After 96h the prevalence of particles at 288nm was 87.7% and 12.3% were 63.05nm in size.
At 0h there was a clear increase in size of nAu aggregations and agglomeration at concentrations of 25mg/l and 40mg/l when compared to other concentrations. But after 96h the 20mg/l and 25mg/l concentrations still showed agglomerations increasing in size and dispersion in the media while concentrations 30mg/l and above showed no or lower measured sizes which is attributed to particle precipitation.

Figure 2.5. Particle size distribution (nm) in gold nanoparticle exposure media showing gold nanoparticle with a percentage intensity of a)more than 60% at 0h and b) less than 40% at 0h as well as c) more than 55% at 96h and d) less than 45% at 96h.
2.3.4. Particle charge (zeta-potential)

The EC differed for the daphnia medium based on the salts used to make up the reconstituted water. For the daphnia medium the zeta potential ranged between -3.07 mV to -17.16 mV, all values across all time intervals were negative. At 5mg/l and 15mg/l exposures the zeta potential decreased even further after 48h while at all other concentrations the zeta potential became less negative (Table 2.3).

Table 2.3 Zeta potential (mV) of the nAu in the daphnia medium across exposure concentrations.

<table>
<thead>
<tr>
<th>nAu</th>
<th>1 mg/l</th>
<th>5 mg/l</th>
<th>15 mg/l</th>
<th>25 mg/l</th>
<th>35 mg/l</th>
<th>45 mg/l</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zeta potential [mV]</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0h</td>
<td>-8.74 ± 2.91</td>
<td>-3.07 ± 1.58</td>
<td>-8.13 ± 9.72</td>
<td>-17.16 ± 22.92</td>
<td>-10.27 ± 2.12</td>
<td>-9.40 ± 0.54</td>
</tr>
<tr>
<td>48h</td>
<td>-2.03</td>
<td>-7.17 ± 4.30</td>
<td>-16.10</td>
<td>-8.50 ± 0.92</td>
<td>-2.78 ± 0.80</td>
<td>-4.14 ± 1.23</td>
</tr>
</tbody>
</table>

For the fish medium there was a distinct difference with a much greater variation (as observed in the increase in the standard deviation) of the measured zeta potential. The standard deviation (Figure 2.5a) ranged between 57.8 mV and -54.9 mV. This is related to the large difference in size distribution as shown in Figure 2.4. In Figure 2.5b the zeta potential data were limited to those that represent 70% of the dominant distribution (i.e. the outliers were removed). The dominant zeta potential at 20mg/l, 25mg/l, 40mg/l and 45mg/l was negative, i.e. -4.09 mV, -6.49 mV, -2.42 mV and -2.88 mV respectively. At all other concentrations the charge was positive with the highest at 10mg/l (3.06 mV).
Figure 2.5. a) The Zeta potential (mV) average peak reading across all concentrations. b) The Zeta potential (mV) reading where the peak had greater than seventy percent distribution measured across all concentrations.

2.3.5. Surface functionality

The FTIR results revealed two distinct peaks (Figure 2.6). One of the peaks was identified as a hydroxyl group and further analyses of the the black particle aggregates was carried out on dried sample to eliminate any interference in the spectrum of the water suspension. A wavenumber of between 1500-2000 cm\(^{-1}\) indicates a double bond present, while 2500-4000 cm\(^{-1}\) indicates a single bond stretch to hydrogen (Pal et al., 2013; Stuart, 1995). The first peak (± 1632 cm\(^{-1}\)) was identified as an organic nitrate or carbon double bond aromatic stretch. Since the reconstituted water did not contain any nitrates a carboxyl double bond was identified. The second peak (± 3343 cm\(^{-1}\)) was identified as a hydroxyl group, H-bonded OH stretch (3570 – 3200 cm\(^{-1}\) (broad) 3400 – 3200 cm\(^{-1}\) or a normal “polymeric” OH stretch, which indicates interaction with the water medium.
2.3.6. Gold nanoparticle aggregation and agglomeration pattern

The TEM results revealed a better understanding to the size distribution patterns. As defined by Klaine et al. (2008) nanoparticle groupings of less than 150nm were referred to as aggregates and those larger than 150nm were seen as agglomerates. The stock solution of 1000mg/l was observed and the citrate capping was visible around the nAu especially at a higher magnification. It was clear that particles were separated from one another while present in the citrate buffer capping agent (Figure 2.7a). A live fast fourier transform (FFT) image was taken to verify that the particles were aligned in a crystal lattice formation as is typical of nano metals (Figure 2.7b). At 0h dispersed nAu were observed in all exposure concentrations. However after 96h this was only possible to identify dispersed particles at 5mg/l and 10mg/l exposure concentrations. For the 40mg/l and 45mg/l exposures it was apparent that after 24h the nAu started to agglomerate and sediment out of solution. At 5mg/l nAu were much more difficult to find on the dried copper grid but were present and showed aggregations of small groups (Figure 2.7c) as well as string like aggregates (Figure 2.7d). While at 10mg/l the string like aggregates were still present but more were present (Figure 2.7e) and had started aggregating at 122.87nm onto the string like structures as though a skeletal backbone had been formed (Figure 2.7f).
In the 20mg/l exposure there were two major types of agglomerates noted; these were skeletal like agglomerates which reached approximately 352.1nm in length (Figure 2.8a) and smaller aggregates which were quite prevalent and easy to find throughout the dried copper grid which showed an approximate size of around 73.67nm. When observing 30mg/l nAu concentrations it was quite clear that the skeletal backbone structures were starting to attach to one another as seen in Figure 2.8c. Seeing that the conformation was stable the smaller agglomerates (152.19nm) started attaching the open spaces between and particle agglomerates were as large as 430.78nm (Figure 2.8d). It appears that at 40mg/l the larger agglomerates as seen in 30mg/l started attaching to one another (Figure 2.8e) the skeletal backbone has now been filled and the nAu aggregate into an accumulation of particles forming large agglomerates of up to 2000nm. At these concentrations agglomerates were visible to the naked eye.
Figure 2.7. a) A TEM photograph of a drop of the concentrated citrate capped 1000mg/l concentration gold nanoparticle solution. b) An FFT image of the nAu to confirm the presences of a crystal lattice. c+d) A TEM photograph of the 5mg/l gold nanoparticle concentration showing average aggregations and measurements. e+f) A TEM photograph of the 10mg/l gold nanoparticle concentration showing average aggregations and measurements.
Figure 2.8. a+b) A TEM photograph of the 20mg/l nAu concentration showing average aggregations and measurements. c+d) A TEM photograph of the 30mg/l gold nanoparticle concentration showing average aggregations and measurements. e+f) A TEM photograph of the 40mg/l gold nanoparticle concentration showing average aggregations and measurements.
2.4. Discussion

When considering nanoparticle ecotoxicity there are several factors to consider, the initial size of the particles, the capping agent, how particles aggregate in an aquatic medium, the charge that exists; all these affect the toxicity and ability for particle uptake in organisms (Oh and Park, 2014; Alanazi et al., 2010; Kim et al., 2010). As with any chemical toxicity the physico-chemical parameters are indicative of any effects, this is not the case with gold nanoparticles as all pH, temperature, conductivity and oxygen measurements stayed within an expected range across all concentrations compared to a control over a 96h period. The changes in zeta potential and aggregation could be related to changes in conductivity and pH over time (Fadeel and Garcia-Bennett, 2010; Schrand et al., 2010). These changes in ionic strength based on the physico-chemical water parameters allow for more binding sites on the particle surface due to an increased negative charge (Cui et al., 2011). Therefore negative zeta potentials have the potential for aggregation to decrease. There is an increase in aggregation as the zeta potential becomes more positive (Weinberg et al., 2011; Griffitt et al., 2008). Self-assembled nAu monolayers form and when a second layer of assembly forms it provide an excellent scaffold for other molecules like peptides and proteins (Abradelo et al., 2007). As opposed to what Goodman et al. (2004) found Choi et al. (2012) and Schaeublin et al. (2011) found that the anionic nAu evoked a greater response in in vitro exposures where in their case sulphur was also present within the medium make up in conjunction with carbon, hydrogen and oxygen.

As all citrate capped gold nanoparticles in the stock solution were around the 14nm range it is evident that from the moment the particles were diluted in medium they started to agglomerate to one another, which could also reflect the 10mg/l loss of nanoparticle concentrations measured due to aggregations as the entire sample was not read only a portion of it. The citrate capping is soluble and by the hydrolysis of water is converted to citric acid (Tabrizi et al., 2009). Sections of the capping agent sodium citrate (Na₃C₆H₅O₇) are washed off in the medium and particles become functionalized. The particles become functionalized in the medium and therefore gain a charge (Yah, 2013) with allows them to stick to one another by van der Waals forces (Oh and Park, 2014). Divalent cations such as Ca²⁺ and Mg²⁺, which are present in the reconstituted OECD medium, effectively neutralize the surface charge of citrate coated silver nanoparticles, thereby compressing the electric double layer (repulsive forces) and enhancing...
aggregation (Baalousha et al., 2013; Delay et al., 2011). This suggests that aggregates of nAu should be regulated by the type and concentration of the solvent and ionic strengths of reconstituted water medium (Elsaesser and Howard, 2012; Römer et al., 2011; Farkas et al., 2010; Abradelo et al., 2007). The Zeta sizer shows three peaks where the distribution of each size is represented as a percentage, it was found that to gain a better understanding of the particle behaviour at each concentration these percentages need to be taken into account.

Functional groups present on the nAu surface play a role in tissue distribution (Fent et al., 2009; Gu et al., 2009). When additional ligands aren't present as is the case with a controlled OECD experiment, the most stable form in solution for the nAu would be the hydrolyzed species, namely functionalized to AuOH (H2O)0 (Stuart, 1995). The OH frequency indicates that OH forms strong H-bonding with groups of citrate caps present on nAu (Pal et al., 2013). However depending on the pH and presence of chloride species this could change, where a pH of less than 4 would cause AuCl2 to form, the pH in this experiment and FTIR results confirmed that this was not the case. When negative charges are found, as seen in 20mg/l, 25mg/l, 40mg/l and 45mg/l, this may change the toxicity of the particle where at a pH above 6 the predominant charge in solution would be AuCl(OH)3, in cases where the pH was above 9 the functionality could change to Au(OH)4. Since the pH had a maximum of 8.7 for the duration of the experiment it is concluded that negative charges were due to the prescence of AuCl(OH)3 ions (Lapresta-Fernandez et al., 2012; Farkas et al. 2010; Stuart, 1995). The cellular uptake is highly dependent on the functional groups attached to the particle as these would affect the charge and aggregation patterns (Yah, 2013).

The particle size is important for the toxicity and therefore it is crucial to quantify the number size distribution of nAu size mixtures (Calzolai et al., 2011; Mahl et al., 2011). At the lowest concentration particles immediately started aggregating to one another where only around 10% remained within the initial range and there were less particles available to keep aggregating to a larger size. Between 20mg/l and 25mg/l there was a diverse distribution as nAu ranged from 17nm to 890nm and were present at equal amounts, the charge was negative at these concentrations and this could be adding to the smaller particles present because negative charges would cause aggregates to repel one another (Weinberg et al., 2011) this is proposed to be the threshold value for exposure concentrations. Where the nAu have a high enough
concentration to have an effect but still small enough size to move through the cellular membrane. After 30mg/l the nAu start forming large agglomerates which when observed under the TEM reveal skeletal like structures which may already be too large to be endocytosized by the membrane and would adsorp to the membrane (Elsaesser and Howard, 2012; Grass et al., 2010; Treuel et al., 2010). Therefore there is a high enough concentration of nAu present for the aggregation to outweigh the charge and therefore all nAu are able to bind to one another and fewer are left in solution. However at 40mg/l and 45mg/l the agglomerates become so large that they are visible to the naked eye (up to 3mm) and therefore when a drop is collected we are only able to see those which have been unable to bind to the larger agglomerate. This would also explain why after 96h it becomes increasingly difficult using TEM to observe nAu in concentrations above 10mg/l as they have already started to sediment out of solution. Therefore the mean size and zeta potential are inaccurate because the distribution of size needs to be evaluated as well as the sizes which are not measured by the current instruments as they are far beyond the nanoscale.

2.5. Conclusion

The concentration of nAu present is directly related to the size and charge of the aggregates which have very likely been functionalized to AuCl(OH)$_3$ and AuOH ($H_2$O)$_0$ in a reconstituted water medium. Divalent cations such as $Ca^{2+}$ and $Mg^{2+}$ present in the reconstituted medium enhance the formation of aggregates in solution. Concentrations below 25mg/l tend to form aggregates, while concentrations of 30mg/l and above form agglomerates which attach to one another in a skeletal like structure and then fill in as concentrations increase to 45mg/l. Therefore the combination of all the above mentioned factors will play a role in the uptake (bioavailability), body distribution, genetic interferences, biomarker responses and mechanical damage.
Chapter 3: Assessing toxic hazard of nanogold using standard aquatic toxicity tests

3.1. Introduction

To manage environmental resources such as water quality laboratory toxicity tests are used world-wide and are deemed as the first step in a tiered approach to set up guidelines for acceptable maximum concentrations of specific pollutants (Kühnel and Nickel; 2014; Muller and Palmer 2004; Chapman 1995, Kimball and Levin 1985). Bioassays are employed to find toxicity which could come from complex mixtures and are a complementary tool to assess toxic effects overcoming certain limitations and are relatively rapid, cost effective and simple to perform (Martins et al., 2007; Grothe et al., 1996; Rand et al. 1995). Bioassays can detect synergistic, antagonistic and additive effects and also explain both point and diffuse sources of aquatic pollution since they are site-specific (Dorn 1996; Martins et al., 2007; Sarakinos et al., 2000; Smolders et al., 2003). Several different bioassays are used simultaneously at different trophic levels of different biological complexity to sufficiently assess if a potential hazard exists (Kühnel and Nickel; 2014; Jergentz et al., 2004) since no single test is suitable to measure all ecological risks due to different sensitivities by organisms (Chapman, 2000; DWAF, 2003; Fernandez-Alba et al., 2001; Rand et al., 1995). Nanoparticle ecotoxicity review studies have referred to the importance of physico-chemical and nanoparticle characterization of the exposure media to be able to link exposure to effect (Chapter 2; Kühnel and Nickel; 2014; Johnston et al., 2012; Shaw and Handy, 2011; Klaine et al., 2008).

Water quality monitoring programs used internationally include the Direct Toxicity Assessment (DTA) used in the UK, Whole Effluent Toxicity (WET) testing used in the USA and the Totale Effluent Milieuhygiene or ‘Whole Effluent Environmental Risk’ approach used in the Netherlands (DWAF 2003). The selection of acute and chronic toxicity testing end points used in these monitoring programs have similar fundamental concepts; some may also include other indirect hazard parameters such as bioaccumulation, oxygen depletion potential and mutagenicity to establish the ecological effects of pollutants.
In South Africa a multifaceted approach is used by The Department of Water Affairs (DWA) and is known as the Direct Estimation of Ecological Effect Potential (DEEEP). The approach incorporates the Totale Effluent Milieuhygiene as a foundation and has become a crucial tool used in ecological hazard assessment (Department Water and Sanitation, 2003). Different trophic levels (fish, invertebrates, algae and bacteria) are used by using representative organisms from each part of the food chain and endpoints are both lethal and sublethal. Protection to aquatic systems is therefore ascertained by defining acceptable ecological hazards due to the overall effect of toxicants (Jooste and Herbst, 2004; Liu and Dutka, 1999). The rapid and relatively inexpensive internationally standardized OECD toxicity bioassays in freshwater, that also form part of the DEEEP approach (Slabbert, 2004), are incorporated into this study to determine the aquatic toxicity of nanogold (nAu) to selected test organisms.

The aim of this chapter was to assess the toxic hazard of nAu to aquatic organisms across different trophic levels using standard aquatic toxicity tests.

### 3.2. Materials and methods

Prior to any acute testing glassware was washed by following the method described by Giesy and Weiner (1977) glassware was soaked in a 2 % non-phosphate soap bath for 24h, followed by soaking in 2 % hydrochloric acid solution for a further 24h. Between and after each washing step glassware was rinsed in double-distilled water. Glassware was allowed to air dry prior to use (USEPA, 1993).
The acute tests that were performed are listed in Table 3.1.

Table 3.1. Species selected to perform the related standardized OECD testing to determine the toxicity of nAu.

<table>
<thead>
<tr>
<th>Species</th>
<th>Common name</th>
<th>Test</th>
<th>OECD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vibrio sp.</td>
<td>Bacteria</td>
<td>Bioluminescence viability test</td>
<td>OECD417</td>
</tr>
<tr>
<td>Pseudokirchneriella</td>
<td>Algae</td>
<td>Growth inhibition test</td>
<td>OECD-TG218/219</td>
</tr>
<tr>
<td>subcapitata</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Daphnia pulex</td>
<td>Water flea</td>
<td>48 hour acute immobilisation test</td>
<td>OECD-TG202</td>
</tr>
<tr>
<td>Daphnia magna</td>
<td>Water flea</td>
<td>48 hour acute immobilisation test</td>
<td>OECD-TG202</td>
</tr>
<tr>
<td>Danio rerio</td>
<td>Zebrafish</td>
<td>96 hour acute lethality test</td>
<td>OECD-TG203</td>
</tr>
</tbody>
</table>

3.2.1. Physico-chemical water properties and characterization of nAu concentrations

Physico-chemical water properties (pH, temperature, conductivity, total dissolved salts, conductivity, oxygen concentration and oxygen percentage) were taken in 24h intervals for the duration of each test. A water sample was collected per each concentration at the start of the test as well as at the end of the test for daphnia and fish acute testing. These samples were used for characterization as well as ICP-OES to confirm that the concentration of exposure substance was maintained throughout the test. All physicochemical chemical water properties and correlating nAu characterization data have been discussed in Chapter 2.
3.2.2. Bioluminescence viability test

The bacterial bioluminescent viability test was done using frozen *Vibrio fischeri* from a 30-min Microtox® kit, ISO 11348-3 test, as described by OECD 417. Frozen bacterial samples were thawed prior to testing in a water bath set to 25°C. A low range finding concentration setup was done using concentrations of nAu and dispersant at 0.1mg/l, 0.21mg/l, 0.42mg/l, 0.83mg/l, and 1.67mg/l. Ionic gold, in the form of chloroauric acid was also tested at concentrations of 6.25mg/l, 12.5mg/l, 25mg/l, 50mg/l and 100mg/l. A bacterial stock concentration was added to each cuvette and samples were read on a bioluminescence spectrometer at zero minutes and again after a period of thirty minutes. There was a direct relation between concentration of nAu (Figure 3.1) and the bacteria test was excluded from the study due to particle interference wavelength readings in a spectrophotometer (Doak *et al.*, 2012; Boodhia, 2013).

3.2.3. Algal growth inhibition test

The algal (*P. subcapitata*) growth inhibition test was performed using AlgaltoxkitF® according standard protocols as set out by the OECD-TG218/219 and to eliminate particle interference as seen in the bacterial test the spectrometer step was replaced by a chlorophyll-a extraction method (Sartory, 1982). For the test only glassware was used as to avoid the nAu particles from adhering to the surface and samples were done in triplicate for each 24h interval, therefore nine samples were prepared in total per concentration.

The culture medium was prepared by placing 800ml of distilled water to a 1L volumetric flask, 10ml of nutrient stock A was added and then 1ml of nutrient stock B, C and D were added. The volume was filled to 1L with distilled water and shaken to mix the solution which was aerated with oxygen for at least thirty minutes. The pH was adjusted to 8.1 using 1M NaOH or 1M HCl. The algal beads were demobilised by throwing off the liquid they were suspended in and then adding 5ml of matrix dissolving medium and vortexing thoroughly until dissolved. The algal beads were then centrifuged (Madell Technology Corporation centrifuge TGL-16M) for 10 minutes at 3000rpm, the supernatant was discarded and 10ml of distilled water was added as a
washing step and samples were centrifuged again at 3000rpm for 10 minutes, 10ml of prepared culturing media was added to resuspend the algae. The suspension was diluted in 25ml of culturing medium and read on a spectrometer and concentration of $1.10^6$ algal cells per millilitre was prepared in a 1000ml flask.

Concentrations ranging from 6.25mg/l, 12.5mg/l, 25mg/l, 50mg/l and 100mg/l for nAu and dispersant and 1mg/l, 2mg/l, 5mg/l and 10mg/l for chloroauric acid were made up using culture medium to which 2ml of nutrient stock solution A was added and 200µl of nutrient stock B, C and D. In each replicate there was 50ml of test sample containing $1.10^6$ algal cells/ml, where three groups were present at 24h, 48h and 72h. Samples flasks were plugged with an aerating cotton wool and were kept at 23°C ±2°C with continuous illumination for 72h.

At each 24h interval chlorophyll-a concentrations were determined using a Hiatashi 150-20 spectrophotometer according to a technique described by Sartory (1982). A volume of 49.5ml of sample was filtered through Whatman GF/C filter paper using a vacuum pump. The chlorophyll which gathered on the filter paper was extracted using 10ml of 95% ethanol in a water bath set to 78°C for a period of 5 minutes. Once removed the samples were left to cool down in a dark room. The samples were read at 665nm and 750nm where 95% ethanol was used as a blank, samples and blank were then acidified with 0.3M HCl and read again at 665nm and 750nm after a period of 2 minutes. The following calculation was used to calculate the chlorophyll-a concentration:

$$\text{Chlorophyll-a (µg/l)} = \frac{[(A_{665}-A_{750}) \times (A_{665a}-A_{750a}) \times 28.66 \times \text{extract volume}]}{\text{volume of sample}}$$

Where $A_{665} =$ absorbency at 665nm; $A_{750} =$ absorbency at 750nm; $A_{665a} =$ absorbency at 665nm after acidification; $A_{750a} =$ absorbency at 750nm after acidification; extract volume = 10mL 95% ethanol; volume of sample = volume of water sample filtered in litres (0.049L).
3.2.4. CytoViva® dark field hyperspectral imaging of algae

One millilitre of algal suspension in the control, 25mg/l and the 50mg/l were placed on a slide with a cover slip and sealed with Entellan® and then viewed using CytoViva® dark field hyperspectral imaging. CytoViva® 150 Unit integrated onto the Olympus BX43 microscope was used where HSI system 1.1 and ENVI software at 60X magnification was used to confirm the nAu particles in the medium by spectral profiles. The same was used to observe nAu particles present and interacting with algal suspensions. Images were captured using the Dagexcel X16 camera and DAGE Exponent software at 60X magnification.

3.2.5. Daphnia pulex and Daphnia magna 48 hour acute immobilisation test

The 48h daphnia immobilization test (OECD-TG202) was performed by making up a stock solution which was prepared by dissolving 2.59 g sodium bicarbonate (NaHCO₃), 1.2 g calcium sulphate (CaSO₄.2H₂O), 0.08 g potassium chloride (KCl) and 2.46 g magnesium sulphate (MgSO₄.7H₂O) in 1L of distilled water in a volumetric flask and filling up to 20L with distilled water. The medium was then aerated for 24 hrs (Truter, 1994) prior to use.

*D. pulex* and *D. magna* were used for acute testing, adults were kept in 2.5L tanks in daphnia media which was replaced three times per week and kept at a temperature of 20°C. Five milliliters of daphnia food was suspended in the daphnia media at each water cycle change. Prior to testing approximately thirty adults were transferred to a new tank and hatchlings less than 24 hrs old were used for the test.

The oxygenated daphnia medium was used to make up the desired concentrations. A stock solution of 1000mg/l of 14nm nAu was sonicated for two hours in an ultrasonication bath prior to use and diluted using the calculation:

\[ C_1V_1 = C_2V_2 \]
For nAu a concentration range of 0.5mg/l, 2mg/l, 5mg/l, 10mg/l, 15mg/l, 20mg/l, 25mg/l, 35mg/l and 45mg/l was made up with dispersant following the same range. The ionic gold concentration range was 0.0005mg/l, 0.005mg/l, 0.5mg/l, 1mg/l, 2mg/l and 5mg/l and a 1g/L potassium dichromate solution was used as the positive control. No food was added to the concentration media for the duration of the test.

Twenty one juveniles were added to each concentration where each 50ml beaker contained no more than five daphnia, except one which contained six. A 16h light and 8h dark cycle was used for the duration of the test and the temperature was maintained between 18°C and 22°C. The physico-chemical water parameters were taken at the start of the test and again at 24h intervals until the test concluded. At 24h intervals the number of immobilized daphnia, any animal that was immobilized for more than 15 seconds, was counted. Any abnormal behaviour was also reported. The test concluded after 48h.

3.2.6. Danio rerio 96 hour acute lethality test

The D. rerio acute lethality test (OECD-TG203) was performed on 14 day old juveniles. Adult breeding stocks were conditioned for breeding in toxicant free water for a period of one month, fed on a diet of blood worm and Tetramin® flakes. In a ratio of five males per one female fish were transferred a new tank in a basket covered in mesh submerged in water. This allowed for fish to breed and the eggs to drop to the tank floor, after a period of approximately thirty hours adult fish were removed and juveniles were left to hatch.

Prior to toxicity testing a fish medium was prepared by dissolving 11.76 g CaCl₂·2H₂O, 4.93 g MgSO₄·7H₂O, 2.59 g NaHCO₃, and 0.23 g KCl separately in distilled water in a 1L volumetric flask. According to OECD (1992) 500ml of each solution was added together and filled up to 20L using distilled water, then aerated for 24h before use.
Similarly to preparation of the daphnia test concentrations exposure medium was prepared and a concentration range of 5mg/l, 10mg/l, 15mg/l, 20mg/l, 25mg/l, 30mg/l, 50mg/l, 100mg/l and 200mg/l was used for nAu. A range of 50mg/l, 100mg/l and 200mg/l were used for dispersant. And 1mg/l, 2mg/l, 5mg/l and 10mg/l were used for ionic gold concentrations in the form of chloroauric acid. Potassium dichromate (1g/l) was used as a positive control. Twenty one fish were used and no more than seven fish were placed in a beaker containing 500ml of exposure medium. A 16h light and 8h dark cycle was used for the duration of the test and the temperature was maintained between 21°C and 25°C. The physico-chemical water parameters were taken at the start of the test and again at 24h intervals until the test concluded. The oxygen concentration was maintained above 60% for the duration of the test. If more than 10% of the juveniles died in the control the test was discarded. At 24h intervals mortalities were counted, if no visible movement was seen including gill movement, the dead fish were removed from the test. The test concluded after a period of 96h.

**3.2.7. Statistical Analysis**

The USEPA methods (1993) were used to calculate EC/LC values. Data which did not fit the assumptions of the Probit method (Version 1.5) were analysed using the Trimmed Spearman-Kärber method (Hamilton *et al.*, 1977).
3.3. Results

3.3.1. Bioluminescence viability test

A direct relation was seen at the start of the test (0min) between exposure concentration and luminescence (Figure 3.1) where the input bacteria concentration related to the luminescence reading. The nAu exposure readings could be misinterpreted as growth but it is rather due to the absorbance as a result of increased agglomeration of nAu at higher exposure concentrations. A shift after 30min shows the same change in absorbance. As for the ionic gold concentrations the bacteria were destroyed at all concentrations measured. The citrate buffer (dispersant) showed no toxicity in the concentrations measured.
Figure 3.1. Bacterial luminescence at 0min and after a period of 30min in a) gold nanoparticles b) ionic gold and c) dispersant.
3.3.2. Growth inhibition test

Due to the potential interference of nAu particles with the measurement of the algal growth endpoint, chlorophyll-a was used as a surrogate. The acute algal test revealed (Figure 3.2) that after 24h there was little chlorophyll-a present, which can be related to limited algal growth. After 48h there was a similar increase in algal growth except for all ionic gold concentrations, which resulted in marked growth inhibition. From 48h it could be seen that 6.25mg/l and 100mg/l had a lower growth rate. After 72h, the lowest concentration (6.25mg/l) had the highest growth inhibition and was more affected than the serial concentrations above it 25mg/l showed the lowest affect but still had approximately 45% less algal growth than the control. The lowest observed effect concentration was 6.25mg/l. nAu concentrations above 25mg/l were darker in colour as can be seen in Figure 3.3. As expected for chemical toxicity in the ionic concentrations as there was an increase in chloroauric acid concentrations there was a decrease in algal growth, where from 5mg/l there was almost no growth seen after 72h. The dispersant concentrations showed no effect on algal growth and were comparable to the control and data did not allow for calculations of an EC50.
Figure 3.2. Chlorophyll-a concentrations in *P. subcapitata* after an incubation period of 24h, 48h and 72h in range finding concentrations of a) nAu, b) chloroauric acid and c) dispersant.
Figure 3.3. The different colours of nAu in range finding concentrations of in algal medium after 72h when compared to a control.

3.3.3. CytoViva® dark field hyperspectral imaging of Pseudokirchneriella subcapitata

The CytoViva® dark field hyperspectral imaging of P. subcapitata revealed that when compared to the control medium where nAu were not present, the nAu particles adhered to the surface of the algae and were not taken up. In the 25mg/l exposure the nAu surface adsorption was less concentrated as when compared to the 50mg/l where more intense agglomerations were seen (i.e. the distinctive red colouration observed in Figure 3.3). In 50mg/l in some cases almost 50% of the algae surface was coated in nAu particles.
Figure 3.4: CytoViva® dark field hyperspectral imaging of *Pseudokirchneriella subcapitata* (algae) in a) control medium. b) in a medium with 25mg/l concentration of nAu particles. c+d) in a medium with 50mg/l concentration of nAu particles.
3.3.4. *Daphnia pulex* and *Daphnia magna* 48 hour acute immobilisation test

The *D. pulex* acute immobilization test (Figure 3.5) showed the highest percentage mortality at 20mg/l, with a percentage of 33.3%. While 15mg/l had 9.5% mortality and 10mg/l had less than 5% mortality as comparable to the control. The LC50 was calculated without confidence limits to be 75.314mg/l, which was comparable to that calculated by Li *et al* (2010a). The LC50 for chloroauric acid for *D. pulex* was 0.01mg/l and the dispersant showed less than 5% mortality and therefore had no effect at all concentrations.

The *D. magna* acute immobilization test (Figure 3.6) showed no mortalities above 25% across all concentrations, where the highest mortality of 23.8% was seen at 25mg/l but 35mg/l showed no mortality. For all other concentrations tested the mortality ranged between ten and twenty percent and therefore an LC50 could not be calculated. The chloroauric acid showed results comparable to those seen in *D. pulex* and an LC50 of 0.15mg/l was calculated. There was less than 10% mortality across all concentrations tested for the dispersant.
Figure 3.5. Percentage mortality in a Daphnia pulex immobilization test after 48h in range finding concentrations of a) nAu, b) chloroauric acid and c) dispersant. (+)ve (positive control).
Figure 3.6. Percentage mortality in a Daphnia magna immobilization test after 48h in range finding concentrations of a) nAu, b) chloroauric acid and c) dispersant.
3.3.5. *Danio rerio* 96 hour acute lethality test

The *D. rerio* acute lethality test was conducted using a wide exposure concentration range (min – max). Results revealed low toxicity for nAu, with the no observed effect below 25mg/l (Figure 3.7). The highest mortality was at 25mg/l where 28.57% mortality was recorded over a period of 96h. The highest concentrations 50mg/l, 100mg/l and 200mg/l showed lower mortality, i.e. 15%, 13% and 19% respectively. Fish appeared to be swimming slower and started twitching in concentrations of nAu above 25mg/l. In the ionic gold concentrations the lowest observed effect was at 1mg/l with 19.05% mortality, while at 10mg/l there was 100% mortality. The LC50 for ionic gold was calculated as 4.85mg/l (±1.23), while the dispersant was showed no mortality across all concentrations. It was not possible to calculate and LC50 for nAu results.
Figure 3.7. Percentage mortality in a D. rerio lethality test after 96h in range finding concentrations of a) nAu, b) chloroauric acid and c) dispersant.
A summary of the acute toxicity results for the different indicator species are presented in Table 3.2. The bacteria data were excluded due to particle interference. The table therefore includes the mean effective concentrations, standard deviations, no observed effect concentration and lowest observed effect concentration is also included.

Table 3.2. Summary of the median effective concentration ($EC_{50}$) and lethal concentrations ($LC_{50}$) for 50% effect and the subsequent 95% confidence levels for $P.$ subcapitata, $D.$ pulex, $D.$ magna and $D.$rerio after exposure to citrate buffer, nAu and HAuCl$_4$.

<table>
<thead>
<tr>
<th>Species</th>
<th>Duration/Endpoint</th>
<th>$EC_{50}/LC_{50}$ (mg/l)</th>
<th>NOEC (mg/l)</th>
<th>LOEC (mg/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Mean std dev</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Citrate Buffer</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$P.$ subcapitata</td>
<td>72h</td>
<td>&lt;5% inhibition</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$D.$ pulex</td>
<td>48h</td>
<td>&lt;5% mortality</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$D.$ magna</td>
<td>48h</td>
<td>&lt;10% mortality</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$D.$ rerio</td>
<td>96h</td>
<td>No Mortalities</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$nAu$</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$P.$ subcapitata</td>
<td>72h</td>
<td>NC</td>
<td>0</td>
<td>25</td>
</tr>
<tr>
<td>$D.$ pulex</td>
<td>48h</td>
<td>75.314*</td>
<td>1</td>
<td>10</td>
</tr>
<tr>
<td>$D.$ magna</td>
<td>48h</td>
<td>NC</td>
<td>0</td>
<td>5</td>
</tr>
<tr>
<td>$D.$ rerio</td>
<td>96h</td>
<td>NC</td>
<td>&lt;15</td>
<td>&gt;15</td>
</tr>
<tr>
<td>Chloroauric acid</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$P.$ subcapitata</td>
<td>72h</td>
<td>&lt;1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>$D.$ pulex</td>
<td>48h</td>
<td>0.01 (±0.01)</td>
<td>&lt;0.0005</td>
<td>0.0005</td>
</tr>
<tr>
<td>$D.$ magna</td>
<td>48h</td>
<td>0.15 (±0.14)</td>
<td>&lt;0.005</td>
<td>0.01</td>
</tr>
<tr>
<td>$D.$ rerio</td>
<td>96h</td>
<td>4.85 (±1.23)</td>
<td>&lt;1</td>
<td>5</td>
</tr>
</tbody>
</table>

* - No Confidence levels could be calculated
NC- Could not be calculated
3.4. Discussion

Since the absorbance that nAu are read on a spectrophotometer is around 540nm (Wang et al., 2011), the increased luminescence which increased as the exposure concentration increased was a result of spiked nAu concentrations. Unpublished data by Boodhia (2013) showed that assays that are based on absorbance to determine end points are not suitable for nanoparticle assessment. A review of the bacterial OECD guidelines needs to be implemented (Doak et al., 2012) as Wang et al. (2011) showed that nAu particles adsorb to the bacterial wall and will thus always cause interference in these assays as both bioluminescence and reflected light will be measured. García et al. (2012) however found low toxicity of nAu to bacterial communities. For these reasons the bacterial bioluminescence viability test was excluded from the current study. It is however important to note that the dispersant showed no toxicity across all concentrations tested, which was concurrent with review articles of studies done by (Klaine et al., 2008; Yah, 2013; ).

The ability for the particles to agglomerate, caused by nAu surface charges, has an effect on the interactions of the particles interacting with one another (see Chapter 2). But the same can be said for the ability of the nAu particles to form interactions with the algal cells present in the media (Zamani et al., 2014; García et al., 2012; Li et al., 2011; Van Hoecke et al., 2011). Renault et al. (2008) showed a strong interaction between amine coated nAu and algae. The ability of nAu particles to undergo plasmon resonance with light is of great importance within the therapeutic fields as it can be used to resonate the electrons to destroy tissue. Gold nanoparticles have the ability to scatter and absorb visible light, this is highly dependent on the size and concentrations of the particles (Lasagna-Reeves et al., 2010; Wang et al., 2008). The larger the agglomeration the longer the wavelength of light absorbed by the particle which leads to shading effects (Kühnel and Nickel, 2014; Wang et al., 2012) and will therefore have the ability to increase the toxicology of the particle on those organisms that are dependent on light for their growth.

When similar absorbance values of spiked samples exist the chlorophyll-a extraction method can be used to account as it would be read at a different absorbance (Burnison, 2011). All nAu
concentrations showed a decrease in algal growth over 72h when compared to the dispersant and control exposures. As the nAu concentration increases so does the amount of surface adhesion of nAu to the algae (Figure 3.4) thereby causing a reduction in the interaction of the algae with the environment, this in turn results in a lower metabolic rate and in turn the ability to for algae to reproduce (Lin *et al*., 2009; Youn *et al*., 2011; Zamani *et al*., 2014). This cellular adhesion may also weaken the wall and influence vital exchanges at this site. In this study there was no evidence that the nAu particles were able to penetrate the plasma of the algae. Positively charged nAu particles adhere to the cell wall by attractive negative charges on the membrane (García-Cambero *et al*., 2013). However it has been proposed that even though nanoparticles do not enter the cell the adhesion to the surface causes the release of free metal ions thereby increasing the toxicity (Schirmer, 2014), which when coupled with shading effects could be detrimental to algal growth (Kühnel and Nickel, 2014; Wang *et al*., 2012).

As was expected the ionic gold had a higher toxicity across all concentrations. García-Cambero *et al*.* (2013) showed an LC50 of 1.91mg/l for algae exposed to chloroauric acid, where as can be seen in this test also showed toxicity from as low as 2mg/l. The bioavailability of the Au$^{3+}$ ions as well as their phototoxicity above 1mg/l provides the rationale for toxicity to aquatic organisms at low concentrations (Wang *et al*., 2011). The long term degradation of nAu particles in the environment is unknown and therefore the toxicity of their metal ions are of concern.

It was apparent that at the concentrations where the daphnia were affected by nAu there was a change in the observed behaviour. This included erratic swimming in circles and remaining at the bottom of the beaker with only the appendages showing movement, while at higher concentrations they all tended to stay in the bottom of the beaker rather than the surface. Klaine *et al*.* (2008) suggests that not enough evidence is available to show the toxic mechanism of nAu in daphnia. Since daphnia are filter feeders, it has been shown that nAu particles line the gut (Luoma *et al*., 2014; García-Cambero *et al*.* 2013; Li *et al*., 2011) and as seen by Fernandes *et al*.* (2010) fluorescent carboxylated nanoparticles were translocated to reserve fat droplets. This translocation occurs via endocytosis across the membrane (Oh and Park, 2014) once it has been ingested. Luoma *et al*.* (2014) found that negatively charged quantum dots were taken up more rapidly. In this study the concentration of exposure was the major contributor to toxicity effect). The nAu is also able to attach to the surface of the daphnia as seen in bacteria (Wang
al., 2011), which could therefore affect the seasonal and reproductive molts and in turn decrease the life span of the adult (Li et al., 2011). In this case in concentrations above 25mg/l up to 45mg/l there were low mortalities and it is suggested that this is related to particle agglomeration in the medium and the nanoparticle bioavailability and size distribution (see Chapter 2) which would have an influence on the biological uptake into the daphnia as well as the particle distribution and effect.

Griffitt et al. (2008) showed that the acute toxicity responses of fish following exposure to silver nanoparticles resulted in different responses such as mortality, accumulation and changes in gene expression were shown. In this study a bimodal mortality response was recorded for D. rerio. This could be attributed to size related uptake (Geffroy et al., 2012) since the size of the particle will affect its uptake and effect (Hirn et al., 2011). At 25mg/l the concentration is high enough to have an effect and particles are small enough to be taken up by the fish rather than precipitation out of solution (see Chapter 2; Wang et al., 2008; Pissuwan et al., 2011; Cui et al., 2011; Alkilany et al., 2012). Dedeh et al. (2014) showed in sediment testing at lower concentrations of nAu that D. rerio showed no mortality but sub lethal effects were apparent. The nAu particles have been specifically designed for uptake and distribution in cells (Yah, 2013), but the binding to other available elements present in the water is what influences their toxicity (Schaeublin et al., 2011). The chloroauric acid however showed 50% mortality at 4.85mg/l due to free Au$^{3+}$ ions present and as already discussed these have a higher effect due to bioavailability.

### 3.5. Conclusion

A new technique needs to be developed for bacterial acute testing, which could possibly involve the use of agar plates to determine the differences in colonies numbers similarly to that of the algae. This study confirms that a chlorophyll-a extraction method can be used for algal testing and that shading effects in conjunction with the membrane attachment interactions as seen in algal acute testing inhibit growth. The daphnia had an LC50 of 75.314mg/l for D. pulex and it was not calculable for D. magna. The effect of changes in molting cycles needs to be addressed to further explain the toxicity in daphnia as well as particle surface interactions. D. rerio showed
low mortality to nAu aquatic exposure except at 25mg/l where particle size distribution and concentration in the medium are suggested as a viable cause for this effect due to lower mortality at concentrations above the previously mentioned one. The ionic gold inhibited growth in algae from as low as 1mg/l, the daphnia had an LC50 of 0.01mg/l with a 95% confidence level and the zebrafish had an LC50 of 4.85mg/l with a 95% confidence level. The toxicity of the ionic gold was attributed to Au$^{3+}$ ions, which have a high bioavailability.
Chapter 4: Gold nanoparticle (nAu) uptake, distribution and effects on reproduction in adult *Daphnia magna*

4.1. Introduction

For nanoparticles to cause toxicity it is assumed that they get taken up and distributed within an organism, therefore a better understanding of how they behave in an *in vivo* is system is necessary (Wepener *et al*., 2011). To date many studies have focused on the *in vitro* toxicity and hazard potentials but bioavailability is rarely directly addressed (Roy *et al*., 2014; Yah, 2013; Linkov *et al*., 2008; Gillis *et al*., 2006). Gold nanoparticles have unique properties which include size distribution, surface charge and the ability to aggregate, therefore the uptake and tissue distribution, will also be unique to these properties. Current techniques employed to study metal accumulation may not be entirely useful and may overlook possible hazards in the use of nanomaterials, which will therefore affect the development of risk assessments and regulation protocols (Luoma *et al*., 2014; Goodman *et al*., 2004).

In an aquatic system there are more factors, which play a role in the particle distribution and this starts with the ability for the nAu to be taken up by the organism (Oh and Park, 2014; Goodman *et al*., 2004). In the case of metal ions they are taken up across the gill, intestine or body surface (van Dyk *et al*., 2009). In the case of nanoparticles, agglomerates are formed in the medium based on forces that exist between water molecules and charges on the particle surface. Variations in surface properties and geometry not only play a role in cellular uptake but also play a role in the bioavailability and biokinetic fate within an organism (Arnida *et al*., 2011; Hirn *et al*., 2011).

For those nAu particles, which do become bioavailable, the size of particles and it's agglomerates would play a role in how the introduced materials are taken up, distributed and eliminated (Schirmer, 2014; Oh and Park, 2014). In citric capped nAu it was found that that 50nm spheres were taken up by endocytosis more rapidly than those particles smaller or larger in size. Therefore effective processing is highly dependent on the size and type of cellular transport across membranes and mechanical barriers and the successful clearance is
dependent on the shape of the particle as well as changes in agglomeration patterns which could occur in the in vivo system (Oh and Park, 2014; Aillon et al., 2009; Goodman et al., 2004).

Since nAu are specifically designed for the therapeutic and drug delivery fields it is essential that they will be able to move across tissue barriers as well as cell membranes (Yah, 2013; Arora et al., 2012; Byrne et al., 2010; Akiyama et al., 2009; Boisselier and Astruc, 2009; Lewinski et al., 2008). However the distribution following uptake is influenced by the normal homeostatic mechanisms, which can either limit or enhance their internal behaviour and effects. Thus an understanding of these factors is essential towards further understanding the toxicity of nAu particles in vivo.

Freshwater daphnids, specifically D. magna and D. pulex, have been used since the 1960’s as standard species in acute and chronic toxicity testing (Persoone et al., 2009). Daphnids are both nationally and internationally recognized as a test organism for small scale toxicity testing where different end points may exist. For ISO and OECD standards an immobilization test is used while the US EPA select lethality as an end point for the test (Persoone et al., 2009; Jonczyk and Gilron, 2005). These invertebrates are routinely used as a model organism since they are relatively easy to culture and maintain and are sensitive to environmental pollutants. Daphnids also provide an alternative for in vivo testing rather than using mammalian systems which require far more space and maintenance (Khan et al., 2014; Gillis et al., 2006; Guilhermino et al., 2000).

As filter feeders D. magna ingest any material ranging between 240 and 640nm within a water column and are able to filter up to 400ml of water per day (Wray and Klaine, 2015; Khan et al., 2014; Zhu et al., 2009). These organisms will cause water currents or stir up sediments using their thoracic appendages to make any particles that have settled out available again (Gillis et al., 2006). Most studies indicate that the ingestion of nanoparticles by D. magna seem to remain in external compartments such as the gut or remain on the external carapace surface (Wray and Klaine, 2015; Khan et al., 2014; García-Cambero et al., 2013; Asghari et al., 2012; Lee and Ranville, 2012; Heinlaan et al., 2011; Zhu et al., 2009; Gillis et al., 2006; Schultz and Kennedy, 1976). Khan et al. (2014) used biokinetic modeling of silver nanoparticles and determined that
70% of the nanoparticle burden was due to ingestion. While Pan et al. (2012) showed that studies on clams also showed that nAu particles were able to accumulate in soft body tissues once filtered.

The aim of this chapter is to determine the nAu particle exposure and fate to adult *D. magna* and the effect on molting and reproduction.

### 4.2. Materials and methods

#### 4.2.1. Bioassay protocol

A stock culture of *D. magna* was maintained in culture medium prepared as discussed in Chapter 3 and media was replaced three times per week. Cultures were kept at 20-22°C and were fed on unicellular algae (*P. subcapitata*) daily as well as a supplemented diet of daphnia food prepared by grinding and settling out trout pellets, yeast and *Spirulina* pellets before using the liquid phase for feeding.

#### 4.2.1.1. Exposure and characterization

Gold nanoparticle stock solutions were prepared and supplied by MINTEK and all characterization was performed and discussed in Chapter 2. The following concentration range was selected and made up using daphnia media: 0.5mg/l, 2mg/l, 5mg/l, 10mg/l, 15mg/l and 20mg/l.

#### 4.2.2. Fourteen day *D.magna* reproduction test

A *D. magna* reproduction test was performed using the OECD 211 guideline (1984) across the above mentioned concentrations and medium to determine whether the nAu particle interaction
had an effect on breeding rates. The test was run for the minimum period of 14 days (OECD 1984, 14-21 days). The medium and concentrations were renewed three times per week and each beaker was supplied with a concentrated drop of *P. subcapitata* with each media change. The temperature was maintained between 18-22°C and there was a 16h light interval and oxygen saturation was maintained above 60% for the duration of the test. Ten adult individuals were used per concentration with a minimum of 40ml of exposure media available to each and offspring as well as molts were counted per concentration per day. Any behavioural changes were noted and only adults were transferred to new beakers at each media change.

**4.2.2.3. CytoViva® dark field hyperspectral imaging**

Several molts were collected throughout the duration of the test. At the conclusion of the test adult *D. magna* and a drop of exposure media were also collected to be further analysed. One drop of exposure media was placed onto a microscope slide allowed to dry for ten minutes, covered with a cover slip and sealed with Entellan®. Exposure media slides were used to set up a baseline to refer to in slides prepared of exposed organisms. Cryopreserve gel (Tissue-Tek® O.C.T.™ Compound) was used to mount adult daphnia and molts onto a microscope slide, slides were covered with a cover slip. Cover slips had to be placed onto the slides very carefully, if this was not done the gut contents would be pushed out of the organism. Slides were then stored at -80°C until further analysis under a CytoViva® 150 Unit integrated onto the Olympus BX43 microscope. Images of the exposure media, molts and *D. magna* were captured using the Dagexcel X16 camera and DAGE Exponent software at 60X magnification.

**4.3. Results**

**4.3.1. External adsorption of nAu to Daphnia magna**

The concentration range used for characterization clearly showed how the stock medium which is initially a dark red colour changed when diluted with daphnia media ranging from a pink to dark purple colour (Figure 4.1.)
The accumulation of nAu in the gut occurred within 24h during both the 14d reproduction and 48h acute immobilisation test (Chapter 3). The accumulation in the gut increased as the exposure concentration increased (Figure 4.2). In concentrations of 15mg/l and above the organisms displayed a purple tinge to their outer surface, which resembles the purple colours observed in Figure 4.1. It was noted that in some concentrations the purple outer coating as seen at 24h was no longer present in some organisms after 48h. Therefore the molting patterns were assessed in combination with the reproduction test.
Figure 4.2: Adult Daphnia magna viewed under a light microscope after a 48h exposure to varying concentrations of nAu a) 0.5mg/l. b) 2mg/l. c) 5mg/l. d) 10mg/l. e) 15mg/l and f) 50mg/l.
4.3.2. CytoViva® dark field hyperspectral imaging of Daphnia magna

CytoViva® dark field hyperspectral imaging was used to further assess the internal particle distribution in daphnia. The medium, which was placed on a slide was studied using dark field hyperspectral imaging to setup a baseline. The presence of nAu particles was confirmed to be bright red to maroon coloured clusters floating at different size agglomerations (Figure 4.3a). When studying the distribution and effect, the control benchmark sample was compared to exposed samples under dark field hyperspectral imaging. There is an accumulation of nAu on the carapace of *D. magna* (Figure 4.3b). Further surface agglomeration of nAu was seen where concentrations of nAu attached onto those already found on the organism. The maxillae used for locomotion and feeding had an accumulation of nAu packing onto one another on the larger portions and then accumulate on and between the finer hairs where it very likely gets trapped during movement (Figure 4.3c+d). A current is formed by the maxillae in the water and food moves into their mouth opening across a pair of toothed or ridged grinding mandibles, photographic evidence (Figure 4.3e) that some of nAu agglomerations are ground onto the mandibles before passing into the gut. A closer magnification of the gut (Figure 4.3f) indicates that the nAu particles stay within the gut lining and pack onto one another as more food is filtered in. The large postabdomen is found at the posterior end of the body and it contains two long abdominal setae, two terminal claws and a series of lateral teeth (Figure 4.4a), as nAu agglomerates are pushed out of the gut while more are being forced in you are able to see as the agglomerates start accumulating between the lateral spines on the terminal claw. The terminal claw is mostly used for cleaning debris from the thoracic legs but may aid in locomotion, in this case it is proposed that particles are able to accumulate between the lateral spines on the terminal claw during this cleaning process. The locomotion of *D. magna* resembled erratic up and down movement, where daphnids swim up and float down toward the bottom, the nAu agglomerates tend to accumulate in higher concentrations around the posterior portion of the organism (Figure 4.4b), when compared to the anterior and this is attributed to the swimming patterns. Between concentrations daphnia appeared to have molted and swimming patterns changed from slow, almost heavy movements to normal locomotion, when molts were observed under hyperspectral imaging it was evident that the nAu agglomerates were present in high concentrations across the carapace and were lost during molts, which included the thoracic legs as seen in Figure 4.4c-f.
Figure 4.3: Daphnia magna exposed to nAu particles in aquatic medium viewed under CytoViva® dark field hyperspectral imaging showing a) aquatic medium, b) body surface, c) appendages, e) mandibles and f) gut.
Figure 4.4: Daphnia magna exposed to nAu particles in aquatic medium viewed under CytoViva® dark field hyperspectral imaging showing a) terminal claw, b) surface spines, c-f) molts.
4.2.3. Daphnia reproduction test

The daphnia reproduction test showed a 97% increase in the number of juveniles in the 2mg/l concentration when compared to control, while the highest concentration of 20mg/l showed an increase of 70%. The cumulative molts (Figure 4.5) and cumulative number of offspring (Figure 4.6) increased at all exposure concentrations when compared to the control. Only the control ($R^2=0.352$), 2mg/l ($R^2=0.596$) and 5 mg/l ($R^2=0.226$) exposure groups showed a significant positive correlation between number of molts and juveniles. The lowest concentration of 0.5mg/l showed a low correlation when compared to control. The number of juveniles was however similar, this was attributed to the control releasing more juveniles and less molts while at 0.5mg/l the opposite trend was observed. At day four the 5mg/l exposure group produced 14 juveniles while the total number of adults that molted was seven. For two consecutive days there were less than two molts but nine juveniles were released at 5mg/l. From day nine the molting pattern continued with up to seven molts per concentration per day, but less than three juveniles were released at 20mg/l. For the control group the molts decreased (less than three) during periods when no juveniles were released. At the higher exposure concentrations similar number of molts (three to seven) occurred regardless of the number of offspring produced.
Figure 4.5 Cumulative number of adult D. magna molts during a fourteen day reproduction test comparing exposure concentrations to a control.

Figure 4.6. Cumulative number of offspring of D. magna during a fourteen day reproduction test comparing exposure concentrations to a control.
Figure 4.7: Results of *D. magna* reproduction test over a duration of fourteen days. a) The sum of juveniles per concentration. b-h) The correlation ($R^2$) between the number of juveniles per concentration and the number of molts shed by adults.
4.3. Discussion

*Daphnia magna* are filter feeders which are able to filter particles up to 640nm (Wray and Klaine, 2015; Khan *et al.*, 2014; Zhu *et al.*, 2009). In this study the largest aggregates seen on the Zetasizer were 986.4nm but sizes predominantly ranged between 20nm and 640nm (Figure 2.4). Lee and Ranville (2012) exposed *D. magna* to nAu agglomerations that had settled out in the container and found that they were still able to ingest and maintain them in the gut, this suggests that aggregation reduces but does not eliminate bioavailability (Khan *et al.*, 2012). Therefore aggregation, size and shape did not seem to play a role in particle uptake (Wray and Klaine, 2015).

Charges present on the nAu surface play a role in tissue distribution and effects (Fent *et al.*, 2009; Gu *et al*. 2009). Uptake modeling studies by Wray and Klaine (2015) predicted that *D. magna* will have a higher body burden of larger cationic nAu at a high exposure concentration and have a higher body burden of anionic particles at lower exposure concentrations. The nAu in this study had anionic charges across all ranges except for higher concentration ranges (15mg/l to 25mg/l) where there seemed to be a mixture of cationic and anionic charges present at 0h. This however changed to anionic charges after 48h across the full range of exposures (Table 2.2.). Wray and Klaine (2015) and Khan *et al*. (2012) showed that surface charge played a major role in where nAu were found once inside the gut, anionic nAu were found associated with or in close proximity to the peritrophic membrane or the microvilli situated beneath it which seemed to inhibit their elimination. Cationic nAu appeared to be aggregated around debris found in the lumen which also slowed down their elimination and ability to be internalized. The particle-particle interactions between nAu will also play in a role in the ability for higher concentrations to have higher nAu body burdens. As the daphnia feed and swim through an exposure medium they would cause particles to collide and further attach onto one another and onto the body surface of the daphnids (Chapter 2; Wray and Klaine, 2015).

In this study the exposure concentration of nAu in daphnia media was directly proportional to the amount of nAu present in the gut as well as the observed adsorption on the carapace and appendages (Figure 4.2). Zhu *et al*. (2009) suggested that this could lead to mechanical disruptions of the feeding appendages or penetration to the gut wall. Mechanical disruptions in
the gut wall could affect the uptake of nutrients and gaseous exchange across the external surface thereby causing cell death (Zhu et al., 2009). Mansfield et al. (2015) showed that titanium dioxide nanoparticles also accumulated in the gut of daphnia until visible to the naked eye. These studies suggest that a number of different types of nanoparticles are able to accumulate in the gut but showed no evidence that uptake of nanoparticles occurs across the gut. There is however a pattern of retention, the foregut and hindgut of daphnia are ectodermal in origin the epithelia are therefore lined with chitin. The midgut which is the site for absorption, digestion and preparation of faeces is mesodermal in origin and is not lined, though there is no internalization this would be an area at risk for mechanical disruption (Khan et al., 2014; García-Cambero et al., 2013; Asghari et al., 2012; Khan et al., 2012; Lee and Ranville, 2012; Heinlaan et al., 2011; Zhu et al., 2009; Gillis et al., 2006).

High concentrations of nAu which accumulate in the gut in this manner or by highly concentrated exposure concentrations which are filter fed may have similar effects as those exposed to sediment (Gillis et al., 2006). Lewinski et al. (2010) found that the nAu that accumulated in to gut remained there even after daphnia were placed in a clean water system for 48h, but when daphnia were fed on algae the nAu in the gut was eliminated out of the organism as ingestion occurred. However Wray and Klaine (2015) and Khan et al. (2014) found rapid removal of nAu present in the gut at lower concentrations (highest concentration of 0.5mg/l and 0.6mg/l respectively) than used in this study (the LC50 for D. pulex was 75.314mg/l; Chapter 3). For lower concentrations irrespective of food availability, it was found that 75% of the elimination within a few hours (less than 24h), while the remaining 25% of nAu that were ingested remained in the hindgut and were eliminated one hundred times slower. García-Cambero et al. (2013) found low lethality in acute testing but the effect over an extended time period may answer important questions about the long term effects of nAu in the gut.

Lovern et al. (2007) also reported changes in swimming behaviour where they would swim on the surface at a faster rate and remain static at the bottom of the beaker, specifically in higher concentrations where daphnia appeared “heavy” (García-Cambero et al., 2013). It was noted (Figure 4.4a+b) that as the swimming pattern had changed into a slower hopping movement the ventral area of the daphnia i.e. the post abdominal claw and lower spine area, had an increased density adhered beneath the surface spines. It is proposed that as more nAu particles are
ingested they are pushed out and as the daphnid swims into again it causes this adherence (Lee and Ranville, 2012). This is magnified as the claw is used to clean appendages used in the feeding process (Wray and Klaine, 2015; Zhu et al., 2009). As seen in Figure 4.4c-f the molts contained a higher intensity of nAu particles on its surface but it would need to be verified using metal analyses.

The reproductive study showed that across all concentrations there were a higher number of cumulative molts (Figure 4.5) released when compared to the control, as well as a higher cumulative number of offspring (Figure 4.6). The role of molting in daphnia is for juveniles to develop into adults as well as aid in the release of the ephipidum or egg. It has also been reported to help regulate internal concentration of cadmium, mercury, nickel and zinc (Lee and Ranville, 2012). The highest total number of juveniles was seen at relatively low exposure concentrations, i.e. 2mg/l (Figure 4.7a). These findings are supported by Ribeiro et al. (2014) who found that the cumulative number of juveniles of *D. magna* exposed to silver nanoparticles was stimulated at the lowest concentration (0.5µg/l) when compared to the control but then decreased as the concentrations increased (1µg/l and 2µg/l). Other studies relate this to the process of hormesis which will be further discussed in Chapter 5 (Nascarella et al., 2012). This study revealed that at 2mg/l ($R^2$ = 0.5967) the cumulative number of molts was similar to the control ($R^2$ = 0.3526), but the number of juveniles produced was higher. As exposure concentrations increased above 2mg/l the rate of molting increased in an unrelated manner (20mg/l $R^2$ = 0.0039) when compared to the number of juveniles released. This could be a direct relation to filtration rates and body burdens (Wray and Klaine, 2015; Gillis et al., 2006). Changes in reproduction could be as a result of energy diversion due to exposure of nanoparticles (Kim et al., 2014).

In an environmental system this alteration in swimming patterns or where organism appeared “heavy” could affect predation (García-Cambero et al., 2013). Changes in molting patterns and a disruption in feeding capacity and digestive function can trigger adverse effects and escalate to processes like growth and reproduction and in turn affect populations and communities (Croteau et al., 2011; Li et al., 2010b; Li et al., 2010c). As nAu interact with organisms from the lowest to the highest trophic level there is a possibility that they would be able to move along the food chain as higher organisms ingest these interactions or nano-coated molts which would lead
to long term effects (Schirmer, 2014). Therefore there is an urgent need to address the effects and fate of nAu in an *in vivo* system due to exposure from release into the environment (Bour *et al.*, 2015).

### 4.4. Conclusion

The ingestion of nAu on D. magna is irrespective of the shape and size of the exposure concentration since the organism are filter feeders which are able to resuspend and ingest any nanoparticles which have settled out of solution. The concentration in the media and the charge of particles play a major part in the uptake and surface adsorption, where higher concentrations aggregate onto the carapace and pack into the gut. There is no evidence of internalization of nAu particles into the daphnia but elimination rates of nAu are dependent on concentrations present within the gut. As exposure concentrations of nAu increase so do the number of molts that occur which becomes unrelated to the numbers of juveniles released, thereby affecting daphnia reproduction.
Chapter 5: Uptake, distribution and sub cellular effects of nAu exposure in *Danio rerio*.

### 5.1. Introduction

There is a lack of understanding of *in vivo* system nanoparticle particle behaviour (Roy *et al.*, 2014; Johnston *et al.*, 2012; Linkov *et al.*, 2008). A standard *D. rerio* (zebrafish) model has been developed for traditional chemical aquatic toxicology to fill the breach in information between cell culture models and mammalian models. The advantage of a rapid pre-clinical model organism such as zebrafish bioassays is that it relatively rapid, inexpensive and straightforward to perform (Shaw and Handy, 2011; De Jong *et al.*, 2008; Fako and Furgeson, 2009). The close homology between the zebrafish genome and the human genome make it an ideal candidate for sub cellular toxicity testing, the genetic parallels include physiological and anatomical similarities between the endothelial cells, blood brain barrier and immunogenic responses. And xenobiotic substances demonstrate a similar response such as oxidative stress and enzyme metabolites (Fako and Furgeson, 2009).

The true ecotoxicological relationships of chemicals are determined not by the external dose but by what reach target tissue within the organism. This is however difficult to determine, therefore in aquatic toxicology the biological effect is assumed to be related to the dose of the chemical that is administered. There are three main assumptions in the standardized OECD toxicity assessment, i.e. there are receptor sites for chemical interaction, the proportional response is related to chemicals at these receptor sites, and the concentration is related to administered dose (Wepener *et al.*, 2011). In the case of nanoparticle exposure assays in aquatic systems there is a lack of evidence to support that a dose dependent response exists due to agglomerations of the nAu particles in the medium. The activity of receptors for nanoparticle uptake will change depending on the size of the agglomerations, the charge and the functionality that exists on the particle surface (Kühnel and Nickel, 2014; Oh and Park, 2014; Hund-Rinke and Klawonn, 2013; Shaw and Handy, 2011; Klaine *et al.*, 2008). The biophysical interaction that exists between the cellular membrane and nanoparticles is important in
determining the toxicity because it can trigger the subsequent penetration an internalization of nanoparticles into living cells (Hartono et al., 2010).

It is evident that acute toxicity bioassays provide an indication of toxicological potency but does not provide any information on the mechanism of toxicity or the sublethal effects of exposure to these nanoparticles. Nanoparticles have been described as being able to be taken up by the body and are able to be redistributed (Balasubramanian et al., 2010; Tedesco et al., 2010) and visualization thereof can be achieved using microscopy. Hyperspectral imaging can give an indication of where these particles accumulate (Arnold et al., 2013). Histology shows the relationship between cell types and tissues, an early detection of changes can be found on a cellular level (Hinton and Laurén, 1990; Van der Oost, 2003). By using muscle tissue to test metal bioaccumulation the differences between exposure groups or organs can be quantified (Balasubramanian et al., 2010).

The application of biomarkers as indicators of effect due to environmental exposure to pollutants is routinely applied internationally (Wepener et al., 2011). Effects assessment makes use of a suite of biomarkers, which indicate responses of the indicator organisms at different levels of biological organisation, ranging from sub-cellular to whole organism level (Wepener, 2008). Microarray is a promising approach to give a large-scale indication of gene expression simultaneously across the whole genome and is a powerful molecular tool, which is becoming increasingly important to identify responsive genes, which can be used as biomarkers (Ruggeri et al., 2008). The gene expression can then be related to the gene ontology (GO) classes for each species. The processes are divided into biological processes, cellular components and molecular functions, which can be used in conjunction with the GO database to source information such as which gene and protein correspond in a particular function (Kashiwada et al., 2012; Kausch et al., 2007; Martyniuk et al., 2007).

Reactive oxygen species (ROS) formation occurs when there is decoupling of the electron transport chain and instead of transferring the electron to ADP it is transferred to oxygen forming a superoxide anion radical. The production and release of ROS from the mitochondria into the cell can have several consequences including DNA/RNA damage, lipid peroxidation,
protein oxidation the and activation of the Ca^{2+}-dependent mitochondrial permeability transition pore which would in turn further affect toxicity and uptake of persistent nanoparticle exposure (Fu et al., 2014; Du et al., 2012; Durazo and Kompella., 2011). Balasubramanian et al. (2010) found that 20nm nAu particles induced oxidative damage in human embryonic lung fibroblasts as well as inhibited cell proliferation and this was further antagonized by the down regulation of cell cycle and DNA repair genes (Oberdörster et al., 2005). In a Drosophila sp. exposure to citrate capped nAu particles there were phenotypic modifications in subsequent generations showing that these genetic mutagenic effects may be transmitted to descendants (Vecchio et al., 2012). Therefore the gene ontology of biological processes, cellular components and molecular functions will give an indication of genetic changes in the organism across the whole genome (Kausch et al., 2007; Martyniuk et al., 2007). Exposure concentrations are genetically compared to one another to determine the effect of all nAu particle characterization parameters (i.e. concentration, charge, functionalization, agglomeration pattern). A focus will be placed on genetic changes in the form of real time polymerase chain reaction (RT-PCR) based on primers designed specifically for each target gene (Miller et al., 2012; Zucchi et al., 2011). Exposure biomarkers were selected to give a better understanding of nAu effects on acetylcholine esterase (AChE), metallothionein (MT - a specific indicator of metal exposure) and cytochrome P450 (CYP450). Oxidative biomarkers of effect will be explored by determining levels of catalase (CAT), superoxide-dimutase (SOD), lipid peroxidation (LP), protein carbonyls (PC) and non-enzymatic reduced glutathione (GSH). The cellular energy allocation biomarker has been used to determine the effect of influence of pollutants on whole body energy reserves (Verslycke et al., 2003).

The aim of this chapter is to relate the uptake of nAu to biological responses ranging from subcellular (genotoxicity and biochemical biomarkers) to organ (histology) level in zebrafish. The responses to nAu are compared to ionic gold exposure to determine whether the toxicity mechanisms are similar.
5.2. Materials and methods

5.2.1. Danio rerio 96h exposures

The exposures were based on the *D. rerio* acute lethality test (OECD-TG203) was performed on adult fish, where five adults were placed in a beaker with a continuous supply of oxygen to maintain the saturation above 60% for the duration of the test. Exposures (i.e. three sets of replicate exposures per concentration) were conducted as described in Chapter 3 and a range of 5mg/l, 10mg/l, 15mg/l, 20mg/l, 25mg/l, 30mg/l, 35mg/l, 40mg/l and 45mg/l was used for the nAu exposures. For ionic gold the exposure concentrations were 1mg/l, 2mg/l, 5mg/l and 10mg/l. On conclusion of the test after 96h, fish were sacrificed by flash freezing in liquid nitrogen and random samples from the three replicates per concentration (i.e. n = 15 per concentration) were collected for the different analyses. Five gill samples were collected for histology, five gills for TEM analysis, three whole body organisms for cryomicrotoming and CytoViva®, ten fish per concentration for muscle digestion, five liver samples were pooled for microarray and RT-PCR, three liver samples were pooled for Cytochrome P450 analysis and whole body samples were frozen at -80°C in respective buffers for biomarker analysis.

5.2.2.1. RNA isolation from Danio rerio liver samples

Liver samples were stored in RNA Later (Ambion) at -80°C pending analysis. An adjusted RNA isolation protocol was used. The TRI Reagent® samples were homogenised using a hand held homogeniser, chloroform was added and samples were vortexed and then centrifuged at 12,000g for twenty minutes at 4°C to separate RNA from the sample. The top layer containing the RNA was placed on a blue ring Nucleospin RNA II kit (Macherey-Nagel) filter membrane and standard protocol provided with the kit was used to remove further DNA, salts and proteins present. The RNA was eluted from the membrane using RNase free ultrapure MilliQ water.
5.2.3. RNA quality

The RNA quality was checked using a NanoDrop 1000A Spectrophotometer to determine the amount of aRNA to be added, while examining the 260:280 absorbance ratio of the graph. In addition gel electrophoresis using a 1kp base pair ladder to identify the 18S and 28S RNA bands was also used. Further confirmation was done using a bioanalyser and RNA Nano Chips (Agilent Technologies) since in vitro studies have shown that particle interference can have an influence on RNA quality (Sanabria et al., 2014). Reagents were brought to room temperature and an Agilent 2100 Bioanalyser was used where a cleaning chip containing 350µl of RNase Zap and a nuclease free chip were used to prepare the instrument for one minute with each chip and a ten second interval period between cycles. Each sample (2µl per sample) as well as a 6000 RNA ladder was heated to 70°C for three minutes and then placed on ice for five minutes. During this time 550µl of gel was prepared by filtering it at 1500g for ten minutes, which could be stored for up to four weeks at 4°C. A 1µl aliquot of light sensitive dye was added to 65µl of gel. The mixture was vortexed and spun at 13000g for ten minutes in a microcentrifuge. A syringe apparatus was set to 1ml to load the gels. To RNA Nano Chip was loaded by adding 9µl of gel and 2ml of the sample loaded in the syringe. The chip was checked for any bubbles and to see if the lines were visible. Nano Marker (5µl) was added to each well, except for the G wells. To each corresponding well number (well 1- 10) respective sample (1µl) was added and 1µl of 6000 RNA ladder was added to the well marker ladder, 9µl of gel dye mix was added to the two additional wells marked G in black. The plate was fixed onto a Bioanalyser chip vortexer (IKA MS3) and shaken for one minute at 2400 shakes. The chip was then inserted into the Agilent 2100 Bioanalyser and the RNA protocol was selected for eukaryote total RNA Nanoscience II. Once completed the cleaning protocol was repeated.

5.2.2.2. Using isolated RNA to make cDNA

The isolated RNA was used to make cDNA by adding 0.5µg of RNA to oligo nucleotides and made up to 3.25µl using milli Q water. The mixture was kept at 70°C for ten minutes in a Thermocycler (Biorad T100) and then placed on ice for five minutes. After this RT buffer, dNTP mix and RT Ace were added and to make a final volume of 10µl. A thermal cycler was used to
keep samples at 42°C for fifty minutes and 99°C for five minutes. Once the run was complete cDNA was diluted to a ten times dilution using MilliQ water for a final volume of 100µl and stored at -80° until used for RT-PCR.

5.2.2.3. GeneChip® Microarray of isolated RNA samples

Once the RNA quality and concentrations were known the microarray protocol was started. Input RNA, which had to be less than 9µl due to the addition of Poly-A controls, was dependent on the previously mentioned RNA quality results but a concentration of 1000ng was selected for all samples. A dilution series was set up for Poly-A controls and 2µl of the fourth dilution was used for the Poly A RNA control. A T7 Oligo (dT) primer was added to the mixture and filled up to 12µl using milli Q water, the mixture was then incubated at 70°C for ten minutes using a thermal cycler and then placed on ice. Reverse transcription to synthesize first strand cDNA was initiated by adding a first strand master mix to the mixture to make a new final volume of 20µl and this was incubated for two hours at 42°C in a thermal cycler.

The sample was then immediately placed on ice and a master mix (80µl) was added to commence second strand cDNA synthesis to a final volume of 100µl. The samples were incubated at 16°C for two hours and then placed on ice and used for cDNA purification. Nuclease free water was heated to 50°C. For cDNA purification a binding buffer was added to each sample and the sample was pipetted onto a cDNA filter cartridge and then centrifuged. A wash buffer was added and the centrifugation step was completed twice. Once to wash the buffer through and a second time to dry the membrane. The cDNA filtration cartridge was transferred to a new elution tube and eluted using 24µl of nuclease free water in a two step process: 12µl was added and incubated for two minutes at room temperature then centrifuging and another 12 µl of preheated nuclease free water was added and centrifuged to elude the cDNA. The eluted cDNA was immediately used for *in vitro* transcription to synthesize labeled aRNA.
An In Vitro Transcription (IVT) master mix was made up at room temperature and was then added to the double stranded cDNA sample (20µl). Gentle vortexing was used to mix the sample and then briefly centrifuged to collect at the bottom of the reaction tube. The sample and IVT mixture were incubated at 37°C for fourteen hours using a hybridization oven. Once incubation time was complete the reaction was stopped by adding 60µl of nuclease free water before moving onto aRNA purification.

Nuclease free water was heated to 50°C to elute aRNA samples once purification was completed. An aRNA binding buffer was added to each sample and straight away 100% ACS grade ethanol was added and mixed by pipetting the sample up and down. Once samples had been mixed each was immediately placed on the aRNA filter cartridge. Samples were centrifuged for one minute at 10 000g, the flow through was discarded and a wash buffer was added. The centrifugation step was completed twice, once to wash the buffer through and a second time to dry the membrane. Preheated nuclease free water (100µl) was added to the membrane and allowed to stand at room temperature for two minutes and then centrifuged to remove all aRNA from the filter. Once aRNA was eluted it was placed on ice. A NanoDrop 1000A Spectrophotometer was used to measure absorbance to determine the amount of aRNA to be added for fragmentation. Twenty µg was used as the maximum amount of input aRNA. A 5x array fragmentation buffer was added and the final concentration was made up to 40µl for a standard (species) GeneChip microarray.

The fragmentation mixture was incubated at 94°C for 35 min and placed on ice immediately after incubation. An electrophoresis gel was used to check the fragmentation comparing the prefragmented aRNA (250- 5000bp) to each sample of fragmented aRNA (35- 200bp). A hybridization cocktail was made by adding 33.3µl of fragmented RNA to 125µl 2x hybridization mix, 50µl nuclease free water, 25µl DMSO and 4.2µl control oligonucleotide B2 (3nM). A 20x hybrization control was heated to 65°C and 12.5µl was added to the mixture to make a total volume of 250µl. The Affymetrix® GeneChip was brought to room temperature prior to hybridization, while the hybridization thermocycler oven was set to 45°C. The hybridization cocktail was heated to 99°C for five minutes and then kept at 45°C for another five minutes. A sterile pipette tip was placed in the top hole in the GeneChip while 200µl of hybridization buffer was added to the GeneChip using the bottom hole. The loaded GeneChip was acclimated in the...
rotating thermocycler set to 45°C for ten minutes. The hybridization cocktail was removed from the 45°C heating plate and centrifuged at 15000xg for five minutes. The hybridization buffer was removed after GeneChip acclimation and the hybridization cocktail was loaded onto the GeneChip in the same method. The GeneChip was incubated for 16hrs at 45°C using a rotation of sixty rotations per minute.

Thirty minutes prior to the end of the 16hr incubation period the Affymetrix® GeneChip® Fluidics Station 450 was primed using distilled water, Affymetrix®wash buffer A and Affymetrix®wash buffer B. Three empty tubes were added to the needles and the fluidics station was set to prime using the Affymetrix® software. Once primed the needles were filled with three eppendorphs containing respectively Affymetrix®staining cocktail 1 and Affymetrix®staining cocktail 2 and needle threes eppendorph was filled with Affymetrix®holding buffer. The fluidics 450 station was then ready to stain the hybridized GeneChips. After the sixteen hour incubation protocol the hybridization cocktail was removed and loaded with Affymetrix®wash buffer A. The GeneChips were placed in the cassette holders and loaded into place. Using the Affymetrix®software the fluidics station was set to staining protocol.

Fifteen minutes prior to the end of the staining protocol the Affymetrix® lazer scanner was switched on to heat into a ready position. Once the staining protocol was completed each GeneChip was scanned individually and six files were given as an output. The ArrayStar12 software was used to analyse data by importing the output file as well as gene annotations available from the Gene Ontology Consortium.

5.2.2.4. Real time polymerase chain reaction using cDNA

Based on the results from the microarray twelve genes were selected for quantification using RT-PCR. The zebrafish genome for each gene was found on the NCBI (National Center for Biotechnology Information) website and copied to be used in Primer3 and Primer Blast. Each nucleotide sequence was pasted into the main tab of the primer3plus interface. Product size, primer size and annealing temperature were displayed for each primer. These primer
sequences were confirmed using available literature (Miller et al., 2012; Zucchi et al., 2011; Lerebours et al., 2009; Martyniuk et al., 2007). Forward and reverse primers (Sigma Aldrich) were diluted from 100µM stock solution to 10 µM using MilliQ water.

Amplification efficiency of the selected primers was determined by using a control cDNA sample. A dilution series was set up where concentrated cDNA was diluted to 5x, 10x, 20x, 40x and 80x cDNA. As each primer had five groups of cDNA only nine genes were analysed per one 96 well plate, where in each well 5µl Fast SYBR Master mix, 0.3µl forward primer (10µM), 0.3µl reverse primer (10µM) and 4.4µl of cDNA were added. The RT-qPCR StepOne software was set to quantitation standard curve and was run at a standard run time of 2hrs including a melt curve stage. Bactin was used as an endogenous control in each 96 well plate.

The C_T value was used to determine amplification efficiency by setting up a graph from the cDNA dilution series. A trendline and R^2 value was added to the graph and the value of the slope was used to calculate efficiency (E).

\[ E = (10^{-1/slope} - 1) \times 100 \]

An amplification efficiency of between 80-105% had to be achieved, while the R^2 value had to be >0.98.

Once amplification efficiency was achieved, the gene expression was determined similarly by adding 5µl Fast SYBR Master mix, 0.3µl forward primer (10µM) and 0.3µl reverse primer (10µM), and 4.4µl of cDNA to each well in the 96 well-plate where Bactin was used as an endogenous control. The run method was set to 95°C for 20s on hold to activate the DNA polymerase, then 40 cycles of 95°C for 3s (to denature cDNA) and 60°C for 30s (for primer annealing and extension). Bactin, MT1, MT2, Sod1, Cat, Gpx1a, EF1a were set to an annealing temperature of 60°C while Cyp1a, Cyp1c1, Cyp11a, Cyp17a and Cyp19a ranged between 53-59°C. The quantitation comparative Ct (ΔΔCt) advanced setup was used. Male sample gene expression was compared to female gene expression samples using ΔCt values.
5.2.3 Biomarker analyses in Danio rerio tissue samples

5.2.3.1. Tissue preparation

Biomarker analysis was performed on whole body and liver (of D. rerio). The head, skin and fins were removed from ten fish. All samples were homogenised on ice in respective buffers and centrifuged using a Madell Technology Corporation centrifuge (Model No. TGL-16M). For acetylcholine esterase (AChE) and malondialdehyde (MDA), 0.06g of sample was homogenised in 300µl of Tris-sucrose buffer and centrifuged at 9500g for 10 minutes at 4°C. For catalase (CAT), non enzymatic reduced (GSH), protein carbonyls (PC) and superoxide dismutase (SOD), 0.1g of sample was homogenised in 1000µl general phosphate-homogenizing buffer and centrifuged at 11 500g for 10 minutes at 4°C. For CEA (cellular energy allocation- protein, lipid and carbohydrate determination) 0.1g of sample which had been homogenised in 500µl cold deionised water and centrifuged at 9250g for 10 minutes at 4°C. Sample for electron transport system (ETS) activity (0.04g) was homogenised in 200µl of ETS buffer (Tris-HCl, 0.2% (v/v) Triton X-100, 15% (w/v) Poly Vinyl Pyrrolidone and MgSO<sub>4</sub>) and centrifuged at 9250g for 10 minutes at 4°C. For metallothionein (MT) concentrations, whole body sample as well as gill samples (from 3 fish per exposure group) were analysed. A sample weight of 0.04g was homogenised in 500µl of homogenising buffer (0.006mM of Leupeptin, 0.0005M Phenylmethylsulphonylflouride and 0.01% βMercaptoethanol), and was centrifuged at 30 000g for 20 minutes at 4°C. The activity of Cyp450 was determined using an Arbor Assays DetectX P450 Demethylation florescent activity kit (Catalog No K011-F1) and three livers samples were homogenised in 200µl of the provided Assay buffer and centrifuged at 11 500g for 10 minutes at 4°C.

5.2.3.2. Biochemical analysis

Protein content in each sample was determined to express biomarker concentrations as activity per mg protein for comparative. The protein content was determined for each of the different biomarkers separately using the method of Bradford (1976) and entails the binding of
Coomassie Brilliant Blue G250 to protein and measuring absorbance at 630 nm where bovine serum albumin (BSA) was used as a standard.

To determine the CAT activity (µmol H₂O₂/mg protein/minute) a protocol adapted from Cohen et al. (1970) was used. Ten µl of supernatant was added to a 96 well microplate in triplicate, hydrogen peroxide (93µl) was added to each well and allowed to incubate for 3min at room temperature. The reaction was stopped by adding 19µl H₂SO₄ and immediately thereafter 130µl of 2mM potassium permanganate (KMnO₄) was added. The quantification of unreacted KMnO₄ was measured spectrophotometrically at 409nm using an automated microplate reader.

The SOD activity (ng SOD/mg protein) protocol was adapted from Greenwald (1989) and 4µl of supernatant was added to each well of the 96 well microplate. Using a multi pipette, 242µl Tris Buffer/DTPA was added and the reaction was initiated by adding 4µl pyrogallol solution. Kinetic readings were taken at 60s intervals for a period of 10min using a microplate reader.

The AChE activity expressed as Abs/min/mg protein was calculated by using a standard curve as adapted from Ellman et al. (1961). The enzymatic activity is measured based on ACh breakdown by a colourimetric method. Prior to addition of the supernatant a reaction mixture consisting of potassium phosphate buffer (210µl), substrate (10µl of s-Acetylthiocholine iodide) and Ellmans reagent (10µl - 2,2'-Dinitro-5,5’dithio-dibenzoic acid), was added to only the first three columns of the 96 well microplate and was incubated at 37°C for 5min. After incubation 5µl of supernatant was added to the reaction wells and read immediately at 60s intervals for 6min. Samples were read at 405nm, using an automated microplate reader (Elx800-Universal microplate reader; BioTek instruments, USA).

The MDA or lipid peroxidation (concentration of thiobarbituric acid reactive substances (TBARS) in nmol/mg protein) was measured according to an adapted protocol Ohkawa et al. (1979) as modified by Üner et al. (2006). A series of glass tubes were used where a reaction mixture of 50µl 8.1% sodium dodecyl sulphate (SDS), 375µl acetic acid, 375µl thiobarbituric acid, and 175µl ultrapure water was added, 25µl of supernatant was added to the mixture and the tubes
were incubated in a hot water bath at 95°C for 30min. After incubation tubes were left to cool to room temperature (22°C) ultrapure water (250µL), and 1250µl of butanol-pyridine solution (15:1) was then added to each sample. Samples were vortexed and then centrifuged at 4000r.p.m for 10min at room temperature. To a 96 well plate 245µl of sample mixture and blank were added in triplicate microplate and read at 540nm using an automated microplate reader.

MT expressed as nM/ mg protein (using the standard curve) were assessed by using an adapted protocol from Viarengo et al. (1997; 1999) using the modifications as indicated by Atli and Canli (2008) and Fernandes et al. (2008). Five hundred microliter of supernatant was added to 500µl of cold (4°C) absolute ethanol and 40µl of chloroform, vortexed and then centrifuged at 7 000r.p.m (4°C) for 10min. After centrifugation three additional volumes of ethanol were added to the mixture, vortexed and incubated at -20°C for 4h until pellet formation. Supernatants were discarded and the pellets were washed twice with 1ml of washing buffer (87% ethanol, 1% chloroform, 12% homogenising buffer), then vortexed and centrifuged at 3000r.p.m (4°C) for 20min. The pellets were dried using compressed air and resuspended in 300µl of Tris-Ethylene diamine tetra-acetate (EDTA) and vortexed. Two hundred and ten µl of Ellman’s reagent and 15µl resuspended pellet supernatant was added to the 96 well microplate in triplicate and incubated at room temperature for 15min. The absorbance of samples was read at 412nm using an automated microplate reader and a calibration curve was prepared from reduced glutathione (GSH) stock solution.

The GSH activity was determined using a protocol of Cohn and Lyle (1966). Seventy five µl of phosphoric acid was added to 500µl of supernatant and left to incubate on ice for 10min to precipitate out the proteins. Thereafter samples were centrifuged at 3000r.p.m. at 4°C for 10min and 100µl of the supernatant was added to 1mL of deionised water and mixed. A 6µl sample reaction mixture, 232µl sodium phosphate buffer and 12µl of O-phalaldehyde was added to each well in a 96 well microplate and incubated in a dark at room temperature for 15min. Sample fluorescence was measured at 420nm resulting from excitation at 350nm on a Multi-Detection microplate reader (Synergy HT; BioTek instruments, USA).
The Cytochrome P450 activity (mM/mg protein) in the fish livers was determined using an ELISA method (Arbor assays- K011-F1). Ninety five µl of the sample supernatant was placed in duplicated in each well in the 96 well microplate provided in the kit, 5 µl of reconstituted NADPH was added to each well and the mixture was left to incubate at 37°C for 30 minutes. Subsequently, 5µl of stop solution was added to stop the reaction. Twenty five µl of DetectX Formaldehyde Detection Reagent was added to each well and incubated for another 30min at 37°C thereafter the fluorescent signal of the samples were read at 510nm with excitation at 450nm using a microplate reader.

The PC concentration (nmol carbonyls/mg protein) was determined using an adapted protocol from Parvez and Raisuddin (2005) as assayed by Levine et al. (1990) and modified by Floor and Wetzel (1998). Then 500µl of sample supernatant was added to 500µl 2,4-Dinitrophenylhydrazine (DNPH) and incubated for one hour at room temperature with vortexing every 10-15 min. Five hundred µl of 6% trichloroacetic acid was added to each sample to precipitate out the proteins. Centrifugation was done for 3min at 24 166r.p.m. and the supernatant were discarded. The pellet was washed three times, allowed to stand for 10min before centrifugation and resuspended in 1ml ethanol to remove any free reagent. Proteins were solubilized by adding 400µl of guanidine hydrochloride to each sample and allowed to stand at room temperature for 15min. The samples were centrifuged at 38 666r.p.m for 5min in order to remove any trace of insoluble material and read in triplicate at 366nm using an automated microplate reader.

The method for CEA (J/g) analysis was adapted from De Coen and Janssen (1997) and De Coen and Janssen (2003). The energy allocation available (Eₐ) to the fish was established by determining the carbohydrate, protein and lipid content found in the muscle tissue. Carbohydrate content was determined at 560nm, by using a glucose test kit (GOD-PAP 1 448 668, Roche) and glucose standard (C FAS 759 350, Roche). Bradford (1976) method was used for protein content. Total lipids were extracted following the protocol of Bligh and Dyer (1959) using tripalmitin as a standard. Two hundred and fifty µl of supernatant was added to 500µl chloroform, vortexed and 500µl methanol and 250µl ultrapure water was added and vortexed again and then centrifuged at 4°C for 10min at 7 250r.p.m. A blank prepared from 100µl chloroform and 100µl of the organic phase was placed in glass tubes and 500µl of sulphuric
acid was added to each tube. The tubes covered with foil and incubated at 200°C for 15min. After the tubes were allowed to cool down 1ml of ultrapure water was added to each tube. Two hundred and forty five µl of the blank and each sample was added in triplicate to polyethylene microplates and the sample absorbancies were read at 360nm using an automated microplate reader. The energy consumption ($E_c$) was determined by measuring the ETS activity, 25µl of ETS buffer was placed in the first 3 wells (A1-3) in a microplate as a blank. Twenty five µl of supernatant was placed in triplicate on a microplate. Buffered substrate solution (BSS; 0.3 % (v/v; 75 µl) Triton X-100, and Tris-HCl), 25 µl NAD(P)H solution and 50µl p-IodoNitro Tetrazolium violet/chloride (INT) was added to each well and the samples were read kinetically using an automated microplate reader. Kinetic readings were taken over a 5min period at 1min intervals and read at 490nm using an automated microplate reader.

The CEA was calculated by converting the energy reserves ($E_a$) into energetic equivalents using the enthalpy of combustion values as indicated by De Coen and Janssen (1997), where 17 500mJ/mg glycogen, 24 000mJ/mg protein and 39 500mJ/mg lipid were used as values. The $E_c$ was determined using the theoretical stochiometric relationship that indicates in the ETS system that per each 2µmol of formazan formed, 1µmol of oxygen is consumed. The amount of oxygen was transformed into energetic equivalents using an average oxyenthalpic equivalent of 484 kJ/mol O$_2$.

The total energy allocated was calculated using the following equations:

$$\text{CEA} = E_a - E_c$$

$$\text{Where: } E_a = E_{glucose} + E_{lipid} + E_{protein} \text{ and } E_c = E_{ETS}$$

5.2.4. Histology of Danio rerio gill and intestinal samples

The gills were fixed in 10% neutral buffered formalin (NBF) for 24hrs, washed in running tap water for 12hrs, and prepared for histological analysis using standard histological techniques (Humason, 1979). The samples were dehydrated for 60min (Van Dyk, 2003) in rising
concentrations of ethanol (30%, 50%, 70%, 80%, 90%, 96%, 100%, and a repeat 100%), cleared in xylene (5–10 min) until transparent and again placed in xylene until translucent and transferred to a 50% xylene liquid paraffin wax mixture (60°C) and three following liquid paraffin wax repetitions.

Samples were all embedded in paraffin wax blocks. Sections of 5 µm were cut from each wax block using a wax microtome (Leica). The sections were then placed on a glass microscope slide and held in place using a solution of albumin and distilled water. The slides were placed on a heating plate to allow sections to stretch out sufficiently (Figure 3.7 E) and air-dried before being put into a dry oven (45°C) for 24 hrs. Samples were stained using an adapted Haematoxylin and Eosin (H&E) method (Van Dyk, 2007) and mounted with Entellan.

The histological slides were assessed under a light microscope (Leica DMLS – ICCA) where four objectives were used for scanning and detailed qualitative histology (4x, 10x, 40x, and 100x-oil). Digital micrographs were taken of the slides using IM50 Image Manager Software (Pixel IT (Pty) Ltd).

5.2.5. Transmission electron microscopy (TEM) and Scanning electron microscopy (SEM)

Five gill samples per concentration and two intestine samples from the highest concentration (45 mg/l) were stored in Todd’s fixative at 4°C overnight to fix the tissue. Todd’s fixative was prepared by mixing 25% glutaraldehyde, 3% picric acid, calcium chloride and paraformaldehyde (heated to 60°C) in a dissolved in a sodium cacodylate buffer (Hyatt, 2000; Todd, 1986). Samples were continuously rotated in a rotator for all the following steps and samples were washed three times for 15 minutes each in a 0.05M cacodylate buffer. The lipids were fixed by placing samples in a 1% osmium tetroxide solution in cacodylate buffer for one hour. Samples were washed three times for 15 min using distilled water and then dehydrated in ethanol (50%, 70%, 90%, 100% and 100%) for 15 minute intervals in each dehydration step. Embedding was done using LR White resin (London Resin Company Ltd) for 15 min and then changed into fresh LR White resin to ensure most ethanol was removed from the sample twice with an incubation
of 45min and then kept in the fridge overnight. Gelatine capsules were filled with LR White resin and samples were placed into capsules bearing sample orientation in mind and covered with the capsule lid. Samples were left in an oven at 65°C overnight to polymerize and cure. A Minora® blade was used to trim the capsules and samples were then microtomed using an Ultracut S (Leica) microtome. Of this histological slides were made for orientation and these were sliced at 0.5µM. And TEM slices of 90nm cut with a diamond knife (Diamant ultra 45°) were placed onto carbon coated copper grids for staining (Toluidine blue and Neufuchsien) and then analysed using a Tecnai G2 microscope and images were captured using digital micrograph FEI Company. For SEM analysis capsule blocks were sliced into squares using a Minora® blade and then coated in carbon overnight using a Emscope TB500 carbon coater and analysed using a FEI Quanta FEG 250 (15kb) microscope with dark field back scatter at an emission current of 380µA. The presence of the nAu particles was confirmed by using EDS (energy dispersant spectrum) and a clear definition between osmium and gold spectrum was achieved where a higher concentration of gold was present i.e. in the gut and this was used throughout the analysis.

5.2.6. CytoViva® dark field hyperspectral imaging

Three adult *D. rerio* per concentration were used. Fish were sacrificed by being placed immediately in ice and then stored at -80°C overnight. Adult samples were embedded into a cryopreserve gel (Tissue-Tek® O.C.T.™ Compound) and stored overnight at -80°C to harden. The cryostat (Cryocut E) was switched on two hours prior to use to ensure the temperature of -20°C was achieved. Samples were cryomicrotomed at 7µm and cooled between slices using cryofreeze aerosol and ribbons were lifted by touching a clean microscope slide kept at room temperature which melted the sample in place. Once on the slide a cover slip was placed over the sample and samples could be stored at -80°C until further analysis under a CytoViva® 150 Unit integrated onto the Olympus BX43 microscope. Images were captured using the Dagexcel X16 camera and DAGE Exponent software at 60x magnification.
5.2.7. **Gold bioaccumulation in muscle tissue**

Upon conclusion of the test the head and internal organs were removed. The sample was rinsed three times in MilliQ water to remove any externally bound nAu and placed in a drying oven at 50°C until a constant mass was achieved. For each sample 0.5g dry weight sampled was accurately weighed (0.01g) out and placed in a digestion tube. Hydrogen peroxide (30%) (1000µl) and 65% nitric acid (7ml) were added to the sample. Samples were loaded into a microwave (Ethos, Milestone Microwave Laboratory Systems). A temperature probe was inserted into the digestion tube which contained the sample. Muscle digestion was undertaken by increasing the temperature to 200°C for 10min and maintaining that temperature for another 20min. The samples are allowed to cool for 10min (Baldwin *et al*., 1994).

Once cooled, samples were poured into a 50ml Erlenmeyer flask. The digestion tube was rinsed out twice with MilliQ water and and to this mixture, 500µl of indium (1000 ppb) was added. Samples were acidified at a 3:1 ratio of HCl:HNO₃ to solublize the gold nanoparticles into ions and Rh was used as an internal standard. The final volume of the sample was achieved by adding MilliQ water to 50ml. All samples were analysed on a NexION X series ICP-MS (Yaru *et al*., 1999).

5.2.8. **Statistical Analysis**

Biomarker concentrations and activity were log transformed and grouped according to exposure concentrations (control, 5mg/l, 10mg/l etc.). Differences between means were determined using Multiple analysis of variance (MANOVA) with Dunnets T3 test (non-parametric) multiple comparison post hoc tests. The significance of results will be ascertained at p<0.05. The graphical representations were performed using the GraphPad Prism software (Prism 5 for windows; Version 5.02) and data were reported as mean and SEM (standard error of the mean).
5.3. Results

5.3.1. Genotoxicity

Genotoxic responses using microarrays were only conducted on the nAu exposures and not the ionic gold exposures.

5.3.1.1. RNA quality

The RNA quality results revealed a RNA integrity (RIN) above 8 across all samples. A RIN of at least 7 is required to do microarray analysis. And the lowest concentration of RNA isolated was 128.99ng/µl (Table 5.1) which was well within the possible 12µl to make up 1000ng concentration of input RNA. There was a clear differentiation between the 18S and 28S RNA bands (Figure 5.1a), which was confirmed by the RNA ladder (Figure 5.1b) used as a marker. Isolated RNA samples were run on a computed gel (Figure 5.1c) during RNA integrity analysis to further confirm the presence of two distinct RNA bands.
Table 5.1. Table showing the initial RNA concentration and RNA integrity number of isolated RNA from Danio rerio pooled liver samples.

<table>
<thead>
<tr>
<th>RNA concentration (ng/μl)</th>
<th>Control</th>
<th>5mg/μl</th>
<th>10mg/μl</th>
<th>15mg/μl</th>
<th>20mg/μl</th>
<th>25mg/μl</th>
<th>30mg/μl</th>
<th>35mg/μl</th>
<th>40mg/μl</th>
<th>45mg/μl</th>
</tr>
</thead>
<tbody>
<tr>
<td>RNA Integrity number (RIN)</td>
<td>8</td>
<td>9.7</td>
<td>9.7</td>
<td>9.7</td>
<td>10</td>
<td>9.7</td>
<td>9.8</td>
<td>9.6</td>
<td>9.7</td>
<td>9.5</td>
</tr>
</tbody>
</table>

Figure 5.1. Results obtained from the bioanalyser of isolated RNA. a) showing the 18S and 28S RNA bands. b) showing the ladder used by the bioanalyser to obtain results. c) showing the computed gel for the 18S and 28S RNA bands.
5.3.1.2. GeneChip® Microarray of isolated RNA samples

Microarray results were used to determine whether any further relationships occurred at the different concentrations across all the genes present within the *D. rerio* genome. A scatter plot (Figure 5.2) was used where each concentration (y axis) was compared to the control sample (x axis) and all genes (15,506 genes) were assessed. As can be seen from the scatter plot, all genes between the three lines in the centre show genes limited to those with an eight fold up or down regulation, the genes with above eight fold showed a visual weight trend. It was clear that the 20mg/l and 25mg/l concentrations were related, but also that the 40mg/l and 45mg/l showed a similar trend in gene expression. All other concentrations, when compared to control, showed a broader scatter of up and down gene regulation where the mentioned concentrations showed either an even higher fold change or gene relation. As this was confirmed by the scatter plot a hierarchical clustering method was used to cluster only genes with an eight fold or higher change as they appeared similar and a better understanding was needed to see if these two were related across concentrations. The clustering established that the control was the least related to all other exposure concentrations and formed a branch on it’s own. While the 5mg/l and 15mg/l were more closely related to one another than to the 10mg/l concentration, these three concentrations were collectively related to the 30mg/l and then 35mg/l concentrations. The 25mg/l, 40mg/l and 45mg/l concentrations respectively were the least related to this grouping and the 20mg/l concentration formed a branch and was a complete outlier when compared to the other groupings showing the least relation to control as well as the other concentrations but the highest relation to the 45mg/l concentration. Genes themselves were clustered according to the up regulation and down regulation as indicated in Figure 5.3. The GO biological process, showed genetic changes relating to development process, establishment of localization, biogenesis, response to stimulus, metabolic process, locomotion and biological adhesion. The cellular component showed responses relating to the membrane part, extracellular region part, cell junctions and synapse. While molecular function showed responses relating to binding, receptor activity including steroid binding, transporter activity, structural molecule activity and enzyme regulator activity (Annexure A-C).
Figure 5.2. Scatter plot of gene up and down regulation in nAu exposures ranging from 5mg/l-15mg/l in the top row, 20mg/l-30mg/l in the middle row and 35mg/- 45 mg/l in the bottom row.
Figure 5.3. Hierarchical clustering of genes patterns (up regulation – blue; down regulation red) at the different nAu exposure concentrations. Only those genes with a fold change greater than 8 are represented.

5.3.1.3. Real time polymerase chain reaction using cDNA

When comparing male and female test organisms, the gene regulation revealed that male *D. rerio* had a higher response across all genes assessed except for Cyp17a1 which had similar up regulation. As was found with the acute bioassays the microarray gene analysis also showed a bimodal response across the increasing concentrations. For the purposes of this study changes less than two fold were seen as inconsequential. Catalase showed a fold change as high at five times (Figure 5.4) when compared to control and this was seen at 30mg/l exposures. The 5mg/l and 15mg/l exposures also resulted in up regulation of greater than 2.5. The SOD gene did not change in females showed but for males there was an increase in up regulation of between four and six fold. The MT gene expression showed a bimodal response, where 20mg/l and 40mg/l
were up regulated in the female (two fold) samples while for males an up regulation of up to seven fold was seen in males samples, the lowest change was twice the normal activity when compared to the control.

Figure 5.4. Graphical representation of fold change in gene regulation from isolated *Danio rerio* liver samples comparing male to female groups for a) catalase (CAT) activity b) superoxide dismutase (SOD) activity c+d) metallothionein (MT) activity.
The Cyp 1 genes (Figure 5.5) also showed a higher response in male fish, where Cyp1a1 had a greater than three fold up regulation across all concentrations. The bimodal response continued to exhibit throughout the Cyp1c1 regulation where 5mg/l to 15mg/l showed up regulation of up to five fold, while between 20mg/l and 30mg/l (as well as 45mg/l) an insignificant fold change was seen. The fold change again increased to four fold in concentrations of 35mg/l and 40mg/l. Cyp11a1, Cyp17a1 and Cyp19a also showed insignificant responses in female samples, but the trend was also a bimodal response which followed the relative effects of the male samples. The Cyp11a1 gene showed inconsequential up and down gene regulation except at four concentrations, these being 20mg/l, 25mg/l, 40mg/l and 45mg/l, where up regulation fold changes were as high as eight fold and no less than six. The female samples for Cyp11a1 also showed up regulation up to four fold at the previously mentioned concentrations, however almost half the activity of the males it still showed a marked effect. A similar trend was exhibited in the Cyp17a1 gene regulation where a clear bimodal response was seen across all groups. However the 20mg/l, 25mg/l and 40mg/l had an up regulation of three fold, while 45mg/l showed almost no activity when compared to the control. Between 5mg/l and 15mg/l as well as 30mg/l and 35mg/l there was a down regulation of up to four fold, indicating an entirely different response across the exposure range. Cyp19a showed inconsequential activity across all concentrations where the highest fold change occurred at 5mg/l, while the male samples had no marked response it was clear that a slight increase in activity was seen from the lowest concentration (5mg/l 1.5 fold change) to the highest concentration (45mg/l 3 fold change) which resembled that of a dose dependent response. The gpx1a gene had a four fold change and greater changes across all concentrations but the highest up regulation was at 5mg/l as well as 15mg/l with a fold change of five and six respectively.
Figure 5.5. Graphical representation of fold change in gene regulation from isolated Danio rerio liver samples comparing male to female groups showing the cytochrome (CYPs) (a-e) activity and glutathione peroxidase (GPX) activity.
5.3.2. Biomarker responses

The biomarkers were analysed in whole body, gill and liver tissue of *D. rerio* following exposure to both ionic concentrations of gold (HAuCl$_4$) and nAu. The changes in biomarker responses were assessed relative to the control exposure, which did not contain any ionic or nAu. In the whole body of *D. rerio* there were no significant differences (p > 0.05) in the CAT biomarker (Figure 5.6a), a similar bimodal reaction, i.e. increase at lower concentrations, with decrease in the mid range, followed by an increase yet again at the highest exposure concentrations. The SOD biomarker (Figure 5.6b) showed the lowest response at nAu 15mg/l which was significantly different to nAu 25mg/l, nAu 35mg/l and ionic 2mg/l. There was also a significant (p < 0.05) difference between the nAu 30 and 35mg/l concentrations whereas at 30mg/l no SOD activity could be measured. The AChE biomarker (Figure 5.6c) showed no significant differences when compared to the control but the ionic exposures showed significant differences from the control at 2mg/l (lower) and 10mg/l which was higher than the control. The MDA (Figure 5.6d) levels did not differ significantly from the control except for the highest exposure group, which was significantly lower (p < 0.05). Although not different from the control, there was a sustained decrease in MDA levels as the exposure concentrations increased. The ionic gold exposure resulted in was significantly lower MDA levels in the 10mg/l concentration compared to the nAu 5mg/l to 20mg/l concentrations. The MT levels (Figure 5.6d+e) were measured in both whole body as well as the gill samples. Although no significant differences were observed between concentrations it was noticeable that for whole body the levels were higher than the control for all nAu exposures, whereas the gills showed increases in the 15mg/l, 30 mg/l and 45mg/l exposures.

There were no significant differences between exposure groups in the ETS activity (Figure 5.7). It was noticeable that the ETS activity in the nAu exposures was lower than the control wit the exception of the 35mg/l exposure. The GSH only showed a significantly higher levels (p < 0.05) at the 15mg/l and 40mg/l exposure concentrations. The CYP450 activity (CYP2a, CYP3a, CYP3b and CYP4a) in liver tissue also showed no significant differences. However interestingly the ionic gold exposures showed values similar to the control with very little variance while from nAu 25mg/l and above showed a marked increase and more variance across samples and groups. The PC biomarker showed the greatest significant differences from the control and
between exposures (Figure 5.7d). The control, nAu 10mg/l and 15mg were significantly different (p < 0.05) when compared to nAu 30mg/l and concentrations up to 45mg/l as well as ionic 1mg/l and 2mg/l concentrations. The nAu 5mg/l, 20mg/l and 25mg/l concentrations were significantly higher than the control, whereas the 5mg/l and 10 mg/l ionic gold exposures were lower than the control PC levels.

The CEA biomarker showed no overall significant changes in available energy when compared to the control group (Figure 5.8f). Even though there was no net energy change, there were some differences that were noted. Although not the major contributor to energy reserves, carbohydrate levels increased at the higher nAu exposures and all the ionic gold exposure groups (Figure 5.8a). It was particularly noticeable that the 15mg/l nAu exposure group had the lowest lipid reserves (Figure 5.8c) and energy consumption (Figure 5.8e).
Figure 5.6. Mean ± SEM activity for CAT, SOD, AChE, MDA and MT biomarkers in whole body and gill tissue of Danio rerio at the different concentration exposures. Common superscripts indicate significant differences (Dunett T3; p < 0.05).
Figure 5.7. Mean ± SEM activity of the ETS, GSH, CYP450 and PC biomarkers in whole body and liver of Danio rerio at the different exposure concentrations. Common superscripts indicate significant differences (Dunett T3; p < 0.05).
Figure 5.8: Mean ± SEM of the individual Cellular Energy Allocation (CEA) components (A-C) measured to calculate available energy (Eₐ-D), energy consumed (Eₑ-E) and the total CEA (F) in whole body of Danio rerio at the different exposure concentrations. Common superscripts indicate significant differences (Dunett T3; p < 0.05).
5.3.3. Histology of Danio rerio gill and intestine

The histology showed slight histopathological changes in the gills of *D. rerio* in the 30mg/l exposure groups where the cytoplasm of the epithelial cells appeared swollen (Figure 5.9b). The 30mg/l to 45mg/l exposure concentrations showed aneurysms and telangiectasia in the secondary gill filaments (Figure 5.9c). The intestine (Figure 5.9d) showed black agglomerates present inside the intestinal lumen.

*Figure 5.9: Histology of the gills (a-c) and a transverse section through the gut (d) of Danio rerio.*
5.3.4. Transmission electron microscopy (TEM) and Scanning electron microscopy (SEM) of gill and intestine tissue of Danio rerio.

The SEM results provided more insight into changes seen in the histology by using the dark field back scatter to capture images as well as scan for EDS. The areas across gill filaments (Figure 5.10a,b) were scanned for energy dispersion, which would provide an indication of the presence of nAu. However no gold could be found. Areas with less blood supply (i.e. cartilage of the gill arch and associated muscle tissue) were also scanned and it was found that gold was present in areas such as cartilage (Figure 5.10c) and muscle (Figure 5.10d). In the cartilage the area scanned showed clear white circular structures, which upon closer inspection were osmium residues left from sample processing. In the 10mg/l exposure group the musculature contained more gold than the cartilage. A general scan of the entire primary filament containing secondary gill filaments and cartilage showed a 0.73% of gold in the area scanned. Figure 5.10e shows a closer view of telangiectasia present in the gill filaments at 30mg/l while Figure 5.10f shows an area where no gold was found but only osmium which was present in mucous introduced during sample processing.

At the highest exposure concentration (45mg/l) (Figure 5.11) the intestine appeared to be eroded away on one side. It is likely that this is the ventral side of the fish and the nAu agglomerates would lay on the surface within the lumen as they move along the intestinal tract. Sections were taken along different portions of the tract and the same trend was observed. The EDS scan revealed that in the area where the intestinal villi remained intact (Figure 5.11b) there was 1.97% of gold present within the villi while the eroded area (Figure 5.11c) showed no gold present. The lumen showed up to 87.7% gold within it, which was clearly seen as large agglomerates. Transmission electron microscopy was used to further confirm the presence of nAu particles in the gut lumen. The nAu agglomerates were found present between organic matter which was present in the gut and were present in agglomerations of up to 800nm (Figure 5.11d). In Chapter 2 it was evident that these agglomerates could be observed with the naked eye and could be up to 3mm in the medium after 96h. Upon further inspection (Figure 5.11e, f) it was observed that some nAu particles were again at their original size of 14nm and ranged up to 30nm but were not part of agglomerates but rather observed as separated nanoparticles.
Figure 5.10: Photomicrographs of SEM using dark field back scatter showing a+b) gill filaments, c) cartilage, d) muscle tissue, e) telangiectasia and f) accumulated osmium in mucous on gill filaments.
Figure 5.11: Photomicrographs of SEM using dark field back scatter showing a-c) transverse section through the gut and d-f) TEM showing characterization of nAu particles in the gut of D. rerio after exposure to nAu in aquatic medium.
5.3.5. CytoViva® dark field hyperspectral imaging

CytoViva® dark field hyperspectral imaging of longitudinal cryo sections of *D. rerio* were only undertaken on the tissues that could be identified easily with this technique, i.e. the intestinal tract and muscle tissue. Similarly to the results obtained for the daphnia and what was confirmed by SEM and TEM for the zebrafish, the gut was filled with nAu agglomerates. The concentration (usually seen as dark red) however appeared to be less in the gut when compared to observations seen in daphnia (Figure 5.12a and Figure 4.3f). Figure 5.12b-f focused on the muscle tissue and it was found that the nAu particles formed pockets within the muscle striations. When compared to the control samples the red colouration was not present in the control. In the 10mg/l exposures (Figure 5.12e) the nAu particles were on the surface of a muscle striation and formed agglomerates around it, while the pockets were also seen but were not as obvious as at the higher exposure concentrations.

5.3.6. Gold bioaccumulation in muscle tissue

The bioaccumulation of gold in muscle tissue following exposure to nAu revealed that the highest accumulation was seen at 15mg/l, 30mg/l and 45mg/l exposures where 5.61µ/g, 5.04µ/g and 5.79µ/g were measured respectively (Figure 5.13). The lowest bioaccumulation was measured in the 20mg/l (2.07µ/g) and 40mg/l (1.92µ/g) exposure groups. Thus a similar multi-modal effect was observed with high concentrations at 15mg/l, 35mg/l and 40mg/l while lower bioaccumulation at 20mg/l and 40mg/l exposures.
Figure 5.12: CytoViva® dark field hyperspectral imaging of D. rerio exposed to nAu in aquatic medium showing a) the gut, b+f) pockets of nAu forming in muscle tissue and c-e) accumulation of nAu between and on the surface of muscle striations.
5.4. Discussion

The microarray results showed that there was a clear relationship between 20mg/l, 25mg/l and 40mg/l, 45mg/l exposure groups. It is proposed that this relationship occurs due to particle agglomeration patterns and surface charge. As discussed in Chapter 2 the 20mg/l and 25mg/l concentrations showed a varied size distribution and particles ranged from small aggregates (17.12nm) to large agglomerates (894.5nm) but had an average of size of 314.6nm across both concentrations. Lapresta-Fernandez and Blasco (2012) also suggested that at a negative charge there is less particle aggregation due to steric stabilization mechanisms making more nAu particles available for biological uptake, distribution and possible toxicity. While at 40mg/l and 45mg/l the particle agglomerates were physically observed and could be at large as 2mm. During these exposures it was observed that fish started feeding on the agglomerates which settled at the bottom of the tank. The presence of the ingested nAu particles in the gut were confirmed through the different imaging analyses undertaken in this study.

Figure 5.13: Bioaccumulation of gold at the different nAu exposure concentrations in using a D. rerio muscle tissue (n=10 pooled samples per exposure).
The GO pathways (Martinovic et al., 2009) which were analysed on genes with an eight fold change revealed a significant response (p<0.05) in genes involved in biological processes (Annexure A) related to development (71%). In vivo treatment of nanoparticles to cell lines show inflammatory/immune responses, up regulation of antioxidants, protein expression, cell cycle responses, defense responses and detoxification of lipid metabolism (Roy et al., 2014; Jovanovic et al., 2011; Li et al., 2010). Other genes that also underwent significant regulation (74.5%) included cellular, chemical, stress and endogenous stimulus responses. Most studies on nAu toxicity focus on in vitro exposure studies and studies by Brandenberger et al. (2010) showed that no oxidative or inflammatory effects were seen due to exposure. Ladhar et al. (2013) showed that dietary cadmium nanoparticle exposure did not change MT2 expression after a thirty day exposure. In this study MT1 and MT2 gene expression in male fish increased 4 fold change from the lower exposure concentrations (5mg/l). When the MT levels in the whole body and gill were measured there were no significant differences following exposure to the different nAu and ionic gold concentrations. However although not significant it is important to note that the MT levels were indeed higher than the control levels, indicating some form of protective mechanism in place. Cho et al. (2010) found increases in MT levels in response to exposure to low concentrations of Au$^{3+}$ ions.

The cellular component (Annexure B) focused on the membrane part and all selected genes were involved in membrane processes (100%). The integral component, plasma membrane part, mitochondrial membrane, transmembrane transporter complex, energy dependent (ATP and NADH) membrane transport complexes, pore complexes and ion channels were all significantly influenced (p<0.05) when compared to control. Bourdineaud et al. (2012) found down regulation in cox1 gene, which had a considerable effect on the respiratory chain of zebrafish in brain tissue after contamination with nAu. In their study they found that the size of the particles resulted in different responses with larger particles having the greatest effect. In this study there was however no change in cox1 gene expression, which is supported by the ETS activity remaining constant during all exposure. The p53 gene is activated when cellular stress occurs in response to nanoparticle exposures (Vecchio et al., 2012). However in this study the microarray data revealed that there was a down regulation of p53 at the lowest exposure concentrations (5mg/l-15mg/l and 35mg/l) and no change when the control activity
was compared to the other exposure concentrations. This indicates that exposure to nAu did not elicit a stress response in zebrafish.

The molecular functions (Annexure C) involved binding, which included protein binding, organic cyclic compound binding, carbohydrate binding, ion binding, small molecule binding and chromatin binding. Most studies on nAu particle toxicity focus on in vitro exposure studies and Brandenberger et al. (2010) showed that no oxidative or inflammatory effects were seen due to exposure to nAu. Free radicals can cause damage to biological components when in excess; this is caused by the oxidation of lipids, proteins and DNA. Physico-chemical properties of nanomaterials in conjunction with phagocytic cell responses can cause oxidative stress (Aillon et al., 2009; Fu et al., 2014). Intracellularly these nAu particles can disrupt or alter cellular function creating ROS and being the main cause for toxicity. Treuel et al. (2010) found that the addition of nanoparticles to a BSA protein solution destroyed parts of the secondary helical structure due to surface adsorption, also suggesting that citrate capped nAu particles showed a stronger interaction with proteins.

The microarray results confirmed that the cascade of reactions related to antioxidant activity in response to ROS formation in zebrafish was initiated. The antioxidant response is traditionally the formation of SOD to break down oxyradicals to OH and H2O2. In turn the H2O2 is broken down into water and oxygen by CAT. Or GSH is used with GPx1 to convert H2O2 to water and oxidized glutathione (Sousa et al., 2012). There was a significant up regulation of SOD (above four fold for all concentrations) CAT (2.5x fold change at 15mg/l and 20mg/l and a 3x, 3.5x and 4x fold change at 15mg/l, 30mg/l and 35mg/l respectively) and GPX1a (4x across all concentrations except 15mg/l which had a 6x up regulation) genes. However the up regulation of these genes was not reflected in same increase in corresponding protein levels in the Zebrafish (Figure 5.6). There was however an increase in the CAT activity in the 10mg/l and 15mg/l exposures. The absence of these enzymes and proteins does not necessarily mean that the antioxidant activity did not take place. The increased GSH production that was found at all exposure concentrations is indicative of peroxidase activity (i.e. as. reflected in the up regulation of the GPX1a gene). The high levels of GSH (Figure 5.7), which are also regarded as biomarkers of Phase II biotransformation is indicative of the potential role of internal biotransformation processes being initiated to excrete the nAu particles (Sousa et al., 2012;
Cazenave et al., 2006). The activation of the biotransformation process is further supported by increased activity of the CYP450 biomarker which is discussed later.

An increase in PC would further indicate damage to proteins via ROS formation; however it could also be caused by mechanical damage due to interactions with proteins and other cellular components as indicated by the microarray analysis. In the 20mg/l and 25mg/l exposures there was an increase in PC formation when compared to the control however at the higher exposures (30mg/l to 45mg/l) there was a significant decrease in PC when compared to the control. Nanoparticles which are often the same size as protein molecules can interfere with cell signaling and interact directly with proteins by changing the conformation or by acting as a chaperone, which could lead to peptide aggregation and fibrillation (Elsaesser and Howard, 2012). Iosin et al. (2009) recorded a noticeable decrease in total absorbance when albumin was added to an aquatic suspension of nAu particles and conformational changes that the protein underwent at the surface of the nAu particle indicated the formation of bioconjugates. Due to this protein binding it is suggested that it aids in endocytosis and toxicity is not caused by cellular uptake (Goodman et al., 2004). Li et al. (2010) showed using western blot analysis that there was oxidative damage showing an increase in MDA. In this study there was an increase in MDA at 5mg/l and 10mg/l but MDA levels started to decrease at the exposure concentrations increased above 25mg/l. The MDA levels increased in the ionic gold exposures. These increases in MDA indicates protein damage or a decrease in ROS formation as agglomerates form in the medium and are unable to be taken up into cells (Oh and Park, 2014; Cho et al., 2009; Chithrani and Chan, 2007).

Interesting to note was that the microarray revealed an increase in steroid hormone activity and structural molecular activity in molecular function. This was clearly shown in the bimodal activity seen in the steroid pathway cytochrome genes (Figure 5.14). The genes involved were CYP11a1 (expressed in the gonads, brain, and interrenal glands), CYP17a1 (expressed in the gonadal tissue, brain, gill, liver and intestine) and CYP19a1 (expressed in the ovary and lower levels in the testis) (Goldstone et al., 2010). The steroidogenesis pathway was stimulated following exposure to 20mg/l and 25mg/l nAu and then again in the 40mg/l and 45mg/l exposures. The CYP11a1 protein converts cholesterol to pregnenolone and once converted to progesterone CYP17a1 (up regulated at the same concentrations) converts this to 17 alpha hydroxyprogesterone (Hoffman and Oris, 2006). The RT-PCR revealed that as can be expected
CYP19a was up regulated in male fish (across all concentrations) where more testosterone would be available, while in female fish there was a two-fold down regulation of the CYP19a gene. It is therefore interesting to speculate that the stimulation in reproductive processes in Zebrafish (similar to the increased reproductive output observed in *D. magna* – Chapter 4) following exposure to nAu may be attributed to the hormesis phenomenon. Nascarella and Calabrese (2012) describe hormesis as a process, which is characteristic of an opposite response, i.e. stimulation in the low dose zone and an inhibitory response at higher doses. The hormetic response is thus the organism’s overcompensation to a disruption in homeostasis (Nascarella and Calabrese, 2012; Iavicoli *et al.*, 2010). It is typically in the presence of a low level stressor where a high dose response is not seen but it is recognizable as a stressor. This hormetic response has been described in nanoparticle exposure (*in vitro* and *in vivo*) by Iavicoli *et al.* (2010) in a review article referring to carbon nanotubes, quantum dots and metal nanoparticles. The bimodal responses of CYP steroidogenesis genes indicate that nAu exposure stimulates a hormetic response in *D. rerio* to rapidly reach sexual maturity in order to breed.
The CYP450 ELISA used in this study is specific for CYP2a, CYP3a, CYP3b and CYP4a. Although not significant, the activity of this biomarker following exposure to all concentrations of nAu, was consistently higher than the control levels. In contrast the CYP450 activity of all the ionic gold exposures was very similar to the control levels. The CYP1, CYP2 and CYP3 proteins are much more complex and are involved in the metabolism of drugs and pollutants (Goldstone et al., 2010). The CYP450s are found in the endoplasmic reticulum of cells and in the inner membrane of the mitochondria and catalyze several reactions and this is why they are important in metabolizing and detoxifying an alarming number of endogenous and exogenous molecules (Li et al., 2010b). Balasubramanian et al. (2010) found that the highest gene expression when exposed to nAu was found in Cyp1a1, but also found a significant up regulation in CYP3 and CYP4, which supports the finding of this study.
The *D. rerio* gill microscopy results suggested that the nAu particles were immediately moved to an area where less blood flow was present, which in the gills would be the cartilage or muscle (Figure 5.10). The suggested limited interaction due to no visible accumulation of the nAu with the gill filaments gives a possible reason for the lack of significantly elevated biomarker responses. Following uptake by the gill and once in the systemic circulation interactions between nAu particles and blood components can cause hemolysis, thrombosis and nanoparticle induced platelet aggregation (Deb *et al*., 2011; Aillon *et al*., 2009). Roy *et al.* (2014) showed that blood cells, which are non-phagocytic, are also able to internalize nanoparticles. Moreover once in the blood stream particles can then be distributed throughout the organism by systemic circulation (Morais *et al*., 2012; Aillon *et al*., 2009; Akiyama *et al*., 2009; Cho *et al*., 2009; Goodman *et al*., 2004). The non-degradable nanoparticles are able to accumulate in organs or intracellularly, as nanoparticles are able to agglomerate in the medium this is also possible within an organism but most have been found in intracellular vesicles (Brandenberger *et al*., 2010) and thus far nAu particles have not been found to enter the nucleus of cells (Oh and Park, 2014; Yen *et al*., 2009).

In relation to other organs the muscle would have the largest surface area and by accumulating and sequestrating the nAu in the muscle, further exposure of more susceptible target organs to nAu would be reduced (Luoma *et al*., 2014). As nAu particles move throughout the circulatory system, pockets or clusters of nAu agglomerates could be formed, either by packing the particles together in an area or by the interactive forces that exist, which would lead to particles attaching within the muscle as blood flows past an area again (Lee and Ranville, 2012; Hainfeld *et al*., 2004). Ladhar *et al.* (2013) found that cadmium nanoparticles accumulated in predominantly fish brain and muscle tissues after a period of 60 days. Etame *et al.* (2012) showed targeted delivery of nAu into the central nervous system, in this study nAu particles accumulation occurred after a period of 96h which could be related to the ability of nAu particles to move across membrane barriers as designed for their delivery (Yah, 2013; Arora *et al*., 2012; Byrne *et al*., 2010; Akiyama *et al*., 2009; Boisselier and Astruc, 2009). It should however be noted that bioaccumulation doesn’t always translate into toxicity (Bour *et al*., 2015) and Alkilany *et al.* (2012) showed that *in vivo* gold nanorods were in a stable state and did not release toxic Au ions as is the case with other inorganic nanomaterials.
There was visibly less packing of nAu into the gut when compared to daphnia and this can be attributed to although the exposure concentration was higher (45mg/l) the length of the gut would spread the particles and therefore appear less concentrated per area but be present in a longer area. Brown et al. (2007) showed that particle uptake was possible in the small intestine by persorption of being extruded from a villus through a single degrading enterocyte and particles of less than 58nm were able to travel through the blood stream. In the case of the highest concentrations (40mg/l and 45mg/l) where nAu agglomerates were ingested it showed a mechanical wearing down as the clumps moved along the intestinal tract. Ensuing damage to the villi could affect the nutrient uptake required for the organism to survive but also make the intestinal barrier more vulnerable, which could lead in further uptake of toxicants. The nAu particles formed smaller agglomerates with previously ingested organic matter and this could result in large nAu agglomerates being broken down into small agglomerates by the normal biological functioning of the body i.e. churning required for digestion (Lee and Ranville, 2012) or enzymatic reactions which could also aid in allowing a higher uptake through the intestinal tract. Wistar rats, which were injected with nAu particles showed that there was an inefficient clearing of nanoparticles from the body by urine and feces one month post exposure and particles of up to 100nm in size could undergo redistribution through the blood (Jenkins et al., 2012; Morais et al., 2012; Balasubramanian et al., 2010; Boisselier and Astruc, 2009; Cho et al., 2009). De Jong et al. (2008) showed that tissue distribution of nAu particles is size-dependent where particles around 10nm showed the highest distribution while those up to 100nm remained in the blood circulation.

5.5. Conclusion

The correlation between the physico-chemical and surface properties of nAu particles and how this affects the toxicity is an important step towards the use and application of these nanoparticles (Paino et al., 2012). In aquatic systems oxidative damage occurs by particle internalization and the mechanical interaction between cell membranes which is related to the particle functionalization and electrostatic attraction (Lapresta-Fernandez and Blaso, 2012). In Chapter 2 it was shown that at 20mg/l and 25mg/l as well as 40mg/l and 45mg/l concentrations that the nAu particles were anionic, the agglomeration patterns and size distribution showed that at 20mg/l and 25mg/l the nAu were still bioavailable after 96h. When size distribution was related
to the concentration and in this chapter the behaviour of fish (eating the nAu particle agglomerations at 40mg/l and 45mg/l) was brought to the forefront where similar genetic responses were see regardless of the method of uptake (Oh and Park, 2014; Schulz et al., 2012; Schaeublin et al., 2011; Alanazi et al., 2010; Schrand et al., 2010). In this study the lack of oxidative stress responses was related to the limited internalisation in tissues and cells that are susceptible to ROS formation and oxidative stress. The CYP genes however showed a hormetic response to exposure to nAu.

Annexure A: Biological process

![Biological process table]
Annexure B: *Cellular component*

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Annexure C: Molecular function

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Chapter 6: Final conclusions and recommendations

6.1. Conclusions

The complexities in aquatic systems add to the multifaceted effects of nanoparticle ecotoxicity studies. As the OECD tests are standardized some of these interferences are eliminated, however in a natural environment they would be present. Since the size and charge of particles play a crucial role in the toxicity it is important to assess particle availability to the organism which can be determined by characterization in a medium. Metal analysis using ICP-MS was applied to confirm that the nominal exposure concentrations of nAu were similar to the measured exposure concentrations. The size distribution, which ranged between 14nm to being visible to the naked eye, of each concentration revealed that the concentration exposed played an important role in agglomeration patterns and the bioavailability of nAu. The concentration of nAu present was directly related to the size and charge of the agglomerates, which occurred in a skeletal like formation as seen by TEM. Additionally in natural aquatic systems there would be higher concentrations of divalent ions, which would also enhance the ability for nAu to aggregate to one another. At the 20mg/l, 25mg/l, 40mg/l and 45mg/l exposure concentrations the zeta potential showed a dominant negative charge, which has very likely been functionalized to a carboxyl double bond present in citrate and a hydroxyl group formed with the media. The negative charges are as a result of the formation of $\text{AuCl(OH)}_3^-$ while all other concentrations formed $\text{AuOH (H2O)}^0$.

Based on the available characterization information it was possible to compare exposure routes within an aquatic organism and determine whether it is across the gill or body surface or by ingestion. To obtain an understanding of the nAu hazard potential, acute LC50 bioassays were conducted. As is the case with traditional chemical exposures there was a dose-dependent response for ionic gold, while thus far in aquatic exposures to nanoparticles there seems to be a bimodal response due to particle agglomeration in reconstituted water (Nascarella and Calabrese, 2012; lavicoli et al., 2010). In this study the same bimodal response was seen and was attributed to hormesis where a low level stressor evokes overcompensation by the organism. There is however a proposed threshold across concentrations where particles are at
a high enough concentration to have an effect but at a low enough concentration to not precipitate out of solution, i.e. as is the case for nAu 20mg/l and 25mg/l which showed this threshold. As shown in Chapter 2 the particle characterization played an extremely important role in the toxicity, but the behaviour in natural aquatic systems would also modify these effects. For example the particle agglomeration could easily be changed by water currents, other chemicals present within a water system or pH changes in the body system, even the swimming patterns and behaviour of fish could cause for further aggregation and agglomeration or on the other hand break up large agglomerates into smaller aggregates. This in turn would affect the route of exposure, where as seen in algae it caused growth inhibition by the blocking of light, in daphnia it covered the surface on the organisms and affected molting and swimming patterns and in zebrafish depending on the concentration it showed different exposure routes, either across the gill surface or by ingestion of large agglomerates which could mechanically be broken apart by the functioning of the digestive system.

The nAu toxicity results showed a bimodal response in both daphnia and fish exposures and therefore traditional dose-response LC50 values could not be calculated. This was in contrast to the ionic gold where the LC50s could be calculated and were determined to be at much lower concentrations. For the daphnia test it was possible to calculate an LC50 but it was above 70mg/l, which is not considered to be at an environmentally relevant concentration. Some conventional OECD/EPA tests rely on the use of absorbance readings as an end point. The bacterial test takes one reading after a period of thirty minutes and this gave false positives. While the algal test kits rely on absorbance readings every 24h, to eliminate false readings a chlorophyll-a extraction protocol was successfully applied which reads at a different spectrum and was able to give a clear indication of toxicity.

However the nAu and body surface interactions are of concern where organisms react to stressors by increasing molting and altering swimming patterns as nanoparticles accumulate on the body surface increasing the body burden. Once ingested the gut clearance rates would depend on the amount of nAu that have been ingested and accumulated in the gut as well as feeding patterns to allow for particles to be removed from the gut by being forced through to the hindgut. However no evidence exists for particle uptake and internalization via the gut, but literature suggests that anionic nAu accumulate in close proximity to the peritrophic membrane.
and microvilli while cationic nAu are found within the lumen interacting with debris (Wray and Klaine, 2015). In daphnia a higher rate of molting was seen across all concentrations when compared to control groups and these increases as daphnia mature. If the molting is continuously increased it would in turn decrease the life span of the organism and if the reproduction rate is not kept at the same level it will in turn affect the entire population growth. This raises concern as this is the same phenomenon observed in other inert nanoparticles and highlights the focus shift in nanoparticle toxicity from than the current chemical to a mechanical focus. Once mechanical interferences were focused on other endpoints to assess toxicity were explored, e.g. CytoViva dark field hyperspectral imaging, TEM, dark field back scatter SEM and EDS.

Mechanical effects are reliant on the in vivo body distribution; since interactions would only occur where nAu are present within the organism. Particles tend to accumulate in areas where less movement of blood occurs like the cartilage and muscle tissue within the gills and muscle tissue within the rest of the body. Many studies show that injected nanoparticles are found at the highest concentration within the liver and spleen (Balasubramanian et al., 2010), but due to small surface areas in standard aquatic species it is very difficult to determine and would require up to five hundred fish to weigh out the 0.5g needed to perform the digestion for ICP-MS. Therefore other methods and larger fish species need to be used to visualize this; these could include CytoViva and TEM in further studies. As particles agglomerate at higher exposure concentrations they are ingested and broken apart by gastrointestinal tract mechanical processes. Following uptake through the gut the nAu are then able to redistribute via the blood it is possible that those particles stored within muscle tissue are also able to redistribute.

The sub-lethal bioassays allowed for a broad-spectrum assessment of genetic changes, as well as the ability to compare the toxicity responses between different exposure concentrations. The microarray results revealed that for the 20mg/, 25mg/l, 40mg/l and 45mg/l exposures there were a linked effect at sub-cellular levels. The data suggest that irrespective whether particles enter the body through gill uptake or are ingested they are able to generate similar responses within the organism and are very likely the result of particle redistribution via the blood stream. Biomarker results showed an alteration in protein damage (as seen in PC) due to mechanical interactions between cellular components and nAu. While standard biomarkers for oxidative
stress showed no significant differences when compared to the control, SOD however showed significant changes between exposure concentrations (15mg/l and 25-35mg/l) and MDA showed an increase at 5mg/l to 15mg/l and decreased as the exposure concentrations increased. There was up regulation of the GPX1 gene across all concentrations (four fold) and also an increase in GSH (25-40mg/l) which is a phase II biotransformation protein. The Cytochrome P450 results showed a response (up to three fold increase when compared to control) following exposure to nAu but no changes when exposed to ionic gold. This was supported by the up regulation in the steroidogenesis pathway that was observed when studied using RT-PCR. Therefore it is crucial to explore nAu effects as particle nanoparticle exposure rather than from a standard metal exposure perspective.

Gold nanoparticles do not go into solution in a water column like metal ions would by forming a uniform dissolved distribution of ionic ions. They have the ability to agglomerate to one another within the solution changing their conformation and size distribution and these abilities also in turn are able to affect the zeta potential (charge). Unlike metal ions they are able to attach to the surface of organisms causing additional mechanical disruptions. Particles can settle on top of sediment and be able to go back into the water column by water currents and animal behaviour, have different zeta potentials and functional groups attached to them. With surface attachment and the ability to be taken up by organisms at lower trophic levels, they would be able to potentially biomagnify in edible tissue and thus be found throughout the food chain. This study showed similar genetic toxicity effects in fish even though particle uptake routes differed. It would suggest that daphnia containing nAu adsorbed to their external surfaces would act as source of particles when ingested by higher trophic level organisms. During the process of digestion the nAu become bioavailable in the gut and cause further toxicity and bioaccumulation.

Based on the results of this study it is proposed that the nanoparticles have a threshold concentration before toxic effects become evident. This is the concentration where a high enough physical number of particles are present, forming aggregates or agglomerates, that are still bioavailable and can be taken up into the body. Concentrations above the threshold would not be passively transported across physical barriers but be taken up by other means, which in the case for aquatic nAu was by ingestion (at exposure concentration higher than 30mg/l).
Concentrations below the threshold (less than 20mg/l) would show no effect as the bioavailable concentration is too low. The proposed threshold for *D. rerio* in an aquatic system for nAu is between 20mg/l and 25mg/l as determined in the microarray experiments.

Aquatic nanoparticle ecotoxicity should be studied using a dual approach (Figure 6.1). The first aspect is to characterize the nanoparticle of interest within the exposure medium. Secondly the interactions of nanoparticles with the organisms present in a medium are assessed. Crucial aspects to consider would be particle availability, exposure routes, biodistribution and then effects. The effects could be at acute or sub-lethal levels. Sub-lethal endpoints that were effective in understanding toxic mode of action of nAu were genetic changes related to gene ontology and biomarker responses. These responses could be attributed to mechanical effects rather than chemical toxicity in the target cell.
The hypothesis for this study was that since nAu is used within the drug and therapeutic fields they will have a low toxicity to aquatic biota. This hypothesis was accepted on a chemical basis, but the hypothesis was rejected when considering the “non-target” mechanical interactions and ensuing negative toxicity effects. These included the attachment to body surfaces, the blocking of light needed for photosynthesis, alterations in molting and swimming patterns, accumulation in muscle tissue, wearing down of villi and microvilli in the gut and blocking surface areas required for food uptake. Therefore these effects collectively show that exposure to aquatic nAu
does result in toxicity to aquatic organisms and further studies are necessary to examine the in depth extent of the proposed mechanical toxicity.

The aims in this study were met given that new characterization data are available for nAu within an aquatic medium using standard physico-chemical as well as determining size distributions, dominant surface charge (zeta potential) and agglomeration patterns. The acute toxicity of nAu and ionic gold was determined across four aquatic trophic levels (bacteria, algae, daphnia and zebrafish). LC50 and EC50 values were calculated for ionic gold but a bimodal response existed for nAu and it could only be calculated for *D. pulex*. By using a combination of genetic and enzymatic biomarkers, histology, CytoViva dark field hyperspectral imaging, SEM, TEM and gold bioaccumulation it was possible to determine nAu uptake, tissue distribution and effect. With *D. rerio* as a model organism for GeneChip® Microarray analysis it was possible to cluster exposure concentrations as well as GO and determine gene regulation using RT-PCR. The genetic regulation could then be related to a standard suite of biomarkers (AChE, MT, CYP450, CAT, SOD, LP, PC, GSH and CEA).

### 6.2. Recommendations

In this study it was demonstrated that nAu build up in the muscle and large adult fish (4-5cm) and it is recommended that future studies include sub-adult fish (1-2cm) to compare the surface area ratio to effects. Therefore a better understanding of the effect of bioaccumulation will be possible.

It was further shown that nAu form pockets within muscle tissue and therefore further studies need be done to test whether these pockets would interfere with charges and synapses sent along the muscle fiber for muscle contraction and relaxation.

The fish circulatory system provides the pathway for biodistribution of nAu. It is however not clear what the mechanical action of the particles distributed has on the blood constituents. It is
recommended that ultra-structural assessments (using TEM) should be used to visualize the isolated effects of pressure on the interaction between nAu and red blood cells as well as other blood constituents. This will give a better understanding of agglomerations within the blood or damage to red blood cells caused by collisions between them and nAu.

Further toxicity responses due to mechanical interaction to consider would be whether the nAu particles line the elastic connective tissue surrounding the swim bladder, which would limit this elasticity or cause breakages within the lining. The biodistribution and uptake in tissues other those assessed in this study should be included, e.g. the liver of aquatic organisms and how agglomeration and bioavailability would differ from the injected dose used on mammals. Modeling of protein and nAu interactions are needed to determine whether active sites would be permanently damaged and provide an indication of the types of nanoprotein interactions are that may be formed.

Future studies should include an assessment of the biomagnification potential of nAu particles. Known concentrations of acute algal tests should be fed to daphnia, known numbers of daphnia should then be fed to fish to determine whether biomagnification occurs across trophic levels or whether the particles are able to be excreted without causing damage to the highest tropic level on the food web. Further use of microarray assessments should be employed across various trophic levels to determine whether the toxic mode of action remains similar.
Chapter 7: References


Chapter 7


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