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Application of Species Sensitivity Distributions in Assessing the Aquatic Toxicity Hazard of Nano-gold

By

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# TABLE OF CONTENTS

List of Figures ......................................................................................................................................... iv  
List of Tables .......................................................................................................................................... vi  
Acknowledgments ................................................................................................................................. viii  
Summary ................................................................................................................................................ ix  
Chapter 1:................................................................................................................................................ 1  
  1.1 RATIONALE OF STUDY ............................................................................................................ 1  
  1.2 OVERALL AIMS AND OBJECTIVES ................................................................................. 5  
  1.3 OUTLINE OF STUDY ........................................................................................................ 5  
Chapter 2:................................................................................................................................................ 7  
  2.1 BACKGROUND ........................................................................................................................ 7  
  2.2 NANOTECHNOLOGY ......................................................................................................... 8  
    2.2.1 History .......................................................................................................................... 8  
    2.2.2 Applications & Benefits of GNPs ............................................................................. 9  
  2.3 GOLD NANOPARTICLES ............................................................................................... 10  
    2.3.1 Characteristics .......................................................................................................... 10  
  2.4 ECOTOXICOLOGY OF NANOPARTICLES ................................................................... 14  
  2.5 TOXICITY OF NANOPARTICLES .............................................................................. 17  
    2.5.1 Toxicity of GNPs vs. metal salts ............................................................................ 17  
    2.5.2 Management of risks associated with nanoparticles ............................................ 18  
  2.6 SPECIES SENSITIVITY DISTRIBUTIONS ................................................................... 19  
Chapter 3:.............................................................................................................................................. 22  
  3.1 SELECTION OF TEST SPECIES ............................................................................... 23  
    3.1.1 Daphnids ................................................................................................................... 23  
    3.1.2 Fish ............................................................................................................................ 25  
  3.2 TEST ORGANISMS AND CULTURE PROCEDURES ................................................. 29
Chapter 5: Discussion ........................................................................................................................... 60

5.1 Characterisations ......................................................................................................................... 60

5.1.1 Particle charge - Zeta potential ............................................................................................ 60

5.1.2 Particle size - hydrodynamic size and surface morphology ................................................. 61

5.2 ACUTE TOXICITY...................................................................................................................... 61

5.2.1 Gold nanoparticles ................................................................................................................ 61

5.3 Species Sensitivity Distributions ............................................................................................... 63

Chapter 6: .............................................................................................................................................. 66

Conclusion............................................................................................................................................. 66

6.1 Conclusion .................................................................................................................................. 66

6.2 Recommendations ..................................................................................................................... 66

References ............................................................................................................................................ 68
## LIST OF FIGURES

Figure 1.1: South African draft framework for ERA (adapted from Murray and Claassen, 1999).............. 4

Figure 2.1: Top-down and Bottom-up strategies (Ju-Nam & Lead, 2008)............................................... 13

Figure 2.2: Release of NP from products and (intended or unintended) applications: (a) Release of free NP, (b) release of aggregates of NP, (c) release of NP embedded in a matrix and (d) release of functionalized NP. Environmental factors (e.g. light, microorganisms) result in formation of free NP that can undergo aggregation reactions. Moreover, surface modifications (e.g. coating with natural compounds) can affect the aggregation behaviour of the NP (adapted from Nowack & Bucheli, 2007) ........................................................................ 15

Figure 3.1: *Daphnia pulex*. Photo was taken with Zeiss A X10 at 10X magnification ............................. 23

Figure 3.2: *Daphnia magna*. Photo was taken with Zeiss A X10 at 10X magnification .......................... 25

Figure 3.3: *Danio rerio* (from www.exoticfishaquarium.com) .............................................................. 25

Figure 3.4: *Poecilia reticulate* (from spiral.univ-lyon1.fr) ................................................................. 26

Figure 3.5: *Labeobarbus aeneus* (Skelton, 2001) .................................................................................. 26

Figure 3.6: *Psuedocrenilabrus philander* (Skelton, 2001) ................................................................. 27

Figure 3.7: *Tilapia sparrmanii* (Skelton, 2001) .................................................................................. 28

Figure 3.8: *Oreochromis mossambicus* (Skelton, 2001) .................................................................... 29

Figure 3.9: Floating cage used to breed *P. reticulata* ............................................................................. 31

Figure 3.10: A double sponge air-driven filter (Oxy Plus Bio Filter II). ................................................... 33

Figure 3.11: A double sponge corner air filter ...................................................................................... 34

Figure 3.12: An example of a series bioassay using *Daphnia* as test organism. Toxicity test series shows triplicates of each test samples as depicted by labels, positive control, chloroauric acid, GNP, dispersant and control ................................................................................................. 38

Figure 4.1: Dispersion of the 14 nm GNP stock solution particles in the citrate buffer solution .......... 48
Figure 4.2: Agglomeration of 5 mg/l (A) and 45 mg/l (B) GNP from stock solution in artificial moderately hard water.................................................................48

Figure 4.3: Comparison between the nominal exposure chloroauric acid concentrations and the mean ± standard deviation of the actual measured concentrations (mg/l) in hard reconstituted water at the onset and termination of the bioassays .........................49

Figure 4.4: Comparison between the nominal chloroauric acid exposure concentrations and the mean ± standard deviation of the actual measured concentrations (mg/l) in moderately hard reconstituted water at the onset and termination of the bioassays .........................49

Figure 4.5: Comparison between the nominal exposure GNP concentrations and the mean ± standard deviation of the actual measured concentrations (mg/l) in hard reconstituted water at the onset and termination of the bioassays ..................................................50

Figure 4.6: Comparison between the nominal exposure GNP concentrations and the mean ± standard deviation of the actual measured concentrations (mg/l) in moderately-hard reconstituted water at the onset and termination of the bioassays ..................................................50

Figure 4.7: Cumulative mortalities of test organisms exposed to GNP over 48 h (daphnids) and 96 h (fish)..................................................................................................................................53

Figure 4.8: Cumulative mortalities of test organisms exposed to ionic gold over 48 h (daphnids) and 96 h (fish)..................................................................................................................................54

Figure 4.9: Species sensitivity distributions and confidence limits for combined data from this study and published literature (Li et al., 2010) and for GNP. ..................................................................................56
Table 3.1: Constituents and physicochemical parameters of hard reconstituted water prepared with deionised water (OECD, 1992) for all the fish species and *D. magna*.................................34

Table 3.2: Constituents and physicochemical parameters of moderately hard reconstituted water prepared with deionised water (USEPA, 1993) for *D. pulex* ...........................................35

Table 3.3: The range of test concentrations used for dispersant, GNP and chloroauric acid in acute bioassays for all organisms studied (all in mg/l) .................................................................39

Table 3.4: Description of hazard concentrations (hazard assessment categories) and perceived conditions for each category. Hazard concentrations are presented as percentile species affected (HC) with a percentage of certainty. The categories are based on those proposed by Ansara-Ross (2010). ........................................................................................................42

Table 4.1: Physicochemical water quality means for selected species in toxicity exposure bioassays with GNP, chloroauric acid and citrate buffer ........................................................................44

Table 4.2: Zeta potential mean and STD values in hard reconstituted water (Fish & *D. magna*) ......46

Table 4.3: Zeta potential mean and STD values in moderately-hard reconstituted water (*D. pulex*)..46

Table 4.4: Particle Size of GNP in hard reconstituted water (Fish & *D. magna*)..........................47

Table 4.5: Particle Size of GNP in moderately-hard reconstituted water (*D. pulex*) ......................47

Table 4.6: Median lethal concentrations for 50 % (LC$_{50}$) effect and the corresponding 95 % confidence intervals for *D. pulex, D. magna, D. rerio, O. mossambicus, P. reticulata, P. philander, T. sparrmanii, L. aeneus* exposed to citrate buffer, GNP and chloroauric acid. .51

Table 4.7: Acute toxicity data from the published literature that was included in the derivation of SSDs for deriving the HAuCl$_4$ guideline value ..................................................................................53

Table 4.8: Predicted hazardous concentration and respective ecological categories (Ansara-Ross, 2010) based on the SSD for GNPs. .......................................................................................58
Table 4.9: Predicted hazardous concentration and respective ecological categories (Ansara-Ross, 2010) based on the SSDs for Chloroaurlacid. ........................................................................................................59

Table 4.10: Median lethal concentrations for 15 % (LC$_{15}$) effect for *D. pulex, D. magna, D. rerio, O. mossambicus, P. reticulata, P. philander, T. sparrmanii, L. aeneus* exposed to GNP. ........................................................................................................................................59
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The production of nanoparticles started as early as 1990s (Alkilany & Murphy, 2010). Nanoparticles are utilised in a range of products such as electronics, optics, textiles, medical, devices, cosmetics, food packaging, water treatment technology, fuel cells, catalysts, biosensors and agents for environmental remediation (Handy et al., 2008). Unlike natural particles, which dissolve or aggregate and are often temporary in the environment, engineered nanoparticles (ENPs) maybe persistent due to the stabilization properties of their capping agent (surfactant or organic material). Thus, there is growing concern about the production and fate of ENPs in the environment (Handy et al., 2008). As ENPs pass through the water system they become exposed to different salinities, ionic concentrations and pH changes (Lapresta-Fernández et al., 2012). During this process the ENPs are degraded, transported, altered and accumulated in various ways. Nanoparticles have been found to aggregate in various organelles, for example endocytotic vesicles (Elsaesser & Howard, 2011; Lapresta-Fernández et al., 2012), cytoplasm and the perinuclear region (Mirkin et al., 2010). This can take place via ingestion, endocytosis and or by diffusion (Nowack & Bucheli, 2007). The major question is are NPs toxic and are they more toxic than their metal salts? While bulk gold is distinguished as a chemically inert and a non-toxic substance, (Alkilany & Murphy, 2010) GNPs may be toxic due to their different physicochemical properties such as small particle size, configuration, charge and specific surface area and easy surface alterations (Cho et al., 2009; Goodman et al., 2004; Lapresta-Fernández et al., 2012).

It is important to be able to determine the risk of new substances and thus an ecological risk assessment (ERA) should be determined. An ERA aspires to evaluate the fate, risks and impacts of chemicals on the ecosystem. To assess the effects of toxic compounds in natural conditions a number of models and indicators have been developed (Breure et al., 2011). The current ERA frameworks and tiered approach have been approved to assess NPs (Handy et al., 2008) since the effects data of NPs are similar to the toxic effect of existing chemicals. Species sensitivity distributions (SSD) are being incorporated into ecological risk assessments (de Vries et al., 2008; Newman et al., 2000; Palmer et al., 2004a) for the development of water-quality guidelines (Hose & Van den Brink, 2004). The SSD is a distribution of statistical data approximated from a set of toxicity data (Maltby et al., 2005; Posthuma et al., 2002) resulting in a hazard concentration (HCp) which is expressed as the
percentage (p) of all species that are presumed to become affected (Newman et al., 2000; Wheeler et al., 2002). The South African water quality guidelines currently incorporate the 95% protection value for the aquatic environments (Roux et al., 1996).

The aim and objectives of this study were to assess the potential of GNPs to aquatic organisms and whether GNPs are more toxic than ionic gold, by determining the acute toxicity of GNPs and ionic gold to aquatic invertebrates (Daphnia magna and Daphnia pulex) and some fish species (Danio rerio and Poecilia reticulate, Oreochromis mossambicus, Labeobarbus aeneus, Tilapia sparrmanii and Pseudocrenilabrus philander) and by applying an SSD approach to compare the relative toxicity of GNPs and ionic gold to the different aquatic test organisms.

An Acute bioassay was conducted on both gold nanoparticles and ionic gold following the guidelines stipulated by OECD (1992). The Invertebrates used in the acute toxicity tests are Daphnia pulex and Daphnia magna (first instar, <24 h old) which were obtained from cultures bred and maintained at the University of Johannesburg’s research aquarium, Gauteng, South Africa. The organisms were maintained according to the standard protocols (OECD-TC202). Six freshwater fish species, namely, Danio rerio, Oreochromis mossambicus, Poecilia reticulate, Labeobarbus aeneus, Tilapia sparrmanii and Pseudocrenilabrus philander (14 days old) were obtained from the University of Johannesburg aquarium, Kirsten Akwakultuur Company and from North West University. The invertebrates were exposed for a period of 48 h and fish for 96 h. Mortality of the test organisms were recorded over a 48 h and 96 h period at 24 h intervals for the duration of the test for invertebrates and fish. The recorded endpoints were morbidity or mortality. Dead individuals were removed from the test vessel to avoid fouling of the test solution that would potentially influence the end results. Behavioural changes of test animals were followed closely and recorded if necessary. A test was regarded as valid if control vessels exhibit <10% mortality.

Surface morphology and GNP diameter were determined using high resolution transmission electron microscopy (HR TEM, Joel Jem 2100). Hydrodynamic size and zeta potential in aqueous GNP suspensions were determined using dynamic light scattering (Malvern Zetasizer Nano series, NanoZS). The gold concentrations (for both the ionic and GNP exposures) were analysed before and after the toxicity tests using inductively coupled plasma atom emission spectroscopy (ICP-OES). Gold nanoparticles of 14 nm were used in this experiment and the characterization of the particles was carried out by a transmission
electron microscope (TEM) (High resolution 200 kV FEI Tecnai G2). The data collected from the exposure tests were analysed by using the EPA Probit Analysis Programme to calculate the LC/EC values (version 1.5). Where data did not fit the assumptions of the Probit method, then the data were analysed using the Trimmed Spearman-Kärber method (Hamilton et al., 1977). The results obtained from the bioassays were used to compare the tolerances of non-indigenous and indigenous macroinvertebrate and fish species using a SSD approach (Posthuma et al., 2002). The SSDs were produced using the CADDIS_SSD Generator (USEPA, 2012). Separate distribution models were constructed with the acute toxicity data collected for a range of species for GNPs and ionic gold from which the average hazard concentration (HC₅) endpoint was extrapolated. The HC₅ (hazard concentration where 5% of species are affected) was determined for freshwater fish and invertebrates with exposure durations from 24 to 96 hours.

The initial zeta potential of the 14 nm GNP stock solution was -33.0 mV (Mintek). This is in accordance with available literature (Pongsuchart et al., 2012) which states that the average zeta potential of 9 nm GNPs is -26.8 mV. The general findings were that zeta potential decreased as the exposure period and GNP concentration increased (i.e. as the GNPs aged). These results are depicted in Table 4.2 and Table 4.3. Substances with negative zeta potentials are not likely to aggregate quickly as like charges repel one another. Negatively charged particles are therefore more stable due to their repulsion. The changes in zeta potential observed during the bioassays could therefore be related to the different ionic strengths of the exposure media used for fish and invertebrates (Cai et al., 2008; Schrand et al., 2010). The particle size results can be found in Table 4.4 and 4.5. It can be seen that the hydrodynamic size is related to the zeta potential thus as the size increases, the stability of the solution increases, and less agglomeration occurs (Alkilany & Murphy, 2010).

Transmission electron microscope (TEM) micrographs (figure 4.1 and figure 4.2), are used not only for visual imaging of NPs but also physical characterization of shape, size, and aggregation. The results illustrate that there is no agglomeration of GNP stock solution and the NP size ranges between 14 nm and 45 nm. The TEM images demonstrated well-defined spherical particle and showed that particles made with the citrate reduction and stabilization method were slightly larger than the defined nanoparticle size range (Figure 4.1 and 4.2).

One can establish from the LC₅₀ values shown in Table 4.6 that GNPs are toxic to various species at very high concentrations. There were definite changes in behaviour and motility observed in each of the species that displayed higher mortalities during the experiments.
The organisms swam erratically and often in a spiral pattern. The LC$_{50}$ values for *D. magna* in studies on GNPs done by Li *et al.* (2010) were ≈70 mg/l. In this study the LC$_{50}$s for *D. pulex*, i.e. 75.31 mg/l, were slightly higher but in general agreement with the results of Li *et al.* (2010). Some of the causes for daphnia mortality could be attributed to impaired natural malting cycles, which could shorten the daphnids lifespan and the inability of nutritional absorption from the gut due to the collection of GNP in the gut (Klaine *et al.*, 2008; Li *et al.*, 2010). The no toxicity to very low toxicity observed for the fish species in this study is in accordance with the results of previous studies using GNPs with different capping agents (Asharani *et al.*, 2011; Browning *et al.*, 2009; Farkas *et al.*, 2010; Furgeson *et al.*, 2009). However, Klaine *et al.*, (2008) found that exposure of *D. rerio* to copper nanoparticles (CNP) resulted in a 48-h LC$_{50}$ of 1.5 mg/l.

Exposure to chloroaauric acid resulted in acute toxicity in all the species tested. The results show that the invertebrates were more sensitive to gold ions than the fish species. Studies by Li *et al.*, (2010) showed that daphnids had toxicity at 2 mg/l in chloroaauric acid, which was lower than the LC$_{50}$s recorded for daphnids in this study. The toxicity results from this study compared to literature by Nam *et al.*, (2014) illustrate that the LC$_{50}$s for invertebrates are much lower in this study (<0.2 mg/l) than in the literature (0.64 mg/l). *Danio rerio, O. mossambicus* and *T. sparrmanii* have LC$_{50}$s of 4.85, 11.3 and 10.78 mg/l which is in par with *Thymallus arcticus* and *Oncorhynchus mykiss* with LC$_{50}$s of 4.1 and 10.7 mg/l (Nam *et al.*, 2014).

Species Sensitivity Distributions can be derived from the numerous results of toxicity test from a toxicity database (Palmer *et al.*, 2004a). In this study the CADDIS_SSD US EPA software was used to ascertain numerous levels of protective concentrations HC$_{cps}$ and related confidence levels. The median (HC$_p$) endpoints for GNPs and chloroaauric acid were acquired from the distribution models constructed with a collection of species. The CADDIS_SSD distribution fits the most frequent distribution, the log-probit, to toxicity data (US EPA, 2012).

The HC$_5$ (50) values for chloroaauric acid (Au $^{3+}$) in table 4.8 show that invertebrates from this study (0.039 mg/l) were more sensitive than fish (6.633 mg/l). When comparing the HC$_5$(5) andHC$_5$(50) values from the local data of all species with the international data it is obvious that the local species are more sensitive with 0.015 and 1.834 mg/l reading than the international data with values of 0.33 and 4.46 mg/l. The Hazardous concentration with a 50
% certainty (HC5, 50) for the combined species tested for local data were estimated to be 1.834 mg/l respectively. Due to the lack of data for GNP no LC50s could be derived but LC15s were calculated for D. pulex, P. reticulate, D. magna, T. sparrmanii and D. rerio, with values of 29.1, 35.1, 55.0, 75.1 and 23472.4 mg/l respectively. The LC15s of GNPs demonstrated the same outcome as chloroauric acid, that the Invertebrates were more sensitive than the fish species. The sensitivities in order from most sensitive to least sensitive were as follows D. pulex < P. reticulate < D. magna < T. sparrmanii < D. rerio. Though P. reticulata was more sensitive than D. magna majority of the fish species was less sensitive and D. rerio was the least sensitive.

Overall it was found that ionic gold showed higher acute toxicity to aquatic species, particularly to D. pulex, than GNPs. Thus the hypothesis that GNPs are more toxic than ionic gold was rejected. The second hypothesis, that there is no difference in the toxicity of GNPs among the species tested is not accepted. The differences between species are presented in Tables 4.8 and 4.9. Single-species acute toxicity tests do not incorporate the interactions that environmental factors may have on toxicants. However the application of SSDs provides a better interpretation of acute toxicity data than the single species tests. The results obtained from this study show that the use of acute toxicity tests with indigenous species can be used in enhancing the ecological risk assessment guide of chemicals such as NPs, as well as being beneficial in including other contaminants such as NPs in South African water quality guidelines.
CHAPTER 1: MOTIVATION OF STUDY
1.1 RATIONALE OF STUDY

The evolution of nanotechnology relies solely on the production of nanoparticles (De Paoli Lacerda et al., 2010). In the last decade there has been major growth in nanoscale science and technology (Kahru & Dubourguier, 2010) thus providing us with a large number of NPs consumer products and industrial product (Farkas et al., 2010). There is such an escalation in research of nanotechnology that there is already over 800 commercial products on the market consisting of suntan lotions, cosmetics, paints, self-cleaning windows and stain-resistant clothing. Not to mention the increasing products such as food packaging, detergents and products made from textiles such as sock and underwear, with silver NPs, this is used as an antimicrobial additive (Kahru & Dubourguier, 2010). Gold nanoparticles (GNP) in particular are widely-use in electronics, catalysis, cosmetics, food quality control and in cancer detection (Tedesco et al., 2010).

A large amount of nanoparticles (NPs) are already present in our environment, consequently both from natural sources (erosion or deliberate introduction during environmental remediation) and manufactured NPs which could create waste-containing NPs Batley & McLaughlin, 2010; Tedesco et al., 2010). Mueller & Nowack, (2008), predicted the current worldwide production of nano TiO2 at 5000 t/a, for nano Ag at 500 t/a, and for carbon nanotubes at 350 t/a and yet there are no routine measurement tools for environmental concentrations of manufactured NPs (Tedesco et al., 2010). However due to this exponential growth of industrial nanotechnology worldwide, a nano risk framework was prepared in 2007 in a partnership between the Environmental Defence Fund and DuPont (Environmental Defence-DuPont, 2007). The US Environmental Protection Agency (US EPA) also has been figuring out how to apply the Toxic Substances Control Act to nanotechnology (Batley & McLaughlin, 2010).

There is growing concern about NPs as there is a lack of quantitative ecotoxicity data and the adverse effects that may arise from the manufacturing of NPs (Klaine et al., 2008). The application of tiered ERAs for NPs has been debated amongst the scientific community and the overall consensus is that it ultimately works (Handy et al., 2008). Many scientists have supported the need for a risk assessment paradigm for nonmaterial specifically NPs due to their unusual physicochemical properties. The properties specific to these particles include the zeta potential, collision efficiency and electrophoretic mobility. As a result of the lack of methodologies for determining the toxicity of NPs a method to satisfy this lack is required (Arvidsson, 2012).
Engineered nanoparticles are applied to a wide range of products including, clothing, engineering, electronics, and environmental protection, medicines, cosmetics, (Brar et al., 2010; Tiede et al., 2008), paints, coatings, Microelectronics, wastewater, nutraceuticals, health supplements and fuel catalyst (Brar et al., 2010) to name a few. There is an expected rapid growth of nanotechnology-derived food ingredients, additives, supplements and contact materials. In over 200 countries this technology has already been incorporated in either agriculture, engineering, processing, packaging or delivery of food and nutritional supplements (Tiede et al., 2008). In some countries NPs such as nano-silver have already been added to foods as an antimicrobial agent. They are even utilised in antibacterial wound dressings and clothing, through to reinforced tennis rackets and to advanced transparent sun protection (Tiede et al., 2008). Nanotechnology does have its advantages, such as improving the environment, both through direct applications by detecting, preventing, and removing pollutants, and indirectly through the use of nanotechnology by inventing cleaner industrial processes and constructing environmentally liable nano-product (Brar et al., 2010).

In addition to the above mentioned products the recent advances in NPs in medicine now comprise of diagnostic, biosensing, therapeutic (Mirkin et al., 2010), and drug delivery and targeting abilities (Chen et al., 2008; Kumar et al., 2012). Over the years there has been a growing interest in polymeric NPs as carriers for proteins, vaccines, and polynucleotides (Cai et al., 2008). Currently ultra-small GNPs are one of the most attractive NPs as they are used in the delivery of drugs directly to cancer cells (Kumar et al., 2012).

A great deal of attention has been given to further development of ecological risk assessments (ERA) in Europe and the United states (Claassen, 2003). Risk assessments first became apparent in the 1980s as a regulatory model (Claassen, 2003). A framework for ERAs (Fig 1.1) was developed by the United States Environmental Protection Agency (USEPA) to assess a smorgasbord of ecological issues (e.g. climate change, habitat loss pesticide and toxic chemicals and many more). The frame work was developed as a means of determining the extent of stress on the environment. A “stressor” refers to any physical, chemical or biological entity that can provoke an adverse effect (USEPA, 1992). Ecological risk assessment is defined, by USEPA (1992) and Claassen (2003), as “a process that evaluates the likelihood that adverse ecological effects may occur or are occurring as a result of exposure to one or more stressors”. An ERA is a process of scientific integrity that helps to determine environmental problems and establish precedence (Claassen, 2003; US EPA, 1992). The first step in ERA makes use of a problem formulation that ascertains the
question to concentrate on during the assessment process. The next step is the hazard assessment which specifies the biological effect of the particular stressor and lastly the characterization of the effect (UNEP/IPCS, 1999). The exposure assessment in an ERA determines the environmental concentration of a precise stressor that biota are exposed to. The characterization of ecological effects is a vital part of the ERA procedure. An accurate concentration or behavioural response of a particular group of species to a toxicant can be determined under appropriate laboratory conditions. These concentrations are most often established through the use of laboratory acute and chronic toxicity tests provide endpoints such as LC\(_{50}\) (median lethal concentration), EC\(_{50}\) (median effect concentration) or no observed effect concentration (NOEC) or by observed in situ toxicity or the ecological field effects on aquatic organisms (UNEP/IPCS, 1999).

South Africa has adopted its international protocols from various countries (Canadian, American and Australian) utilizing their test criteria (Ketse & Muller, 2004; Roux et al., 1993). The standard test species used in these protocols lack any local depiction. Thus there is some speculation as to whether international data from standard test species that are mostly based on temperate species, would be a good source of information for tropical and sub-tropical ecosystems (Kwok et al., 2007) as most species used are temperate species.

Local species may differ in their sensitivity to environmental pollutants as the effect on organisms is directly dependant on the environmental conditions, such as temperature and exposure time (Malaj et al., 2012), homeostatic regulatory and membrane permeability also play a role in sensitivity (Brix et al., 2001). Thus species sensitivity distributions (SSD) can be utilized to describe the differences in sensitivity to contamination (Posthuma et al., 2002). Unfortunately there is little to no information on the toxicity of NPs worldwide on freshwater species.
Figure 1.1: South African draft framework for ERA (adapted from Murray and Claassen, 1999).

Main Research Questions and Hypotheses

The use of NPs is growing larger in domestic (e.g. cosmetics & deodorants) and industrial processes (e.g. mining, milling & electronics). There is a huge gap in the knowledge of the toxicity of NPs and their effect on the aquatic organisms. Therefore the main research questions are, 1) are GNP$s more toxic than their ionic gold equivalent? 2) Does the toxicity of GNP$s differ amongst different aquatic organisms?
The Hypotheses of the study are therefore as follows:

• Hypothesis: Gold nanoparticles are more toxic in comparison to ionic gold.
• Null-hypothesis: There is no difference in the toxicity of GNPs among the species tested.

1.2 OVERALL AIMS AND OBJECTIVES

The aims of this study are to determine if GNPs are toxic to aquatic organisms and whether GNPs are more toxic than ionic gold. A second aim is to determine if there is a difference in toxicity of GNPs between different species.

To meet the aims the following objectives were set:

• Determine the acute toxicity of GNPs and ionic gold to aquatic invertebrates using standardized OECD protocols for the standard (*Daphnia magna*) and indigenous (*Daphnia pulex*) test organisms
• Determine the acute toxicity of GNPs and ionic gold to different fish species using standardized OECD protocols for the standard fish (*Danio rerio* and *Poecilia reticulata*) and indigenous (*Oreochromis mossambicus*, *Labeobarbus aeneus*, *Tilapia sparrmanii* and *Pseudocrenilabrus philander*) test organisms.
• Apply an SSD approach to compare the relative toxicity of GNPs and ionic gold to the different aquatic test organisms.

1.3 OUTLINE OF STUDY

Chapter 1 – This chapter illustrates the overall motivation of the study accompanied by the aims and objectives and hypotheses of the project.

Chapter 2 – The chapter provides a general background of nanotechnology, GNPs, aquatic toxicology and species sensitivity distribution.

Chapter 3 – In this chapter a description of the materials and methods and the characterization of the exposures as well as the statistical analyses are given.

Chapter 4 – This section provides a summary for the results illustrating the overall outcome of the acute toxicity test along with the SSD results.

Chapter 5 – The chapter discusses the results of the acute toxicity test and the SSDs.
Chapter 6 – This chapter presents the conclusions of the study and provides recommendations for further research.

Chapter 7 – The references used in this study are provided in the final chapter.
CHAPTER 2: INTRODUCTION
2.1 BACKGROUND

The earth is made up of ≈ 24% of metals. Metals make up about two thirds of the periodic table thus showing the importance metals have in human civilization, they are used in a wide variety of products ranging from household items to space ships (Sau & Rogach, 2010). Gold, like that of silver and copper, is a group 11 transition metal, it is highly malleable and ductile and it makes up 4 ppb of the earth’s crust (Stewart, 2012). Gold is one of the most precious commodity which was discovered about 6200 years ago (Stewart, 2012). Gold is a frequently used in jewellery, coins, dental work and as gold plating for decoration (Stewart, 2012). Gold has a resistance to oxidation, electrical, magnetic, optical and physical properties and for these reasons it is frequently used metal which forms various compounds. The oxidation state of compounds dominant in Au (I) and Au (III) ranges from -1 to +5, The Au (I) ion, or more frequently referred to aurous ion, is the most frequently encountered form of gold used in drugs, this is due to the fact that it reacts with ligands such as thioethers and tertiary phosphines. Elemental gold is inert to all bodily chemicals, thus only gold salts and radioisotopes are of interest in pharmacological practices (Lapresta-Fernández et al., 2012).

The use of gold by humans has been well known for many years (since 4500-4000 BC) (Stewart, 2012). Records show that gold has been used in human culture as far back as 4000 BC to 2500 BC gold has been used by the Eastern Europeans, southern Iraqis and Egyptian Dynasties as a fashion accessory, gold jewellery and the Egyptians were even buried in gold jewellery. For many centuries gold was used as currency by almost every country. Then in 1927 A.D. the French proved that gold could be used to treat rheumatoid arthritis. In 1971 Amersham Corporation of Illinois introduced gold markers used to tag/label proteins for treatment of diseases (Coleman, 2004). This, however, is not the first time gold is used as a treatment for many diseases Havel et al., (2009) showed that as early as 2500BC gold was used in many primitive medicines by the Chinese. In early Indian culture gold was also medicinally used in the form of red colloidal gold, known as ayurvedic medicine, as a rejuvenation and revitalization treatment during old age (Havel et al., 2009).

The gold drug called “Nervin” was used for an extended period to treat people who suffered from nervous disorders. In the 16th century it was thought that colloidal gold could cure a number of diseases, namely heart and venereal problems, dysentery, epilepsy and tumours. A German chemist by the name of Johann Kunckels devoted a chapter in his book to “drinkable gold that contains metallic gold in neutral, slightly pink solution that exert curative properties for several diseases”. In the early 18th century Jeremias B. Richters proposed an
explanation for the change in colour of “drinkable gold”, suggesting that it was a different form of gold (GNPs). Preparation of the elixir with the finest degree of subdivision produced a pink or purple colour whilst a yellow solution is observed once the particles have aggregated (Daniel & Didier, 2004).

In 1857, Michael Faraday reduced chloroaurate (AuCl₄⁻) with phosphorus in CS₂ which resulted in the characteristic deep red solution of colloidal gold. Gold colloids were used in the diagnosis of syphilis until the 20th century as the test proved to not be completely reliable (Daniel & Didier, 2004). In the 1920s gold cyanide was initiated as a treatment for tuberculosis. However, to date gold and its compounds are still most commonly employed as a treatment of rheumatic diseases (Havel et al., 2009).

2.2 NANOTECHNOLOGY

2.2.1 History

The production of NPs started as early as 1990s (Alkilany & Murphy, 2010). Now a days there are a wide variety of NPs that are described as particles with lengths ranging from 1 to 100nm in one, two or three dimensions (Alkilany & Murphy, 2010; Blaise et al., 2008; Drezek et al., 2008; Handy et al., 2011; Sau & Rogach, 2010). Even today nanoparticles are utilised in a range of products such as electronics, optics, textiles, medical, devices, cosmetics, food packaging, water treatment technology, fuel cells, catalysts, biosensors and agents for environmental remediation (Handy et al., 2008). The use of industrial and medical products is growing rapidly. In the next 17 years production of NPs is expected to increase by 35 000 tons (Drezek et al., 2008).

There are two types of NPs: naturally occurring, ultrafine nanosized particles and engineered NPs (Crosera et al., 2009). Nano size particles are a component of atmospheric particles created by natural events such as volcanic eruption and forest fires (Farré et al., 2009; Handy et al., 2011). Nanoparticles have existed since the beginning of time. Ten thousand years ago NPs were found in glacial ice cores (Handy et al., 2008). The fumes created during welding, metal smelting, automobile exhaust, and industrial processes also contain NPs (Farré et al., 2009). There are a variety of practices that produce NPs in the environment ranging from geological to biological mechanisms which produce either inorganic or organic NPs (Handy et al., 2008). There is a growing number of engineered NPs used for anti-reflection coatings, nanomagnetic particles, electrocatalysts, antibacterial silver coatings on
wound dressings, sensors for disease detection and inorganic sunscreens to list a few (Farré et al., 2009). There is some concern about the production and fate of engineered nanoparticles (ENPs) in the environment. Unlike natural particles, which dissolve or aggregate and are often temporary in the environment, engineered NPs maybe persistent due to the stabilization properties of their capping agent (surfactant or organic material). ENPs may contain toxic substances at high concentration compared to nature and they may react with other environmental pollutants (Handy et al., 2008).

The large number of nanotechnology products that exist can be divided into several different classes such as carbonaceous NPs; metal oxides; semiconductor materials, including quantum dots; zero-valent metals such as iron, silver, and gold and nanopolymers, such as dendrimers (Klaine et al., 2008). These categories are developed according to their composition (mono-, bimetallic, metal oxide, magnetic, semiconductor, hybrid & composite) and/or shape or morphology and they can be further categorized into anisometric NPs according to their dimension of growth (Sau & Rogach, 2010). The spectrum of NPs is endless, namely nanospheres, nanocubes, nanorods, nanowires, nanodumbells, octahedra, prisms, stars, nanocages and many more (Sau & Rogach, 2010).

2.2.2 Applications & Benefits of GNPs

Engineered nanoparticles are used in a wide range of biochemical and industrial applications such as electronics, biomedicine, pharmaceuticals, cosmetics, environmental analysis and remediation, catalysis and material science because of their unique physicochemical properties (opticaloccilations, surface area & size) (Ju-Nam & Lead, 2008). There is also a large biomedical application of NPs as drug-delivery agents, biosensors (Cho et al., 2009), or as imaging contrast agents which are either ingested or injected into the body (Drezek et al., 2008). Gold nanoparticles negotiate tumour cell necrosis as photothermal agents (Cho et al., 2009). Gold nanoparticles are used in cancer research as they accumulate in tumour cells and are observed as bright scattering (Blaise et al., 2008; Patra et al., 2007).

Many drugs used in biomedical applications are often coated with biocojugates of GNPs, to facilitate drug delivery to specific target cells (Drezek et al., 2008; Yih & Al-Fandi, 2006). Gold and gold compounds are used in treatment diseases such as psoriasis, palindromic rheumatism, juvenile arthritis and discoid lupus erythematosus (Havel et al., 2009). Gold is used as an anti-inflammatory agent for rheumatoid arthritis treatment (Auranofin® and
Tauredon®) (Alkilany & Murphy, 2010). During treatment the body is exposed to large amounts of gold composites, which can diffuse into a range of organs (liver, kidney and spleen). Prolonged exposure to these drugs has brought about effects such as skin irritation, mouth ulcers, liver toxicity and blood disorders (Havel et al., 2009).

Radioactive GNPs have been found to destroy bacteria and cancer cells (Alkilany & Murphy, 2010; Havel et al., 2009). However, they are deemed to be passive medicines and are used as delivery systems in an effort to supply targeted sites with chemicals to destroy cancer cells (Havel et al., 2009). Gold thiolates used to treat arthritis have also been explored for their anti-tumour and anti-HIV activity (Tiekink, 2002).

Therapeutic agents are generally taken orally; unfortunately there are a few disadvantages to the ingestion of such medication. The oral agents are often destroyed in the intestine or absorption is insufficient. Furthermore, the body may be harmed due to the increasing concentrations as the agent is uninhibited. None the less, these drawbacks can be avoided by adding the therapeutic agents to GNPs. This will allow for successful drug accumulation at affected sites in the body (Yih & Al-Fandi, 2006).

### 2.3 GOLD NANOPARTICLES

Bulk gold is distinguished as a chemically inert and non-toxic substance; however NPs physicochemical properties differ greatly from their bulk counterparts. The toxicity of NPs is not yet established as there is an array of elements that influence the NPs physico-chemical properties for instance size and aggregation state, surface charge, and surface chemistry (Alkilany & Murphy, 2010). It has been shown that even a slight deviation to the geometric structure of these NPs will affect the properties of NPs (Havel et al., 2009). Proteins attach themselves to NPs, ultimately changing their surface chemistry (Alkilany & Murphy, 2010). Cai et al., (2008) confirmed that the sulfonic acid functional group in poly (styrene-co-4-styrene-sulfonate) (PSS) caused a strong increase is negative charge, transforming the charge density of NPs. Gold nanoparticles also differ in colour, from their inert bulk substance, being wine red in colour and of course ionic gold being yellow in colour (Havel et al., 2009).

#### 2.3.1 Characteristics
Due to their importance, as indicated above, GNPs have a more dynamic field due to their small particle size, physicochemical properties (i.e. configuration, size, charge and specific surface area) and easy surface alterations. It is also easy to integrate secondary tags (Nanoparticles) such as targeting of peptides to specific cell types (Cho et al., 2009; Goodman et al., 2004; Lapresta-Fernández et al., 2012). Within this part of the introduction, the general characteristics of GNPs will be discussed.

**Colour**

Gold nanoparticles are blue, green or red in colour whereas their bulk counterparts are yellow in colour, this is because NPs (ranging from 5-100nm) can support conduction bands. These are free moving electrons in the outermost orbitals, but in contrast to visible light wavelengths they are relatively small. Collective oscillations of electrons are a result of irradiation with light; these “plasma oscillations” or “plasmons” are often seen as washing over the surface of particles. The plasmons, in resonance with the incident electromagnetic radiation, dictate the optical properties of NPs (Murphy et al., 2008). The colour change demonstrates a change in optical properties of GNPs. As the variation in the colours of the NPs is visible to the unaided/naked eye, the changes in colour can be used as an indication for reactions with a number of different compounds (Ai et al., 2009). Nanoparticles have a high surface area to volume ratio and the plasmon frequencies coating the surface are highly sensitive to the surrounding medium, changes to these surroundings is the cause of the changes in the colour of the nanoparticle solution. This is similarly noted in instances where particle aggregation and/or surface modifications take place (Murphy et al., 2008). The physicochemical properties and the characteristics of the water column/medium which are in direct interaction with the particles thus has an effect on factors such as the degree of aggregation, behaviour and size range of the NPs (Lapresta-Fernández et al., 2012).

**Capping**

In the case of spherical NPs a capping agent is used to protect the particles high surface energy. If a particle is not coated the interaction between particles will reduce the surface energy and result in aggregation. There are a number of different capping agents, ranging from organic molecules, polymers and biological agents. These agents alter the charge or stabilize the core particle so as to prevent aggregation. Capping agents may also be used as delivery system for further applications (Ju-Nam & Lead, 2008). This application is used in the Frans, (1973) and the Turkevich, (1951) methods, which produce a citrate-capping agent. The surface of NPs can be adapted with many biomolecular materials such as DNA,
proteins and drugs (Yih & Al-Fandi, 2006). There has been remarkable growth in the use of functionalised peptide GNPs in the pharmaceutical field for delivery of drugs and genes to intercellular regions (Kumar et al., 2012). For the purpose of this project the GNPs were synthesised with sodium citrate by following the methods by Frans, (1973) and Turkevich, (1951).

**Synthesis**

There are a large number of procedures for manufacturing of GNPs. The method used for the preparation of these particles is dependent on the use of the NPs, this determines what the chemical, physical and structural properties that are required (Ju-Nam & Lead, 2008; Philip 2009). The procedures used in the manufacturing of the GNPs can be divided into two groups, namely top-down and bottom-up (Figure 2.1) (Ju-Nam & Lead, 2008). Top-down strategies are methods that manufacture NPs directly from bulk materials (e.g. products made from TiO$_2$, SiO$_2$ and aluminium and iron oxides) by using various distribution techniques to produce isolated atoms. The techniques most commonly used for top-down preparations are physical methods such as milling or attrition and repeated quenching, which fracture the bulk material and liberate free atoms. Bottom-up approaches on the other hand, comprise chemical reactions such as, nucleation and growth processes which begin with molecular components which are employed to encourage the development of more complex clusters (Ju-Nam & Lead, 2008; Narayanan & Sakthivel, 2010; Sau & Rogach, 2010). Bottom-up is one of the more commonly used methods for producing a wide range of NPs (Sau & Rogach, 2010).

The chemical technique to producing colloidal gold generally involves the biochemical or chemical reduction of metal salts such as chloroauric acid (HAuCl$_4$) (Sau & Rogach, 2010). The reduction methods used range from photochemical and electrochemical pathways to thermal decomposition of the metal with the use of organic solutions (Sau & Rogach, 2010). There are a wide number of reagents used for the synthesis of GNPs (Sau & Rogach, 2010). Both the Frans, (1973) and Turkevich, (1951) methods produce 15 nm gold particles in boiling water through the reduction of gold chloride by citrate (Murphy et al., 2008).
The GNPs used in this study were synthesised with a citrate buffer, 95 ml of chloroauric acid solution was boiled. Added to the boiling solution was 5 ml of 0.01 M sodium citrate, at this point the citrate becomes citric acid. After one minute a colour change was observed, the yellow solution become transparent. A purple-blue colour forms representative of gold nuclei forming. The solution slowly turns a wine red due to the development of the nanoparticle. The reaction was completed by continues stirring of solution while the solution cooled (Kumar et al., 2007; Tabrizi et al., 2009; Turkevich et al., 1953). The reduction of gold salts with most solvents produces GNPs with a zero valence state. Unfortunately this decreases the particle life span and they have a tendency to agglomerate (Ju-Nam & Lead, 2008). Citrate buffers are most commonly used ligand molecules as they keep the GNP particles in a suspended solution (Ju-Nam & Lead, 2008) acting as both the reducing agent and the stabilizer (Kumar et al., 2007).

There is a growing interest in biosynthesis of GNPs as most synthesis methods used contain toxic chemicals, high pressure and temperatures, and may cause the materials to become unstable or agglomerate upon interaction with biological media or biomolecules (Suresh et al., 2011). The following methods are some chemical or biochemical procedures used to synthesize NPs. Performed – Seed-Mediated Growth Method is where reduced ions of metal particles, otherwise known as seed particles, are added to a growth solution with the same
or different metal ions along with ligands & surfactants (Sau & Rogach, 2010). Sau & Murphy, (2004), prepared cylindrical rod shaped GNPs with the use of the seeded growth method. The production of NPs with the use of electrochemical methods is a classical method that involves the use of voltage to induce a chemical reaction in an electrolyte solution. Photochemical Reduction is a technique whereby particles are be produced by passing through UV light irradiation; the synthesis of NPs may also employ the use of enzymes and peptides (Sau & Rogach, 2010). Such Biosynthetic methods utilise live plant and plant extracts, fungi, microbes to reduce metal ions and produce NPs (Sau & Rogach, 2010). There are a few articles which deal with biosynthesis of metal NPs for instance edible mushroom (Volvariella volvacea) extract (Philip, 2009); microbes (Narayanan & Sakthivel, 2010); Plant extract, beans (Macrotyloma uniflorum) (Aromal et al., 2012) and palm oil mill effluent (POME) (Gan et al., 2012).

2.4 ECOTOXICOLOGY OF NANOPARTICLES

Transportation

Natural NPs have existed since the creation of earth, they are found in volcanic dust and as colloids in sediment and natural waters. Fish have lived in these conditions with natural colloids for centuries. However, regarding the effect of ENPs at the organismal and physiological levels, such effects are still not fully known (Handy, et al., 2011). The nanoscale pollutants may originate from either point sources such as production facilities, landfills or wastewater treatment plants; or from a nonpoint sources such as particle emissions from vehicles and frictional erosion of road surfaces (Handy et al., 2011; Nowack & Bucheli, 2007)

Aggregation

As ENPs pass through the water system they become exposed to different salinities, ionic concentrations and pH changes (Lapresta-Fernández et al., 2012). During this process the ENPs are degraded, transported, altered and accumulated in various ways. As a result of the large surface areas and binding contents, NPs, are able to undergo aggregation or self-aggregation whereby they become absorbed to smaller NP colloids or when they become exposed to changes in water parameters (Lapresta-Fernández et al., 2012). Within the aquatic environment when these processes take place the particles (aggregates) become deposited in the sediment after separating out of water column. Alternatively, the particles may remain in suspension where they can then become accumulated / ingested by
organisms and are dispersed through the food chain (Lapresta-Fernández et al., 2012). Such factors thus effect the concentration of free NPs. Other factors (Figure 2.2) influencing the concentrations of free NPs like that of concentration of free NPs change as aggregated particles are trapped or destroyed via sedimentation affects (Nowack & Bucheli, 2007). The degree, behaviour and size range of aggregation is dependent on the physicochemical properties (i.e. composition, size, capping & shape.) of the ENPs and the characteristics of the water column (i.e. salinity, temperature pH, ionic strength & hardness.) (Lapresta-Fernández et al., 2012). Humans and animals are affected by NPs indirectly by absorption through the skin from contact with air, water or soil or directly by consumption of contaminated plants or animals (Krysanov et al., 2010; Nowack & Bucheli, 2007).

**Figure 2.2:** Release of NP from products and (intended or unintended) applications: (a) Release of free NP, (b) release of aggregates of NP, (c) release of NP embedded in a matrix and (d) release of functionalized NP. Environmental factors (e.g. light, microorganisms) result in formation of free NP that can undergo aggregation reactions. Moreover, surface modifications (e.g. coating with natural compounds) can affect the aggregation behaviour of the NP (adapted from Nowack & Bucheli, 2007)

**Accumulation**

Nanoparticles accumulate in various parts of the cell, for example endocytotic vesicles (Elsaesser & Howard, 2011; Lapresta-Fernández et al., 2012), cytoplasm, perinuclear region (Mirkin et al., 2010). This can take place through a number of different pathways, such as ingestion, endocytosis and or by diffusion (Nowack & Bucheli, 2007), to name a few. A study by Cho et al., (2009) found aggregation of 13 nm PEG-coated GNP s in the liver, spleen and
blood of rats. The authors further showed specific deposits of the PEG-coated GNP in cytoplasmic vesicles and lysosomes of Kupffer cells and macrophages of the spleen.

Assessing the toxicity of nanoparticles

Toxicology is a science that deals with the adverse effect of a chemical or physical agent that is harmful to a living organism (Browne, 2005; Elsaesser & Howard, 2011; Hodgson, 2004). Ecotoxicology is a branch of environmental toxicology which is interested in the toxic effects of physical and chemical substances on living organisms (Browne, 2005). Aquatic ecosystems are unpredictable, uncertain and complex, ecotoxicology utilises a risk-based approach by accepting these issues and bettering the functioning of ecosystem protection (Browne, 2005). The study of toxicology functions as a way to protect humans and the environment from the detrimental effects of toxicants. “A toxicant involves a qualitative biological aspect because a compound, toxic to one species or genetic strain, may be relatively harmless to another” (Hodgson, 2004). It is also useful in the formulation of specific toxicants such as anti-cancer and other clinical drugs, and pesticides (Hodgson, 2004). Toxicological responses to doses may be non-linear and complex as the measurements may be acute or chronic and may vary from organ to organ and with age, genetics, gender, diet, physiological condition, or the health status of the organism (Elsaesser & Howard, 2011; Hodgson, 2004). The main parameter for toxicology is concentration and time. These characteristics are easily measured for a single substance, by determining the nature of the chemical’s dose response, a hazard level can be produced thus labelling a compound either “safe” or dangerous” (Elsaesser & Howard, 2011).

The lethality of a toxicant to an organism is easily measured with toxicity tests (Nussey et al., 1996). Organisms have different sensitivities as they react differently to aqueous ligand (pH, water hardness) and are adapted to different temperatures and factors, salinity, and many more (Malaj et al., 2012; Posthuma et al., 2002). There are three major hazardous traits that chemicals pose on the environment: the tendency to accumulate in living things, environmental persistence, and high toxicity. Many substances degenerate in the environment thus possessing no real threat but chemicals such as DDT, PCBs and TCDD persist in the environment for an extended period of time. Compounds continuously dumped into the environment accumulate and at a specific level become toxic to the environment, thus presenting hazard even after their disposal into the ecosystem has stopped (Hodgson, 2004; Le Blanc, 2004). There are a number of water quality factors that may alter the toxicity of a metal, An increase of calcium in the soft water, a decrease in dissolved oxygen and a
change in temperature may cause an increase in the toxicity of a pollutant as well as the concentration of hydrogen ions (pH) that can change the ionisation and solubility of a metal (Malaj et al., 2012; Nussey et al., 1996).

### 2.5 TOXICITY OF NANOPARTICLES

Toxicity studies, for example (Drezek et al., 2008; Lai, 2012; Handy et al., 2011; Schrand et al., 2010) have demonstrated that accumulation of NPs results in the formation of ROS (Reactive oxygen species). The activation of ROS causes interference with a variety of natural cell functions, such as oxidative stress, DNA damage, and proliferation of pre-neoplastic cells resulting in pulmonary toxicity and formation of neoplasms (Lai, 2012). The toxicity of NPs is dependent on characteristics such as composition, charge, shape, surface area, solubility and coating agent (Lai, 2012; Pan et al., 2007; Schrand et al., 2010).

Nanoparticles like those of Ag, Cu and Al may cause oxidative stress in that the free radicals created possibly interrupt the epithelial cell membrane, allowing NPs to cross the blood-brain barrier and enter the central nervous system (Schrand et al., 2010). The intrusion of the blood-brain barrier may have many side effects such as reduction in growth rate and embryonic development (Li et al., 2010; Schrand et al., 2010).

Pan et al., (2007) proved that the toxicity of GNPs is highly dependent on the size of the particles. The authors found that 15 nm triphenylphosphine coated GNPs were non-toxic, whereas 1.4 nm particles cause rapid necrosis of cells. A study done on size range and different capping agents showed that NPs capped with biotin and citrates were not toxic (Pan et al., 2007). Cell membranes are composed of a phospholipid bilayer and are selectively permeable (Elsaesser & Howard, 2011), however, because of small size (nanometers) and specific coating, these particles are able to cross the phospholipid bilayer and result in the intrusion cells by NPs (Elsaesser & Howard, 2011). On the other hand, Drezek et al., (2008) found that the gold salt (AuCl₄) was 90 % toxic. In addition, studies have shown that the toxicity of NPs could be affected by the charge of the particle such that cationic (positively) charged GNPs are more toxic than anionic (negatively) charged GNPs of the same size (Goodman et al., 2004).

#### 2.5.1 Toxicity of GNPs vs. metal salts

There are a number of gold radioactive isotopes such as, gold-198, which are used in the treatment of cancer (Stewart, 2012) and gold sodium thiosulfate (AuNa₃O₃S₄), used as a
The isotope chloroauric acid (HAuCl$_4$) is used to preserve photographs by replacing the silver atoms present in an image (Gagnon, n.d.). Gold salts such as, gold chloride, are neurotoxins and known to cause peripheral neuropathy (Havel et al., 2009).

Ispas et al., (2009) performed an experiment comparing the toxicity of nickel NPs with nickel salts on zebrafish embryos. It was concluded that the NPs were of the same or lower toxicity to the embryos than the nickel salts. The toxicity of GNPs and Au ions (aurochloric acid) were tested on daphnia and found that GNPs have a toxicity of about 2 mg/L after 48 hours and showed that ionic gold is significantly more toxic than GNPs (Li et al., 2010).

2.5.2 Management of risks associated with nanoparticles

Ecological risk assessment (ERA) aspires to evaluate the fate, risks and impacts on the ecosystem of any compound entering the environment. To assess the effects of toxic compounds in normal environmental conditions a number of models and indicators have been developed (Breure et al., 2011). A large number of species indigenous to South Africa, namely freshwater insect larvae and fish species have been used as bioindicators for inclusion in an ERA Framework (Wepener, 2008).

The overall thought is that the current ERA frameworks and tiered approach can be used to assess NPs (Handy et al., 2008) since the effects data of NPs are similar to the toxic effect of existing chemicals, for instance respiratory distress and oxidative stress responses in adult fishes, or developmental effects on embryos (Handy et al., 2011). Some factors affecting the outcome ERAs on NPs are the large gap in data on NPs and their hazards (Handy et al., 2011), their physicochemical differences (Aschberger et al., 2011; Handy et al., 2008; Klaine et al., 2008) and the array of NPs that need to be grouped and ranked. Thus focus should be put on the differences between the NPs and their bulk materials (Handy et al., 2008).

General aquatic exposure assessments following traditional aquatic toxicology principles are classified according to the soluble portion of the contaminant, thus this approach may not be suitable for assessing NPs as these materials are insoluble in water (Klaine et al., 2008). The quantitative ecological risk approach requires the Predicted Environmental
Concentration (PEC) and the Predicted No-Effect Concentration (PNEC) for each environmental compartment (air, water, and soil) to be established (Aschberger et al., 2011). To aid in this approach data of concentrations and physicochemical properties would be required (Klaine et al., 2008). Information from colloid chemistry, mammalian toxicology, including material and geological sciences, will enable ecotoxicology studies to move forward (Handy et al., 2008) and more and more data to be collected.

The ability to predict new and upcoming risks is an essential aspect of a risk management program no matter where the changes occur, in technology, equipment, or the induction of new materials in a working environment. Continuous observation of recognisable risks should be a necessity by gathering information regularly and evaluating features that may cause potential harm (Schulte et al., 2008). Any new materials should be assessed for risks under frameworks such as REACH (Registration, Evaluation, Authorisation and Restriction of Chemicals) (Handy et al., 2008). A project ENRHES (Engineered Nanoparticles: Review of Health and Environmental Safety) funded by the EU funded to determine if the REACH regulation might apply to ENPs, could not be completed as there was a lack of data (Aschberger et al., 2011). However, there has been some apprehension to the use of these regulations as they are only applicable to production of materials over a tonne annually and not all NPs may reach this target level (Handy et al., 2008).

2.6 SPECIES SENSITIVITY DISTRIBUTIONS

SSDs are being integrated into ecological risk assessments to evaluate the toxicity of particular chemicals (Palmer et al., 2004a). The SSD is a distribution of statistical data approximated from a set of toxicity data and observed as a function of accumulative distribution (Maltby et al., 2005; Posthuma et al., 2002). Species sensitivity distributions are used to calculate the concentration at which a specified proportion of species will be affected. The SSD provides a plot of logarithmic toxicity endpoints, specifically LC$_{50}$ (the lethal concentration of toxin that cause 50 % death of test organisms at a specific period of time) against rank-assigned percentiles for each endpoint value to which a statistical distribution is fitted (Wheeler et al., 2002). A resulting hazard concentration (HC$_p$) is produced from the SSD which is expressed as the percentage ($p$) of all species that are presumed to become affected. A 95 % confidence level of the specified percentage is employed to correctly verify the level of protection required (Newman et al., 2000; Wheeler et al., 2002). Toxicological databases are key factors in the derivation of water quality guidelines, thus SSDs are more commonly used in ecological risk assessments and
generating water quality guidelines (Browne, 2005; Wheeler et al., 2002). The South African water quality guidelines currently incorporate the 95 % protection value for the aquatic environments (Roux et al., 1996).

One of the methods to assess ecological risk is the use of Species Sensitivity Distributions (SSD). The local and international data on the toxicity and bioavailability are integrated into statistical representations to envisage the toxic effects on the ecosystem (Breure et al., 2011). The concentration and speciation, the environmental characteristics, determining bioavailability and particularly the sensitivity to metal pollutants of the intended species influence the effects that metals might have on the biota (Malaj et al., 2012). The variation of these sensitivities is constructed by the parametric distribution function like that of triangular, normal, or logistic distribution, this is known as a SSD model (Posthuma et al., 2002). An SSD describes the mean sensitivity and range of sensitivity among biota for a specific stressor (de Vries et al., 2008). For the purpose of this dissertation mortality mean (LC50) was calculated for an array of species and plotted to produce a SSD. From the distribution graph a protective concentration of 95 % of species (PC95) estimated, this is commonly known as the hazardous concentration (HC5) (Newman et al., 2000). The hazard concentration is protective of all but p % of species; this is commonly a mean value of 5 % (HC5) or 10 % (HC10) protection value. The HCp is accompanied by the 95 % lower confidence level, this is an important aspect and it allows for more information as the lower confidence levels increase as more data is added (Newman et al., 2000).

There are two ways in which to carry out a SSD model in a (1) forward sense where the tiny percentage of species affected at a specific concentration is approximated; and in a (2) inverse sense which estimates the fraction of species sensitivities for some iota of species not protected. The latter assigns the HC5 values to be used in setting the ecological quality guidelines (Aldenberg et al., 2002). Data used in SSDs are generally acquired from the USEPA ECOTOX database. This data base provides international toxicity information for a large number of species and chemicals (USEPA, 2012). The use of USEPA’s toxicity database provides a large source of information that is often used to determine ecological risk assessments (Posthuma et al., 2002). Majority of the information published on ecological risk assessments using SSDs have centred on freshwater environments for which there is a great quantity of good quality data, primarily for pesticides (Wheeler et al., 2002). However a search of the database shows a lack of data for nanoparticle species which presents a problem when applying the data to the SSD approach.
Species sensitivity distributions are being incorporated into ecological risk assessments (Newman et al., 2000; de Vries et al., 2008) for the development of water-quality guidelines (Hose & Van den Brink, 2004). ERAs are being continuously developed; they can be estimated with the use of the hazard quotient (HQ) approach. When the HQ value is above one the level of stressor present poses a risk (Brix et al., 2001). ERAs are developed by determining endpoints which are measurable features of important components of ecosystem considered to be a risk. These endpoints are typically aspects of toxicity evaluations done in the laboratory, field observations or literature comparisons (Claassen, 1999). The transport of a chemical agent through a specific organism is an important aspect of environmental bioavailability (Breure et al., 2011).

An ecological risk assessment is much like human risk assessment, in that it classifies pathways and mechanisms of exposure to physical chemical or biological factors of concern. However ERA’s depend on information for the evaluation of risks to relevant individuals, populations, communities or ecosystems, where as human health risk focuses on the most sensitive individuals (Claassen, 1999). The European Union (EU) perform toxicity tests of environmental substances to guard human and aquatic health under Commission Directives to facilitate the risk assessments of (EU) under Commission Directives that directs the risk assessment of new substances, existing substances, and biocidal products (Chapman et al., 2011). Under Directive 93/67/EEC, risk assessment must at least have hazard identification and where appropriate, concentration– response (effect) assessment, exposure assessment and risk characterisation for both human health and the environment (Chapman et al., 2011).

The Australian water-quality guidelines for toxicants are based on the use of the HC₅ values and SSD curves (Hose & Van den Brink, 2004). Water quality guidelines are produced by placing local and international data into the Burr Type III program to produce a distribution graph to determine the acceptable level of toxicants and to provide a specified level of protection (Palmer et al., 2004a). In South Africa the direct estimation of ecological effects potential (DEEEP) is a common approach in the management of industrial wastes, which involves an assortment of toxicity tests on these waste products (Wepener, 2008). Thus for the purposes of this project, the DEEEP methodologies for aquatic macroinvertebrates and fish were used to determine the hazard potential of ionic gold and GNPs. The data generated from these methodologies were then subjected to SSD models to calculate hazard.
CHAPTER 3:
MATERIALS AND METHODS
3.1 SELECTION OF TEST SPECIES

3.1.1 Daphnids

*Daphnia* spp. are commonly referred to as “water fleas”. They can survive in brackish and marine water but they are predominantly freshwater organisms (Miller, 2000). These daphnids are small crustaceans of the family Daphniidae which falls under the order Cladocera. Daphnids feed on algae, bacteria, fungi and phytoplankton floating in the water. They produce via parthenogenicity and yielding ten live young per individual. Daphnids are found throughout the world. They are kidney shaped, have a single compound eye and two leaf-like limbs inside the carapace (Clare, 2002; Miller, 2000).

*Daphnia pulex*

*Daphnia pulex* (Figure 3.1) is the most common species of daphnids and is found worldwide (Miller, 2000). The size of *D. pulex* range according to gender, males are generally about 1.5 mm and females are 2.5-3.5 mm. They vary in colour from yellow to almost red. *Daphnia pulex* is very similar to *D. magna* but they differ in size, as *D. pulex* is smaller than *D. magna*, they poses an appendage near the post-abdominal region of the carapace that is covered in setae and ephippium (egg) is more triangular in shape with only extension from the top edge and the embryos are not parallel. *Daphnia pulex* also flourish in organically enriched waters (Clare, 2002; Miller, 2000).

![Daphnia pulex](image)

*Figure 3.1: Daphnia pulex. Photo was taken with Zeiss A X10 at 10X magnification*
**Daphnia magna**

This species of daphnia is commonly found in Western Europe including England, Belgium, the Netherlands, Finland, in the black sea bordering the Ukraine and some Baltic Islands. It has also been seen in rock pools of the Atlantic coastlines if north-eastern United States (Elenbaas, 2013). *Daphnia magna* (Figure 3.2) males are 2 mm in length and the females are 3-5 mm in size. Depending on their food source they range in colour from yellow to salmon-pink. *Daphnia magna* has a large range of temperature tolerance but their optimal temperature is between 18 °C to 22 °C. The ephippium of *D. magna* is a curved-edge rectangle that is saddle shaped, with parallel embryos and two extensions from the top long side. *Daphnia magna*, in general, are often considered to be more sensitive than *D. pulex* (Clare, 2002; Elenbaas, 2013).

Due to their sensitivity towards pollutants such as metals, crustaceans like *D. magna* and *D. pulex* are approved models for release of toxicants into the environment by the USEPA and other regulatory agencies (Li *et al.*, 2010). *Daphnia magna* is often used as toxicity test species (Baun *et al.*, 2008; Li *et al.*, 2010; Martins *et al.*, 2007; Rosenkranz *et al.*, 2009; Soucek *et al.*, 2000) because they are essential species in the aquatic food web. They are ideal representatives of zooplankton due to the fact that any harmful effect on these species may cause community or ecosystem – level response (Baun *et al.*, 2008). They are sensitive to many chemicals and other stressors and are easy to cultivate (Taylor, 2010). *Daphnia magna* is commonly one of the species in the standard OECD toxicity guidelines (Baun *et al.*, 2008) and the US EPA incorporate both *D. magna* and *D. pulex* as standard test species in their toxicity guidelines (US EPA, 2002).
3.1.2 Fish

Danio rerio

Danio rerio (Figure 3.3) is from the family Cyprinidae and they inhabit slow streams, canals, ditches and ponds but prefer fast flowing waters. These fish feed on worms, small crustaceans and insect larvae. They can withstand temperatures ranges of 15 °C to 43 °C but their optimal temperatures are between 25 °C to 29 °C. Danio rerio can grow up to 60 mm but on average they only reach 45 mm in size (Ross, 2004).

Danio rerio is often utilised as sentinels and models for toxicity tests because these species are obtainable, inexpensive, easy to maintain and under the correct conditions will produce a large number of eggs (Ross, 2004).

Poecilia reticulata (Peter, 1859)

P. reticulata (Figure 3.4) from the family Poeciliidae are commonly known as Guppies were introduced and released into Gauteng and Kwazulu-Natal in 1912 to control mosquitos. Most of the feral populations are released from aquariums. They are found in only a minute region of South Africa; they have been seen along the coastal reaches of Kwazulu-Natal spanning Durban southwards as well as in Namibia in the Kuruman Eye and Lake Otjikoto (Skelton, 2001).
This species of fish is tolerant to wide range of salinities but does require warm waters, with optimal temperature being between 23 °C and 24 °C. Guppies need vegetative waters to survive. They feed on daphnia, mosquito larvae and small worms (Skelton, 2001).

Figure 3.4: *Poecilia reticulate* (from spiral.univ-lyon1.fr)

*Labeobarbus aeneus* (Burchell, 1822)

*L. aeneus*, (Figure 3.5) from the family Cyprinidae, are distributed in South Africa along the Orange-Vaal system; and transferred to larger Cape coastal rivers such as the Gourits, Great Fish and Kei rivers; along with the Mutirikwe and Limpopo Dam in Zimbabwe (Skelton, 2001).

This species resides in dams and clear flowing waters with sandy or rocky substrates. *Labeobarbus aeneus* is largely omnivorous, generally feeding on benthic invertebrates, including bivalves, vegetation, algae and detritus. Mature males are about 200m and females are 240m in size. These fish are important angling fish.

Figure 3.5: *Labeobarbus aeneus* (Skelton, 2001)
Psuedocrenilabrus philander (Weber, 1897)

These fish are from the family Cichlidae and are found north of South Africa from Orange and most of Kwazulu-Natal region and extends into Africa and through Southern Congo tributaries and Lake Malawi. *Psuedocrenilabrus philander* (Figure 3.6) favour vegetative zones of wide variety of habitats from flowing waters to lakes and isolated sinkholes. They prey on insects shrimp and small fish. *Psuedocrenilabrus philander* is a brooding fish used for evolutionary and behavioural research (Skelton, 2001).

![Psuedocrenilabrus philander](image)

Figure 3.6: *Psuedocrenilabrus philander* (Skelton, 2001)

Tilapia sparrmanii (A. Smith, 1840)

From The Cichlidae family this species of Tilapia are distributed throughout the Orange River and KwaZulu-Natal south coast, spanning northwards to the southern Congo tributaries, Lake Malawi and the Zambezi system. They were extensively transferred to the Cape (Skelton, 2001). *Tilapia sparrmanii* (Figure 3.7) is tolerant of a wide variety of habitats but prefer stationary waters with submerged or emerged vegetation. They are omnivorous mostly feeding on algae, soft plants, small invertebrates and small fish. Originally these fish were distributed as fodder for bass (Skelton, 2001).
Figure 3.7: *Tilapia sparrmanii* (Skelton, 2001)

**Oreochromis mossambicus (Peter, 1852)**
These species are also from the family Cichlidae. *Oreochromis mossambicus* (Figure 3.8) is restricted to closed estuaries and coastal reaches of rivers. They span the east coastal rivers from the lower Zamezi system south to Bushmans system, Eastern Cape. They are also distributed south of the Phongolo system. They are also circulated amongst inland regions toward south-west and west coastal rivers together with the lower Orange River and rivers of Namibia (Skelton, 2001).

They thrive in standing and still waters and tolerate fresh, brackish or marine waters and higher concentrations of salinity. They survive low temperature of 15 °C in high salinity waters and favour temperatures of 22 °C but can tolerate temperatures as high as 44 °C (Skelton, 2001). *Oreochromis mossambicus* feed on algae; diatoms, and detritus, larger organisms may consume insects and other invertebrates. This species is commonly used in aquaculture and commercial and subsistence fisheries. These species has been extensively used in biological, physiological and behavioural research (Skelton, 2001).
3.2 TEST ORGANISMS AND CULTURE PROCEDURES

3.2.1 BREEDING OF INVERTEBRATES

The invertebrates used in acute toxicity tests were *D. pulex* and *D. magna* (first instar <24 h old). The *D. magna* ephippia were obtained from MicroBioTests Inc. and hatched according to their protocols (DAPHTOXKIT F™ MAGNA). The *D. pulex* were obtained from the University of Johannesburg aquarium. Both cultures were bred and maintained at the University of Johannesburg’s Aquatic Research Facility, Gauteng, South Africa, as part of this study. After each species was successfully bred, a portion of the instars were used in the exposure process. The organisms were spawned within a 24hr period and therefore were of similar age and size. The *D. pulex* and *D. magna* cultures were maintained at 22 ± 2 °C in Daphnia medium (Table 3.1 & 3.2) and were fed a mixture of trout pellets, yeast and alfalfa (TYA) food following standard protocols (USEPA, 1992).

*Daphnia pulex*

A minimum of eight culture vessels were prepared by rinsing each vessel three times with hot deionised water and once with the culture medium before preparing new cultures. Two and a half litres of exposure medium (Table 3.1 & 3.2) was placed in the beakers and 5 ml of daphnia food was pipetted into medium (IWQS, 1998). About 30 adults and a few young, from an old culture, were carefully transferred to the new vessels using a 10 ml gradient pipette. All daphnia that were translocated were younger than three weeks and had no ephippia. Each beaker was covered to prevent any dust from contaminating the water. New
stock solutions were prepared every three or four days and the process was repeated to prevent overcrowding and to guarantee a constant supply of healthy organisms (IWQS, 1998).

**Daphnia magna**

The content of the vials that the ephippia were stored in were emptied into a microsieve, they are rinsed thoroughly with tap water to rid all trace of storage medium. The ephippia are then transferred to a 10 cm petri-dish with 50 ml of pre-aerated standard freshwater (Table 3.1). The dishes are covered and incubated for 72 hours at 20-22 °C (MicroBioTests Inc., 2011; USEPA, 2002).

### 3.2.2 BREEDING OF FISH

Fresh water fish species used in this experiment were *D. rerio* and *O. mossambicus* bred and maintained at the University of Johannesburg; *P. reticulata*, which were acquired from Kirsten Akwakultuur Company; *T. sparrmanii, P. philander* and *L. aeneus* were cultivated and obtained from North West University. The fish were fed Tetramin® flake food and supplemented with frozen *Chironomus* sp. (bloodworms) every other day. Temperatures varied for each species, with *P. reticulata* being maintained at 22 ± 2 °C and the other species of fish were kept at 26 ± 2 °C. A percentage of offspring of each species was used in the exposure test to later determine the different sensitivities between the species. All tests were done as a static acute toxicity test, primarily based on the guidelines for fish acute toxicity tests according to (OECD, 1992) regulations.

**Danio rerio and Poecilia reticulata**

Adult *D. rerio* and *P. reticulata* were obtained from the University of Johannesburg and a commercial aquarium in Pretoria, after they were, they were left to acclimate and until they had reached maturity. Since adult *D. rerio* eat their eggs a breeding method was created to prevent this. A plastic breeding mesh cage was wrapped with a mesh of specific size to allow eggs to pass through it. The breeding cage was designed to be easily suspended in the surface of a tank, with the lower portion of the cage submerged 5cm below the surface of the water (Figure 3.9). A mesh cover was secured to the top of the cage to prevent the fish from jumping out the tank. To reduce stress the adult fish were placed into the breeding tank 2 days prior to spawning induction, in a ratio of 1.5 males to 1 female. The breeding cycle is typically over after one to two hours after lights have turned on. The whole breeding tank is
then removed and the adult fish were transferred to a clean tank. *Poecilia reticulata* is bred in much the same way but once the larvae hatched they were transferred to a tank with gravel substrate fitted with under gravel filters to maintain a more stable environment.

![Figure 3.9: Floating cage used to breed *P. reticulata*](image)

The embryos are then seen at the bottom of the tank until they hatch within 72-96 h. Fungus between the embryos can be treated by adding 5-10 drops of methylene blue (Rid-All® Methylene Blue 1 % B.V.F) to 100 litres of tank water. Soon after hatching the larvae, along with their yolk sacks will adhere motionless to the sides of the tank for the next 24-48 hours after which they will become free swimming. Free swimming juveniles were fed INTERPET Liquifry No 1 two to four times daily by mixing 1 drop of Liquifry into 4 litres of water. Once the larvae are big enough they can eat finely ground flake food, like that of TetraMin *Baby®*.

**Oreochromis mossambicus and Psuedocrenilabrus philander**

Both species were left undisturbed in tanks. The ratio of *O. mossambicus* males to females was 4:16 and the temperature was maintained at 27 ± 2 °C for *P. philander* the ratio was three females to one male and the breeding temperature was set at 25 °C. There is no specific procedure to breeding these species; the adult fish were monitored in a tank containing of about 20 mm in length. The embryos were removed from female fish to allow them to breed again. The embryos were removed by opening the female’s mouth and rinsing
them into a net suspended in water. In the *P. philander* the embryos were removed once the female’s throat changed a darker colour, indicating the hatching of larvae (Ross, 2004). The larvae were then translocated into large funnels with a flow system, allowing them to be circulated during the incubation period. The *O. mossambicus* embryos were also transferred into the same type of funnels with a flow system for incubation period. As the embryos hatch they move up the funnel and spill over, with the out flow, into larger containers (Ross, 2004). They were removed and reared in aquaria fitted with sponge filters. A portion of each species’ juveniles were used in the toxicity test. The juveniles were fed small amounts of TetraMin *Baby®* and Daphnia.

*Tilapia sparrmanii*

A male was selected according to their colour distinction and placed in the breeding tanks along with three females. The males were observed for any aggressive behaviour towards females. Inferior females were removed from the tank. Once a male had selected an apt mate, the breeding was initiated and about 400-500 eggs laid. Immediately after the eggs were laid they were removed and artificially incubated. The breeding pairs were removed from tanks and placed back into the stock group to prevent any further aggressive behaviour amongst the spawning stock. The eggs were usually laid on rocks these rocks were removed from the tanks and placed into a tank with a flow through system, allowing water to wash over the eggs. Once the eggs had attached, within two to three days, the rock was removed and their water was cleaned sporadically. The free-swimming larvae were fed TetraMin *Baby®* and a percentage of juveniles were used for toxicity tests.

### 3.3 MAINTENANCE

According to the standard protocol for undertaking acute toxicity tests (OECD, 1992) all fish were obtained and held in the aquarium for a minimum of 12 days before they are used for testing. They must be held in water of the quality to be used in the test for at least seven days immediately before testing and under the following conditions:

- Light: 12 hours photoperiod daily;
- Temperature: appropriate to the species;
- Oxygen concentration: at least 80 per cent of air saturation value;
- Feeding: three times per week or daily until 24 hours before the test is started.
Toxicity of chemicals to aquatic organisms is affected by age, size and health of the organisms. Young organisms are usually more sensitive to toxicants than adults. As a result the juveniles were used for vertebrate and invertebrate exposures. All fish and invertebrate cultures were maintained in a climate controlled room separate from the test facility with a photoperiod of 12:12 h light: dark cycle. All species acquired from an external source was acclimated, at their specific temperatures, in tanks for two to three weeks while they reach the approximate ages. Once externally and internally acquired fish had acclimated and reach the correct age they were moved and allowed to acclimate to exposure conditions 24 h to 48h prior to start of test. A minimum of seven individuals of mixed gender were selected at random and used in the exposure test. Only apparently healthy organisms were used in the exposure experiments.

During the maintenance period a 10-20 % water change was carried out once a week to ensure that any excess nutrients and other undesired wastes were removed. Water replacement keeps the nutrient levels from building up and exceeding maximum concentrations and the temperature of the tank water maintained according to each species. Each holding tank was equipped with an air stone, a double sponge filter and or an air driven corner filter (Figure 3.10 & 3.11). The air stone supplied dissolved oxygen to the water and the double filter facilitates maximum filtration. The two sponges act as biological filters and the air-driven corner filter, which contain filter floss and activated charcoal as a biological/mechanical filter as well as a chemical filter medium. A few stones were placed in the bottom of the filter to anchor it and to act as a “reservoir” for denitrifying bacteria. Each filter was checked and cleaned on regular bases.

![Diagram of a double sponge air-driven filter](image)

**Figure 3.10:** A double sponge air-driven filter (Oxy Plus Bio Filter II).
Figure 3.11 A double sponge corner air filter

The tanks were emptied and sterilized regularly to reduce any spread of diseases. For sterilization a mixture of 10% bleach (sodium hypochlorite) solution was made up and left in the tank for 24 hours. Once the tanks were sterilized they were rinsed thoroughly to remove any toxicants from the tank.

3.3.1 Preparation of the exposure media

Subsequent solutions and testing concentrations were diluted with moderately hard to hard reconstituted water, prepared according to OECD standard methods (1992) and USEPA (1993). The reconstituted waters were also used for the control exposures. Properties of hard reconstituted test water used for the *D. magna* and fish bioassays are given in Table 3.1, while the moderately hard water used in the *D. pulex* bioassays is presented in Table 3.2.

Table 3.1: Constituents and physicochemical parameters of hard reconstituted water prepared with deionised water (OECD, 1992) for all the fish species and *D. magna*

<table>
<thead>
<tr>
<th>KCl</th>
<th>MgSO₄</th>
<th>NaHCO₃</th>
<th>CaCl₂·2H₂O</th>
<th>pH</th>
<th>Hardness as mg/l CaCO₃</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.23g</td>
<td>4.93g</td>
<td>2.59g</td>
<td>11.76g</td>
<td>6-8.5</td>
<td>180-250</td>
</tr>
</tbody>
</table>
Table 3.2: Constituents and physicochemical parameters of moderately hard reconstituted water prepared with deionised water (USEPA, 1993) for *D. pulex*

<table>
<thead>
<tr>
<th>KCl</th>
<th>MgSO₄</th>
<th>NaHCO₃</th>
<th>CaSO₄·2H₂O</th>
<th>pH</th>
<th>Dissolved Oxygen</th>
<th>Hardness as mg/l CaSO₄</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.08g</td>
<td>2.46g</td>
<td>2.59g</td>
<td>1.2g</td>
<td>7.4-7.8</td>
<td>&gt;4.0 mg/l</td>
<td>80-100</td>
</tr>
</tbody>
</table>

3.3.2 *Daphnia media*

*Daphnia pulex*

The medium used was moderately to hard reconstituted water (Table 3.2) made by mixing 1 l of stock solution with 19 l of deionised water. The stock solution was prepared by dissolving 2.59 g sodium bicarbonate (NaHCO₃), 1.2 g calcium sulfate (CaSO₄·2H₂O), 0.08 g potassium chloride (KCl) and 2.46 g magnesium sulphate (MgSO₄·7H₂O) in 1 l of deionised water in a volumetric flask. The medium was then aerated for 24 hours (Truter, 1994).

*Daphnia magna*

The medium used was hard reconstituted water (Table 3.1). The following stock solutions, CaCl₂·2H₂O, MgSO₄·7H₂O, NaHC03 and KCl solutions were prepared by dissolving 11.76 g calcium chloride (CaCl₂·2H₂O), 4.93 g magnesium sulphate (MgSO₄·7H₂O), 2.59 g sodium bicarbonate (NaHC0₃), and 0.23 g potassium chloride (KCl) prepared in deionised water. Then a volume of 25 ml of each stock solution was added to a container and made up to 1 l with deionised water (OECD, 1992). The solution was aerated for 24 h prior to toxicity testing.

3.3.3 *Fish media*

The medium used was hard reconstituted water (Table 3.1). The following stock solutions, CaCl₂·2H₂O, MgSO₄·7H₂O, NaHC03 and KCl solutions were prepared by dissolving 11.76 g calcium chloride (CaCl₂·2H₂O), 4.93 g magnesium sulphate (MgSO₄·7H₂O), 2.59 g sodium bicarbonate (NaHC0₃), and 0.23 g potassium chloride (KCl) prepared in deionised water. Then a volume of 25 ml of each stock solution was added to a container and made up to 1 l with deionised water (OECD, 1992). The solution was aerated for 24 h prior to toxicity testing.
3.4 GOLD NANOPARTICLE, DISPERSANT AND IONIC GOLD STOCK SOLUTIONS

3.4.1 Gold nanoparticle stock solution

This stock solution was supplied by Mintek Analytical Services Division (ASD) at a 500 mg/l concentration in a 0.1 M citrate buffer as dispersant. The required concentrations for the toxicity test was obtained by diluting the stock solution with either the hard or moderately-hard medium (Table 3.1 & 3.2) depending on the species being tested.

These concentrations were calculated with the use of a dilution formula:

\[ C_1 V_1 = C_2 V_2 \]

A range finding test was initially conducted to determine the range of concentrations that the conclusive tests should include. Based on the results of the range finding test the following concentrations were used: 1 mg/l, 5 mg/l, 15 mg/l, 25 mg/l, 35 mg/l, 45 mg/l. Each concentration was made up by mixing 2 ml (1 mg/l), 10 ml (5 mg/l), 30 ml (15 mg/l), 50 ml (25 mg/l), 70 ml (35 mg/l) and 90 ml (45 mg/l) of GNP stock solution with medium and made up to 1 l in a volumetric flask.

3.4.2 Citrate Buffer stock solution

The dispersant stock solution was also supplied by Mintek at a 0.1 M concentration, which is the same concentration of buffer in the GNP stock. The citrate buffer concentration used were the same as the concentrations utilised for the GNP. The concentrations were prepared in the same way as the GNP concentration presented above.

3.4.3 Ionic gold (chloroauric acid) stock solution

A stock solution of 1000 mg/l was prepared by dissolving 1.73 g of HAuCl₄ (supplied by Sigma-Aldrich Pty. Ltd.) in the specified medium in a volumetric flask and made up to 1 l with the same reconstituted water. This stock solution was further diluted to produce the different concentrations used in the toxicity tests. There was a wide variety of concentrations ranging from 0.5 – 45 mg/l for fish species, 0.005 – 25 mg/l for D. magna and 0.0005 – 25 mg/l for D. pulex. The volume of stock needed to make each concentration was determined with the dilution formula:

\[ C_1 V_1 = C_2 V_2 \]
The specific volume was diluted to 1 l in a volumetric flask with the each medium depending on which species was being tested.

3.4.4 Potassium dichromate stock solution

This solution was made up by dissolving 1 g of KCrO$_4$ in either medium to produce a stock solution of 1000 mg/l. No further dilution was necessary for this solution as it was the positive control.

3.5 TEST CHEMICALS AND TEST WATER

The GNP solution in a citrate buffer and trisodium citrate buffer (Na$_3$C$_6$H$_5$O$_7$·2H$_2$O) was obtained from Mintek. These solutions were prepared by purification of chloroauric acid (HAuCl$_4$·3H$_2$O) and trisodium citrate (Na$_3$C$_6$H$_5$O$_7$·2H$_2$O) according to methods by Enustun & Turkevich (1963) and Frens (1973). Analytical grade chloroauric acid was used as one of the testing agents and it was obtained from Sigma-Aldrich chemical company.

3.6 TOXICITY TESTS: EXPERIMENTAL SETUP

3.6.1 Water quality

To determine if water characteristics would influence the toxicity of the GNP and gold ion the following physicochemical water parameters were measured: temperature, pH, electrical conductivity (EC), total dissolved solids (TDS), dissolved oxygen concentration and percentage dissolved oxygen using calibrated handheld (Eutech pH 110 RS232C meter, Eutech CON 110 RS232C conductivity and TDS meter, and Eutech DO6 dissolved oxygen and temperature meter) water quality meters. These parameters were measured daily throughout exposure period. If the specific limits determining the validity of the test (OECD, 1992) were not satisfactory, the test was discarded and repeated. These criteria include:

- Dissolved oxygen percent should not drop below 60 %.
- The concentration of the substance being tested should be maintained at a constant and not be allowed to decline below 4.0 mg/l.
- The pH may vary between 6.0 and 8.5, and
- The mortality of the control group of organisms must not exceed 10 %.

3.6.2 Test protocol and duration
Acute lethality bioassays were carried out using static toxicity tests. Multiple exposure concentrations were used to calculate relevant endpoints. The fish and invertebrate toxicity tests were performed according to the OECD (1992) guidelines. The invertebrates were exposed for a period of 48 h and fish for 96 h.

### 3.6.3 Test chambers

Fish bioassays were conducted in standard, 600 ml glass laboratory beakers, with total test volume being made up to 300 ml in each beaker. For invertebrates, 50 ml beakers were utilized and final test volume was made up to 40 ml. Prior to use all glassware was washed following the method described by Giesy and Weiner (1977) and Van Vuren *et al.*, (1994) by soaking it in 2% non-phosphate detergent solution for 24 h, followed by soaking in 2% hydrochloric acid for additional 24 h. Between soakings the glassware was rinsed in double-distilled water. Following the acid wash the glassware was rinsed in double distilled water and allowed to air dry prior to use. This guaranteed the elimination of all metals and organic compounds USEPA 1994.

### 3.6.4 Replicates, numbers and dilutions

Each bioassay was carried out in triplicate containing seven fish or invertebrate individuals per replicate beaker. This gave twenty one organisms per concentration. The bioassay series (Figure 3.12) consisted of a range of no less than five concentrations of the test solutions, an untreated (negative) control and a positive control (potassium dichromate). Stock solutions were prepared by dissolving preferred quantities in specified test medium.

![Figure 3.12: An example of a series bioassay using Daphnia as test organism. Toxicity test series shows triplicates of each test samples as depicted by labels, positive control, chloroauric acid, GNP, dispersant and control.](image-url)
A range-finding test was performed prior to definitive test to assist in determining an appropriate concentration exposure range. The nominal exposure concentration used for the dispersant (citrate buffer) and GNP suspension (1.0 – 45 mg/l), and ionic (chloroauric acid) (0.5µg/l – 45 mg/l) for each species are given in Table 3.3.

Before adding the relevant exposure concentration, the organisms were allowed to acclimate in a tank to test conditions for 24 h. The test individuals were not fed during this period or throughout the subsequent exposure period. Active and healthy test organisms were relocated to appropriate sized beakers using disposable Pasteur pipettes to minimize dilution of test solutions with culture medium. The different concentrations of the toxicants were added to each vessel. For each concentration of the toxicants a 1l solution was mixed, by diluting the toxicant with reconstituted water. This concentration was then evenly distributed into the beakers.

### Table 3.3: The range of test concentrations used for dispersant, GNP and chloroauric acid in acute bioassays for all organisms studied (all in mg/l)

<table>
<thead>
<tr>
<th>Toxicants</th>
<th>Dispersant</th>
<th>GNP</th>
<th>Chloroauric acid</th>
</tr>
</thead>
<tbody>
<tr>
<td>D. pulex</td>
<td>1.0 - 45</td>
<td>1.0 - 45</td>
<td>0.0005 - 5</td>
</tr>
<tr>
<td>D. magna</td>
<td>1.0 - 45</td>
<td>1.0 - 45</td>
<td>0.005 - 25</td>
</tr>
<tr>
<td>D. rerio</td>
<td>1.0 - 45</td>
<td>1.0 - 45</td>
<td>1.0 - 45</td>
</tr>
<tr>
<td>O. mossambicus</td>
<td>1.0 - 45</td>
<td>1.0 - 45</td>
<td>1.0 - 46</td>
</tr>
<tr>
<td>P. reticulata</td>
<td>1.0 – 45</td>
<td>1.0 - 45</td>
<td>1.0 - 47</td>
</tr>
<tr>
<td>P. philander</td>
<td>1.0 - 45</td>
<td>1.0 - 45</td>
<td>0.5 - 45</td>
</tr>
<tr>
<td>T. sparrmanni</td>
<td>1.0 - 45</td>
<td>1.0 - 45</td>
<td>1.0 - 35</td>
</tr>
<tr>
<td>L. aeneus</td>
<td>1.0 - 45</td>
<td>1.0 - 45</td>
<td>0.5 - 12</td>
</tr>
</tbody>
</table>

### 3.6.5 Mortality

Mortality of the test organisms were recorded over a 96 h period for fish and 48 h period for invertebrates at 24 h intervals. The mortality endpoints were regarded as immobility or an absence of response to stimuli in addition to an opaque colouring. Dead organisms were removed from the vessels to prevent contaminating the test solution and influencing results. Behavioural changes were examined and recorded if necessary. A mortality of < 10 % of the individuals in control group rendered the test as valid. The test was negated if a mortality of > 10 % was recorded for control organisms and was repeated.
3.6.6 **Characterisation of the exposure material**

Surface morphology and GNP diameter were determined using high resolution transmission electron microscopy (HR TEM, Joel Jem 2100). Hydrodynamic size and zeta potential in aqueous GNP suspensions were determined using dynamic light scattering (Malvern Zetasizer Nano series, NanoZS). An aliquot of water from each concentration for each species was collected at 0 and 96 h for the fish and 0 and 48 h for the daphnia bioassays.

The gold concentrations (for both the ionic and GNP exposures) of each sample for each species were analysed before and after the toxicity tests using inductively coupled plasma atom emission spectroscopy (ICP-OES). The samples were treated as follows: 11 ml of each sample was mixed with 3 ml 30 % HCl and 1 ml of 65 % HNO₃ and then analysed using ICP-OES. Once all the raw results were collected the values were back calculated to the original concentration based upon the dilution from the treatment of samples.

**Transmission electron microscopy (TEM)**

Gold nanoparticles of 14 nm were used in this experiment and the characterization of the particles was carried out by a transmission electron microscope (TEM) (High resolution 200 kV FEI Tecnai G2).

3.7 **STATISTICAL ANALYSES**

3.7.1 **ACUTE TOXICITY**

The data collected from the exposure tests were analysed by using The EPA Probit Analysis Programme to calculate the LC/EC values (version 1.5). Where data did not fit the assumptions of the Probit method, then the data were analysed using the Trimmed Spearman-Kärber method (Hamilton *et al.*, 1977). To determine whether there were any significant differences between the LC₅₀ values for the different species, the following formula was used (APHA, 1992): f₁₂ = antilog √((log f₁)² + (log f₂)²), where f is the factor for 95% confidence limits of the LC₅₀ and is calculated by dividing the upper confidence limit by the LC₅₀. If the ratio of the higher LC₅₀ to the lower LC₅₀ exceeds the value for f₁₂ for both the upper and lower 95% confidence limits the LC₅₀s are considered to be significantly different. The nominal concentrations where used to determine the LC₅₀ values. In the absence of
chronic data, the lowest observed effect concentration (LOEC) and no observed effect concentration (NOEC) can be useful in deriving water quality criteria. The NOEC was taken as the maximum concentration of the test materials that produced no significant harmful effect on the test organisms. The LOEC was determined as the lowest concentration that has a significant harmful effect on test organisms.

3.7.2 SPECIES SENSITIVITY DISTRIBUTIONS

The results obtained from the bioassays were used to compare the tolerances of non-indigenous and indigenous macroinvertebrate and fish species using a SSD approach (Posthuma et al., 2002). The SSDs were produced using the CADDIS_SSD Generator (USEPA, 2012). Separate distribution models were constructed with the acute toxicity data collected for a range of species for GNPs and ionic gold from which the average hazard concentration (HCp) endpoint was extrapolated. The HC5 (hazard concentration where 5% of species are affected) was determined for freshwater fish and invertebrates with exposure durations from 24 to 96 hours. The comparisons in sensitivity will be drawn between toxic responses of South African organisms and other non-South African standard test organisms. The taxonomic groups were analysed separately for the SSD model to illustrate the difference in tolerance between the taxa. A cumulative SSD model of all data for fish and invertebrate’s endpoints were also constructed for both gold solutions to ascertain the hazard threshold values.

The resulting hazard threshold values were interpreted within an ERA framework using the method described by Ansara-Ross (2010). Generally the hazard concentration, i.e. HC5, 50 that equates to the 95% protection of organisms with 50% confidence value is calculated. Different hazard concentrations based on acute LC50 data (i.e. HC1 and HC5) with varying levels of certainty were proposed by Wepener et al., (2006) as a classification scheme to assess the hazards posed by chemicals in estuarine systems. Palmer et al., (2004b) developed a classification scheme in which they interpreted hazard concentrations using the national water classification terminology, i.e. excellent/good class as 95% protection, for the good/fair boundary as 90%, and for the fair/poor boundary as 80% protection. Ansara-Ross (2010) proposed a hazard assessment classification scheme that combined the aforementioned classifications. For the “Natural” boundary a 99% level of protection with 50% certainty (HC1, 50) was proposed, for the “Good” boundary a protection level of 95% level of protection and between 75 and 95% certainty (HC5, 5-25), for the “Fair” a protection level of 95% level of protection and between 50 and 75% certainty (HC5, 25-50), and a
protection level of 95% protection with 50% and less certainty (HC₅, ≥50), was chosen to represent an unacceptable – “Poor” boundary category. This classification scheme was also used in this study for the ERA of GNP and ionic gold. The categories with their respective percentage hazard concentrations (HCp’s) and different levels of certainty are presented in Table 3.4.

**Table 3.4**: Description of hazard concentrations (hazard assessment categories) and perceived conditions for each category. Hazard concentrations are presented as percentile species affected (HC) with a percentage of certainty. The categories are based on those proposed by Ansara-Ross (2010).

<table>
<thead>
<tr>
<th>Hazard Score</th>
<th>Hazard categories/ Boundary categories</th>
<th>Hazard Category</th>
<th>HCp Hazard concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>Natural. No hazard due to toxicity</td>
<td>A</td>
<td>&lt; HC₁ (50)</td>
</tr>
<tr>
<td>1</td>
<td>Good. Slight hazard due to toxicity</td>
<td>B</td>
<td>&lt; HC₅ (5) &gt; HC₁ (50)</td>
</tr>
<tr>
<td>2</td>
<td>Moderate. Moderate hazard due to toxicity</td>
<td>C</td>
<td>&lt; HC₅ (25) &gt; HC₅ (5)</td>
</tr>
<tr>
<td>3</td>
<td>Fair. High hazard due to toxicity</td>
<td>D</td>
<td>&lt; HC₅ (50) &gt; HC₅ (25)</td>
</tr>
<tr>
<td>4</td>
<td>Poor. Very high hazard due to toxicity</td>
<td>E</td>
<td>&lt; HC₅ (75) &gt; HC₅ (50)</td>
</tr>
<tr>
<td>5</td>
<td>Very Poor. Unacceptable hazard due to toxicity</td>
<td>F</td>
<td>&gt; HC₅ (75)</td>
</tr>
</tbody>
</table>
CHAPTER 4: RESULTS
4.1 TEST CONDITIONS AND CHARACTERIZATION

According to the standard guidelines, there should not be mortalities in the control organisms of more than 10% in order for the acute toxicity to be valid (OECD, 1992). This criterion was adhered to for all test implemented. Furthermore all the water quality parameters were in their specified ranges as prescribed by OECD (1992). The physicochemical properties of the test media are represented in Table 4.1. The measured pH ranged from 5.81 and 8.95. The Dissolved oxygen (DO) remained above 60% and ranged from 4.0 to 9.78 mg/l. The electrical conductivity ranged from 198 to 420 µS/cm for invertebrates and between 545 and 874 µS/cm for fish. The mean temperature of test solutions range from 19.3 to 23.9 °C depending on the species tested and the TDS ranged from 104 to 595 mg/l before and after experimental period for all toxicants tested. Although the concentration of DO drops below 5mg/l. The TDS and EC results for Daphnia is lower due to the smaller volume of solution tested. The primary water quality parameters for exposures and controls did not show great variance throughout exposure and remained within 10% of the initial values.

Table 4.1: Physicochemical water quality means for selected species in toxicity exposure bioassays with GNP, chloroauric acid and citrate buffer

<table>
<thead>
<tr>
<th>Species</th>
<th>pH (min-max)</th>
<th>D.O. (mg/l) (min-max)</th>
<th>O₂ (%) (min-max)</th>
<th>EC(µS/cm) (min-max)</th>
<th>Temp. (˚C) (min-max)</th>
<th>TDS (mg/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Citrate Buffer</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>D. pulex</td>
<td>7.85 (6.57-8.36)</td>
<td>5.43 (4.44-6.08)</td>
<td>64.6 (61.7-67.4)</td>
<td>339 (309-374)</td>
<td>20.5 (20-21.2)</td>
<td>171 (156-189)</td>
</tr>
<tr>
<td>D. magna</td>
<td>7.70 (7.6-8.37)</td>
<td>7.26 (6.63-8.72)</td>
<td>77.0 (62.6-91.4)</td>
<td>310 (297-362)</td>
<td>19.3 (20-21.9)</td>
<td>155 (148-181)</td>
</tr>
<tr>
<td>D. rerio</td>
<td>8.26 (7.10-8.65)</td>
<td>4.77 (4.0-6.15)</td>
<td>69.6 (60.0-87.2)</td>
<td>708 (621-809)</td>
<td>23.3 (22.7-23.9)</td>
<td>350 (309-398)</td>
</tr>
<tr>
<td>O. mossambicus</td>
<td>7.98 (7.39-8.34)</td>
<td>5.04 (4.26-5.9)</td>
<td>66.9 (60.2-78.08)</td>
<td>708 (530-841)</td>
<td>21.4 (20.6-22.1)</td>
<td>365 (321-407)</td>
</tr>
<tr>
<td>P. reticulata</td>
<td>7.74 (6.57-8.22)</td>
<td>4.85 (3.5-5.77)</td>
<td>67.7 (60.7-77.1)</td>
<td>748 (696-810)</td>
<td>22.2 (21.6-23.0)</td>
<td>373 (316-405)</td>
</tr>
<tr>
<td>P. philander</td>
<td>6.93 (6.01-7.75)</td>
<td>5.62 (4.3-6.47)</td>
<td>71.7 (60.89-89.5)</td>
<td>732 (665-874)</td>
<td>23.1 (22.9-23.2)</td>
<td>366 (333-402)</td>
</tr>
<tr>
<td>T. sparrmanni</td>
<td>6.57 (5.81-7.39)</td>
<td>4.85 (4.00-5.77)</td>
<td>67.9 (60.7-77.1)</td>
<td>674 (612-704)</td>
<td>22.1 (21.3-22.6)</td>
<td>372 (313-595)</td>
</tr>
<tr>
<td>L. aeneus</td>
<td>7.06 (5.99-7.93)</td>
<td>5.93 (4.89-7.07)</td>
<td>67.0 (60.3-80.5)</td>
<td>680 (638-729)</td>
<td>22.5 (21.5-23)</td>
<td>346 (324-369)</td>
</tr>
</tbody>
</table>
### Table 4.1: continued....

<table>
<thead>
<tr>
<th>Species</th>
<th>pH (min-max)</th>
<th>D.O. (mg/l) (min-max)</th>
<th>O₂ (%) (min-max)</th>
<th>EC(μS/cm) (min-max)</th>
<th>Temp. (°C) (min-max)</th>
<th>TDS (mg/l)</th>
</tr>
</thead>
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<tr>
<td><strong>GNP</strong></td>
<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>D. pulex</strong></td>
<td>7.66</td>
<td>5.05</td>
<td>63.1</td>
<td>328</td>
<td>20.7</td>
<td>167</td>
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<tr>
<td></td>
<td>(7.05-8.7)</td>
<td>(4.4-6.1)</td>
<td>(60.7-72.6)</td>
<td>(208-420)</td>
<td>(20-21.9)</td>
<td>(104-220)</td>
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<tr>
<td><strong>D. magna</strong></td>
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<td>7.23</td>
<td>78.4</td>
<td>309</td>
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<td>155</td>
</tr>
<tr>
<td><strong>D. rerio</strong></td>
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<td>4.49</td>
<td>68.1</td>
<td>674</td>
<td>22.6</td>
<td>336</td>
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<tr>
<td></td>
<td>(7.13-8.42)</td>
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<td>(642-765)</td>
<td>(22.4-23.9)</td>
<td>(321-412)</td>
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<td>4.68</td>
<td>63.9</td>
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<td>20.8</td>
<td>340</td>
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<td>(7.13-7.97)</td>
<td>(4.11-6.24)</td>
<td>(60.0-82.9)</td>
<td>(621-827)</td>
<td>(21.1-21.1)</td>
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<tr>
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<td>(6.31-8.19)</td>
<td>(4.84-8.26)</td>
<td>(60.0-79.6)</td>
<td>(658-824)</td>
<td>(21.3-22.6)</td>
<td>(329-412)</td>
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<td><strong>P. philander</strong></td>
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<td>73.9</td>
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<td>350</td>
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<td>(6.23-7.79)</td>
<td>(5.14-6.76)</td>
<td>(60.8-92.20)</td>
<td>(580-762)</td>
<td>(22.9-23.2)</td>
<td>(328-379)</td>
</tr>
<tr>
<td><strong>T. sparrmanni</strong></td>
<td>7.51</td>
<td>5.69</td>
<td>66.6</td>
<td>703</td>
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<td>335</td>
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<tr>
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<td>(6.85-8.01)</td>
<td>(4.37-8.26)</td>
<td>(60.7-79.6)</td>
<td>(617-754)</td>
<td>(21.2-22.7)</td>
<td>(308-378)</td>
</tr>
<tr>
<td><strong>L. aeneus</strong></td>
<td>7.55</td>
<td>5.55</td>
<td>55.9</td>
<td>556</td>
<td>22.9</td>
<td>275</td>
</tr>
<tr>
<td></td>
<td>(6.29-7.90)</td>
<td>(4.08-7.08)</td>
<td>(60.0-80.7)</td>
<td>(628-762)</td>
<td>(21.6-21.3)</td>
<td>(314-381)</td>
</tr>
</tbody>
</table>

### Chloroaeric acid

<table>
<thead>
<tr>
<th>Species</th>
<th>pH (min-max)</th>
<th>D.O. (mg/l) (min-max)</th>
<th>O₂ (%) (min-max)</th>
<th>EC(μS/cm) (min-max)</th>
<th>Temp. (°C) (min-max)</th>
<th>TDS (mg/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>D. pulex</strong></td>
<td>7.90</td>
<td>5.35</td>
<td>66.3</td>
<td>344</td>
<td>20.6</td>
<td>176</td>
</tr>
<tr>
<td></td>
<td>(6.93-8.95)</td>
<td>(4.35-6.13)</td>
<td>(62.7-71.5)</td>
<td>(198-382)</td>
<td>(20-22)</td>
<td>(104-267)</td>
</tr>
<tr>
<td><strong>D. magna</strong></td>
<td>8.22</td>
<td>7.57</td>
<td>83.3</td>
<td>332</td>
<td>21.6</td>
<td>168</td>
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<tr>
<td></td>
<td>(7.59-8.43)</td>
<td>(6.4-9.78)</td>
<td>(62.5-99.7)</td>
<td>(296-381)</td>
<td>(20.4-22.4)</td>
<td>(148-259)</td>
</tr>
<tr>
<td><strong>D. rerio</strong></td>
<td>7.92</td>
<td>4.98</td>
<td>72.0</td>
<td>677</td>
<td>23.0</td>
<td>350</td>
</tr>
<tr>
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<td>(6.24-8.67)</td>
<td>(4.2-6.044)</td>
<td>(62.8-85.3)</td>
<td>(621-739)</td>
<td>(21.8-23.7)</td>
<td>(311-485)</td>
</tr>
<tr>
<td><strong>O. mossambicus</strong></td>
<td>7.56</td>
<td>5.86</td>
<td>72.3</td>
<td>700</td>
<td>21.3</td>
<td>350</td>
</tr>
<tr>
<td></td>
<td>(6.45-7.98)</td>
<td>(4.23-7.85)</td>
<td>(60.2-84.8)</td>
<td>(620-806)</td>
<td>(20.6-21.6)</td>
<td>(310-403)</td>
</tr>
<tr>
<td><strong>P. reticulata</strong></td>
<td>7.33</td>
<td>5.63</td>
<td>73.6</td>
<td>711</td>
<td>21.7</td>
<td>355</td>
</tr>
<tr>
<td></td>
<td>(6.26-8.27)</td>
<td>(4.8-7.67)</td>
<td>(60.9-81)</td>
<td>(616-838)</td>
<td>(20.9-22.4)</td>
<td>(308-419)</td>
</tr>
<tr>
<td><strong>P. philander</strong></td>
<td>7.62</td>
<td>6.82</td>
<td>77.0</td>
<td>702</td>
<td>23.1</td>
<td>352</td>
</tr>
<tr>
<td></td>
<td>(7.36-8.04)</td>
<td>(4.49-8.26)</td>
<td>(62.1-99)</td>
<td>(603-736)</td>
<td>(22.3-23.5)</td>
<td>(301-400)</td>
</tr>
<tr>
<td><strong>T. sparrmanni</strong></td>
<td>7.24</td>
<td>5.61</td>
<td>73.4</td>
<td>633</td>
<td>21.6</td>
<td>388</td>
</tr>
<tr>
<td><strong>L. aeneus</strong></td>
<td>7.77</td>
<td>5.90</td>
<td>76.7</td>
<td>700</td>
<td>23.1</td>
<td>350</td>
</tr>
<tr>
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<td>(7.36-8.04)</td>
<td>(4.46-8.24)</td>
<td>(61.8-99.4)</td>
<td>(603-794)</td>
<td>(22.3-23.6)</td>
<td>(301-398)</td>
</tr>
</tbody>
</table>
4.2 NANOPARTICLES CHARACTERISATION

The zeta potential of the GNP stock solution was -33.5 mV with average diameter of 13.9 ± 2 nm (Mintek, Certification of analysis). Particle size and zeta potentials were determined at the onset and upon termination of each bioassay. Data are reported as the average of the replicates for each exposure concentration. The zeta potential results for all fish and *D. magna* is depicted in Table 4.2 and for *D. pulex* in Table 4.3. The general findings were that zeta potential decreased as the exposure period increased (i.e. as the GNPs aged). There was also a decrease in zeta potential with an increase in the GNP exposure concentration.

<table>
<thead>
<tr>
<th>Sample GNP</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>
<th>500 mg/l</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Zeta potential [mV]</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0 hr</td>
<td>-1.85 ± 0.59</td>
<td>-7.14 ± 0.23</td>
<td>-8.32 ± 0.35</td>
<td>-9.26 ± 0.39</td>
<td>-9.35 ± 0.70</td>
<td>-10.49 ± 0.67</td>
<td>-27.40 ± 0.53</td>
</tr>
<tr>
<td>48 hr</td>
<td>-9.52 ± 2.50</td>
<td>-11.13 ± 0.74</td>
<td>-13.53 ± 1.26</td>
<td>-11.40 ± 0.40</td>
<td>-4.40 ± 14.72</td>
<td>-13.30 ± 0.96</td>
<td>-28.60 ± 2.31</td>
</tr>
<tr>
<td>96 hr</td>
<td>-6.05 ± 0.49</td>
<td>-6.44 ± 0.58</td>
<td>-11.50 ± 0.52</td>
<td>-11.73 ± 0.21</td>
<td>-12.43 ± 0.85</td>
<td>-12.73 ± 0.12</td>
<td>-25.50 ± 2.57</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Sample GNP</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>
<th>500 mg/l</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Zeta potential [mV]</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0 hr</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>
<td>-14.77 ± 3.38</td>
</tr>
<tr>
<td>48 hr</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>
<td>-18.47 ± 1.07</td>
</tr>
</tbody>
</table>

As the stock solution was placed in exposure medium the particle size increased outside the nanoscale, i.e. >100 nm. The particle size then also increased over time (Tables 4.4 and 4.5). The zeta size data for all fish and *D. magna* are represented in Table 4.4. The data illustrate that the zeta size at the lowest concentrations (1 and 5 mg/l) over time gives a drastic increase in size. However, the size starts to decreases through the concentrations.
and then inverts the results towards the higher concentrations. The values for *D. pulex* is portrayed in Table 4.5. These values are similar to those of the concentrations in the hard reconstituted water but where the fish species concentrations have a drastic increase in size the particles in each concentration for *D. pulex* only have a slight increase in size and then a drastic decrease in size at 35 and 45 mg/l. For all the species the 500 mg/l (100 %) concentration seems to remain at a consistent size.

**Table 4.4: Particle Size of GNP in hard reconstituted water (Fish & *D. magna*)**

<table>
<thead>
<tr>
<th>GNP</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>
<th>500 mg/l</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Particle size [d.nm]</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0 hr</td>
<td>292.2 ± 76.0</td>
<td>785.5 ± 74.5</td>
<td>344.7 ± 11.6</td>
<td>385.5 ± 9.9</td>
<td>574.0 ± 4.3</td>
<td>584.9 ± 15.7</td>
<td>47.7 ± 2.1</td>
</tr>
<tr>
<td>48 hr</td>
<td>546.2 ± 68.3</td>
<td>1112.1 ± 151.0</td>
<td>345.4 ± 6.7</td>
<td>365.5 ± 8.6</td>
<td>388.1 ± 10.5</td>
<td>465.8 ± 24.9</td>
<td>63.3 ± 0.13</td>
</tr>
<tr>
<td>96 hr</td>
<td>722.3 ± 133.9</td>
<td>3586.0 ± 1374.1</td>
<td>389.0 ± 22.7</td>
<td>501.6 ± 17.2</td>
<td>342.2 ± 20.1</td>
<td>464.8 ± 25.9</td>
<td>36.3 ± 0.27</td>
</tr>
</tbody>
</table>

**Table 4.5: Particle Size of GNP in moderately-hard reconstituted water (D. pulex)**

<table>
<thead>
<tr>
<th>GNP</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>
<th>500 mg/l</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Particle size [d.nm]</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0 hr</td>
<td>76.4 ± 32.1</td>
<td>181.2 ± 29.3</td>
<td>148.3 ± 12.9</td>
<td>799.5 ± 16.8</td>
<td>1107.0 ± 25.5</td>
<td>1997.0 ± 123.0</td>
<td>61.4 ± 1.0</td>
</tr>
<tr>
<td>48 hr</td>
<td>119.8 ± 52.65</td>
<td>252.5 ± 141.5</td>
<td>221.1 ± 8.06</td>
<td>1061.0 ± 25.9</td>
<td>285.5 ± 55.6</td>
<td>3042.5 ± 375.5</td>
<td>60.0 ± 2.4</td>
</tr>
</tbody>
</table>

**Transmission electron microscopy (TEM)**

The TEM image of GNPs show spherical NPs (figure 4.1 and figure 4.2), it also illustrates that there was no agglomeration of GNP stock solution. Nanoparticles size ranges between 14 nm and 45 nm; most of the particles were in the 14 nm range. TEM results of 5 mg/l in Figure 4.2A demonstrate a string like agglomeration of NPs of all sizes. The 50 mg/l concentration in Figure 4.2B showed the clustering of particles thus showing an increase in agglomeration compared to the 5 mg/l concentration in moderately hard water.
Figure 4.1: Dispersion of the 14 nm GNP stock solution particles in the citrate buffer solution

Figure 4.2: Agglomeration of 5 mg/l (A) and 45 mg/l (B) GNP from stock solution in artificial moderately hard water

4.3 GOLDCONCENTRATIONS (IN BOTH IONIC AND NANO EXPOSURES)

Gold concentrations were measured in the different exposures after the 96 h exposure period. Measured Au concentrations for both the ionic and GNPs exposures in hard (Figures 4.3 and 4.5) and moderately-hard reconstituted water (Figures 4.4 and 4.6) were very similar to the nominal exposure concentrations.
Figure 4.3: Comparison between the nominal exposure chloroauric acid concentrations and the mean ± standard deviation of the actual measured concentrations (mg/l) in hard reconstituted water at the onset and termination of the bioassays.

Figure 4.4: Comparison between the nominal chloroauric acid exposure concentrations and the mean ± standard deviation of the actual measured concentrations (mg/l) in moderately hard reconstituted water at the onset and termination of the bioassays.
Figure 4.5: Comparison between the nominal exposure GNP concentrations and the mean ± standard deviation of the actual measured concentrations (mg/l) in hard reconstituted water at the onset and termination of the bioassays.

Figure 4.6: Comparison between the nominal exposure GNP concentrations and the mean ± standard deviation of the actual measured concentrations (mg/l) in moderately-hard reconstituted water at the onset and termination of the bioassays.
4.4 ACUTE TOXICITY

4.4.1 Test Organism responses to aqueous gold ions and GNP

The calculated LC\textsubscript{50} values together with the upper and lower 95 % confidence levels (CI) for the dispersant (citrate buffer), GNP, and chloroauric acid (ionic gold) for the eight test organisms are presented in Table 4.6. Less than 5 % mortality was observed in both the control and the dispersant groups.

Table 4.6: Median lethal concentrations for 50 % (LC\textsubscript{50}) effect and the corresponding 95 % confidence intervals for D. pulex, D. magna, D. rerio, O. mossambicus, P. reticulata, P. philander, T. sparrmanii, L. aeneus exposed to citrate buffer, GNP and chloroauric acid.

<table>
<thead>
<tr>
<th>Species</th>
<th>Duration/Endpoint</th>
<th>LC\textsubscript{50} (mg/l) (95 %CI)</th>
<th>NOEC</th>
<th>LOEC</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Mean Upper &amp; lower confidence limits (mg/l)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Citrate Buffer</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>D. pulex</td>
<td>48 h</td>
<td>&lt; 5 % mortality</td>
<td></td>
<td></td>
</tr>
<tr>
<td>D. magna</td>
<td>48 h</td>
<td>&lt; 5 % mortality</td>
<td></td>
<td></td>
</tr>
<tr>
<td>D. rerio</td>
<td>96 h</td>
<td>No Mortalities</td>
<td></td>
<td></td>
</tr>
<tr>
<td>O. mossambicus</td>
<td>96 h</td>
<td>No Mortalities</td>
<td></td>
<td></td>
</tr>
<tr>
<td>P. reticulata</td>
<td>96 h</td>
<td>&lt; 10 % mortality</td>
<td></td>
<td></td>
</tr>
<tr>
<td>P. philander</td>
<td>96 h</td>
<td>&lt; 10 % mortality</td>
<td></td>
<td></td>
</tr>
<tr>
<td>T. sparrmanii</td>
<td>96 h</td>
<td>&lt; 10 % mortality</td>
<td></td>
<td></td>
</tr>
<tr>
<td>L. aeneus</td>
<td>96 h</td>
<td>&lt; 10 % mortality</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GNP</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>D. pulex</td>
<td>48 h</td>
<td>75.314 *</td>
<td>1</td>
<td>10</td>
</tr>
<tr>
<td>D. magna</td>
<td>48 h</td>
<td>could not be calculated</td>
<td>0</td>
<td>5</td>
</tr>
<tr>
<td>D. rerio</td>
<td>96 h</td>
<td>could not be calculated</td>
<td>&lt;15</td>
<td>&gt;15</td>
</tr>
<tr>
<td>O. mossambicus</td>
<td>96 h</td>
<td>No Mortalities</td>
<td></td>
<td></td>
</tr>
<tr>
<td>P. reticulata</td>
<td>96 h</td>
<td>52.57 *</td>
<td>&lt;35</td>
<td>35</td>
</tr>
<tr>
<td>P. philander</td>
<td>96 h</td>
<td>could not be calculated</td>
<td>5</td>
<td>10</td>
</tr>
<tr>
<td>T. sparrmanii</td>
<td>96 h</td>
<td>12.083 *</td>
<td>5</td>
<td>25</td>
</tr>
<tr>
<td>L. aeneus</td>
<td>96 h</td>
<td>could not be calculated</td>
<td>1</td>
<td>5</td>
</tr>
</tbody>
</table>
No mortalities were recorded for the negative and solvent controls (i.e. mortalities were below 10 %). It was not possible to calculate LC<sub>50</sub> values for 48 h <i>D. pulex</i> and 96 h <i>D. rerio</i>, <i>O. mossambicus</i>, <i>P. philander</i>, and <i>L. aeneus</i> (Table 4.6). The LC<sub>50</sub> values for these test organisms were 0.01, 0.15, 4.85, 11.30, 20.58, 7.53, 10.78 and 0.93 mg/l for chloroauric acid respectively. For the GNP, out of the three species <i>T. sparrmanii</i> was the most sensitive aquatic organism tested (96 h LC<sub>50</sub> = 12.08 mg/l). For the chloroauric acid, <i>D. pulex</i> was the most sensitive aquatic organism tested (48 h LC<sub>50</sub> = 0.01 chloroauric acid mg/l). When looking at the LC<sub>50</sub> values of the entire test species it was evident that the species are much more sensitive to chloroauric acid than GNP. The toxicity of chloroauric acid was significantly greater (p<0.05) to <i>D. pulex</i> compared to all the species tested. Both the <i>Daphnia</i> species were significantly more sensitive to chloroauric acid than the fish species. For the fish species <i>L. aeneus</i>, <i>D. rerio</i> and <i>P. philander</i> were significantly (p < 0.05) more sensitive than the other species.

### Table 4.6: continued…

<table>
<thead>
<tr>
<th>Species</th>
<th>Duration/EndPoint</th>
<th>LC&lt;sub&gt;50&lt;/sub&gt; (mg/l) (95 %CI) Mean</th>
<th>Upper &amp; lower confidence limits (mg/l)</th>
<th>NOEC</th>
<th>LOEC</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>LC&lt;sub&gt;50&lt;/sub&gt; (mg/l) (95 %CI) Mean</td>
<td>Upper &amp; lower confidence limits (mg/l)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chloroauric acid</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;i&gt;D. pulex&lt;/i&gt;</td>
<td>48 h</td>
<td>0.01</td>
<td>0</td>
<td>&lt;0.0005</td>
<td>0.0005</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0.02</td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;i&gt;D. magna&lt;/i&gt;</td>
<td>48 h</td>
<td>0.15</td>
<td>0.29</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0.08</td>
<td>&lt; 0.005</td>
<td>0.01</td>
</tr>
<tr>
<td>&lt;i&gt;D. rerio&lt;/i&gt;</td>
<td>96 h</td>
<td>4.85</td>
<td>3.62</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>6.49</td>
<td>&lt;1</td>
<td>5</td>
</tr>
<tr>
<td>&lt;i&gt;O. mossambicus&lt;/i&gt;</td>
<td>96 h</td>
<td>11.30</td>
<td>15.89</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>8.03</td>
<td>&lt;1</td>
<td>5</td>
</tr>
<tr>
<td>&lt;i&gt;P. reticulata&lt;/i&gt;</td>
<td>96 h</td>
<td>20.58</td>
<td>24.15</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>17.54</td>
<td>&lt;10</td>
<td>10</td>
</tr>
<tr>
<td>&lt;i&gt;P. philander&lt;/i&gt;</td>
<td>96 h</td>
<td>7.53</td>
<td>8.18</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>6.94</td>
<td>&lt; 10</td>
<td>10</td>
</tr>
<tr>
<td>&lt;i&gt;T. sparrmanii&lt;/i&gt;</td>
<td>96 h</td>
<td>10.78</td>
<td>13.03</td>
<td>1.5</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>8.92</td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;i&gt;L. aeneus&lt;/i&gt;</td>
<td>96 h</td>
<td>0.93</td>
<td>1.08</td>
<td>&lt;1</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0.80</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*No Confidence levels could be calculated*
Table 4.7: Acute toxicity data from the published literature that was included in the derivation of SSDs for deriving the HAuCl$_4$ guideline value.

<table>
<thead>
<tr>
<th>Test species</th>
<th>Endpoint</th>
<th>Duration (h)</th>
<th>HAuCl$_4$ (mg/l)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Invertebrates</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>D. magna</em></td>
<td>LC$_{50}$</td>
<td>48</td>
<td>2</td>
<td>Li <em>et al.</em> (2010)</td>
</tr>
<tr>
<td><em>D. magna</em></td>
<td>LC$_{50}$</td>
<td>48</td>
<td>0.64</td>
<td>Nam <em>et al.</em> , (2014)</td>
</tr>
<tr>
<td><em>M. macrocopa</em></td>
<td>LC$_{50}$</td>
<td>48</td>
<td>0.62</td>
<td>Nam <em>et al.</em> , (2014)</td>
</tr>
<tr>
<td>Fish</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>T. arcticus</em></td>
<td>LC$_{50}$</td>
<td>96</td>
<td>14.4</td>
<td>Nam <em>et al.</em> , (2014)</td>
</tr>
<tr>
<td><em>O. mykiss</em></td>
<td>LC$_{50}$</td>
<td>96</td>
<td>10.7</td>
<td></td>
</tr>
<tr>
<td><em>O. kisutch</em></td>
<td>LC$_{50}$</td>
<td>96</td>
<td>14.1</td>
<td></td>
</tr>
</tbody>
</table>

Figure 4.7: Cumulative mortalities of test organisms exposed to GNP over 48 h (daphnids) and 96 h (fish).
The cumulative mortality rates of the organisms over the 72- and 96 h exposure periods for both GNP and ionic gold are depicted in Figures 4.7 and 4.8 respectively. The cumulative percentages are based on the mortalities recorded at all exposure concentrations during the particular exposure period and provide further indication of the sensitivity of the particular species. The low toxicity of GNP exposure is clearly visible (Figure 4.7) with only _T. sparrmanii_ displaying a 20% response to toxicity after 36-48 hr exposure and maximum mortality of 48% after 96 h. The rest of the species had a mortality rate lower than 10%.

Figure 4.8 indicates that for most species 50% mortality was reached between 12 h and 24 h with _O. mossambicus_, _D. rerio_ and _L. aeneus_ displaying the highest immortality of 57%, 68% and 78%. Both species of Daphnia show 75% mortality after 24 h.

**Lowest observed effect concentration and no effect concentration**

The NOEC and LOEC data were established from the mortality results and are depicted in Table 4.6. Following exposure to the dispersant, _D. rerio_ and _O. mossambicus_ showed no mortalities across all the concentrations, whereas the mortalities were below 10 % for the all
other species. The LOEC observed for GNP for *D. pulex, D. magna, D. rerio, P. reticulata, P. philander, T. sparrmanii* and *L. aeneus* were 10, 5, >15, 35, 25 and 5 mg/l respectively with *O. mossambicus* showing no mortalities at any concentration. No effect concentrations were seen at 1, 5, <15, <35 mg/l respectfully. The LOEC regarding the chloroauric acid were 0.0005, 0.01, 5, 5, 10, 10, 2 and 1 mg/l respectively for *D. pulex, D. magna D. rerio, and O. mossambicus*. *Poecilia reticulata* and *P. philander, T. sparrmanii and L. aeneus*. The NOEC for chloroauric acid were all below the LOEC data.

### 4.5 SPECIES SENSITIVITY DISTRIBUTIONS

Since it was not possible to calculate GNP LC$_{50}$ values for all species tested, an SSD was constructed based on the toxicity data that were determined. This is however not ideal since at least seven data points are required to generate a meaningful SSD for any given chemical (Wepener et al., 2006). The SSD for GNP was generated based on the limited acute toxicity data generated during this study and the few data published in literature (Figure 4.9).

Species sensitivity distributions were used to relate the toxicity of chloroauric acid to the different species tested locally and internationally. The SSD toxicity plots for chloroauric acid of all species in this study, from the published literature (Li et al., 2010; Nam et al., 2014) and of combined results are depicted in Figures 4.10, 4.11 and 4.12 respectively.
Figure 4.9: Species sensitivity distributions and confidence limits for combined data from this study and published literature (Li et al., 2010) and for GNP.

Figure 4.10: Species sensitivity distributions and confidence limits based on bootstrap regressions applied to chloroauric acid for invertebrates and fish species.
Figure 4.11: Species sensitivity distributions and confidence limits from published literature for HAuCl₄ (Nam et al., 2014).

Figure 4.12: Species sensitivity distributions and confidence limits for combined data of chloroauric acid from this study and published literature.

Figure 4.12: Species sensitivity distributions and confidence limits for combined data of chloroauric acid from this study and published literature.
The SSDs for GNPs and chloroaucric acid with their calculated hazardous concentrations and levels of assurance are represented in Tables 4.8 and 4.9 respectively. The SSD derived hazard concentrations and levels of assurance were then also related to ecological protection categories proposed by Ansara-Ross (2010).

With regards to the HC\(_p\) categories for the GNPs (Table 4.8) it can be seen that all the species from the combined data have a low sensitivity to GNPs with an HC\(_5\)\(_{50}\) of 42.78 mg/l. Natural levels are assumed at anything below 8mg/l and hazardous effect only occur from 18.4mg/l (category C) which is relatively a large quantity of GNPs. The HC\(_5\)\(_{50}\) values for chloroaucric acid (Table 4.9) showed that invertebrates from the local data (0.039 mg/l) were more sensitive than fish (6.633 mg/l). When comparing the HC\(_5\)\(_{50}\) values from the local data of all species (1.834 mg/l) to the data from the published literature (4.46 mg/l), it was obvious that the local species were more sensitive to chloroaucric acid than the international data.

**Table 4.8**: Predicted hazardous concentration and respective ecological categories (Ansara-Ross, 2010) based on the SSD for GNPs.

<table>
<thead>
<tr>
<th>Criteria 1: combined international and local data (Li et al., 2010)</th>
<th>HC(_p)</th>
<th>HC(_p) Categories</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>HC(_1)(50)</td>
<td>A</td>
<td>&lt;8.0</td>
</tr>
<tr>
<td>HC(_5)(5)</td>
<td>B</td>
<td>8.22</td>
</tr>
<tr>
<td>HC(_5)(20)</td>
<td>C</td>
<td>18.39</td>
</tr>
<tr>
<td>HC(_5)(50)</td>
<td>E</td>
<td>42.78</td>
</tr>
<tr>
<td>HC(_5)(95)</td>
<td>F</td>
<td>222.77</td>
</tr>
</tbody>
</table>
Table 4.9: Predicted hazardous concentration and respective ecological categories (Ansara-Ross, 2010) based on the SSDs for Chloroauric acid.

<table>
<thead>
<tr>
<th>HCp</th>
<th>Categories</th>
<th>Criteria 1: local data of all tested species</th>
<th>Criteria 2: local data of tested species</th>
<th>Criteria 3: all international data</th>
<th>Criteria 3: combined international and local data</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Fish &amp; Arthropod</td>
<td>Arthropod</td>
<td>Fish</td>
<td></td>
</tr>
<tr>
<td>n</td>
<td></td>
<td>8</td>
<td>2</td>
<td>6</td>
<td>5</td>
</tr>
<tr>
<td>HC₁(50)</td>
<td>A</td>
<td>&lt;0.01</td>
<td>&lt;0.001</td>
<td>&lt;0.9</td>
<td>&lt;0.3</td>
</tr>
<tr>
<td>HC₅(5)</td>
<td>B</td>
<td>0.015</td>
<td>0.001</td>
<td>0.965</td>
<td>0.33</td>
</tr>
<tr>
<td>HC₅(20)</td>
<td>C</td>
<td>0.157</td>
<td>0.007</td>
<td>2.474</td>
<td>0.294</td>
</tr>
<tr>
<td>HC₅(50)</td>
<td>E</td>
<td>1.834</td>
<td>0.039</td>
<td>6.633</td>
<td>4.46</td>
</tr>
<tr>
<td>HC₅(95)</td>
<td>F</td>
<td>224.511</td>
<td>1.052</td>
<td>45.576</td>
<td>153.5</td>
</tr>
</tbody>
</table>

Table 4.10: Median lethal concentrations for 15 % (LC₁₅) effect for D. pulex, D. magna, D. rerio, O. mossambicus, P. reticulata, P. philander, T. sparrmanii, L. aeneus exposed to GNP.

<table>
<thead>
<tr>
<th>Species</th>
<th>Duration/Endpoint</th>
<th>LC₅₀ (mg/l) (95 %CI)</th>
<th>Upper &amp; lower confidence limits (mg/l)</th>
<th>NOEC</th>
<th>LOEC</th>
</tr>
</thead>
<tbody>
<tr>
<td>GNP</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>D. pulex</td>
<td>48 h</td>
<td>75.314 *</td>
<td></td>
<td>1</td>
<td>10</td>
</tr>
<tr>
<td>D. magna</td>
<td>48 h</td>
<td>could not be calculated</td>
<td></td>
<td>0</td>
<td>5</td>
</tr>
<tr>
<td>D. rerio</td>
<td>96 h</td>
<td>could not be calculated</td>
<td></td>
<td>&lt;15</td>
<td>&gt;15</td>
</tr>
<tr>
<td>O. mossambicus</td>
<td>96 h</td>
<td>No mortalities</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P. reticulata</td>
<td>96 h</td>
<td>52.57 *</td>
<td></td>
<td>&lt;35</td>
<td>35</td>
</tr>
<tr>
<td>P. philander</td>
<td>96 h</td>
<td>could not be calculated</td>
<td></td>
<td>5</td>
<td>10</td>
</tr>
<tr>
<td>T. sparrmanii</td>
<td>96 h</td>
<td>12.083 *</td>
<td></td>
<td>5</td>
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CHAPTER 5: DISCUSSION
5.1 Characterisations

5.1.1 Particle charge - Zeta potential

The zeta potential of the 14 nm GNP stock solution was initially -33.0 mV (Mintek). This is in accordance with available literature (Pongsuchart et al., 2012) which states that the average zeta potential of 9 nm GNPs is -26.8 mV. Over time the zeta potential increased to average of -27.4 mV in the fish and D. magna exposures and increased even further to -14.77 mV in the D. pulex experiments. Even though the different GNP solutions were treated in a similar manner, i.e. prior to adding the stock to the specific exposure medium, it was agitated using a sonication bath. Therefore the sample was well dispersed prior to adding the relevant stock concentrations to the exposure media of the bioassays. The 500 mg/l stock solution remained quite stable and there were no major changes in the zeta potential. In a previous study by Hahn et al., (2012) the citrate buffer was found to be more stable than a cysteine buffer due to its high negative charge. Thus the citrate acts as a stabilizing agent. The reason for agglomeration of the solutions in the reconstituted media water can be attributed to the sodium citrate buffer, which is soluble in water, being converted to citric acid through hydrolysis reactions in water (Tabrizi et al., 2009).

Substances with negative zeta potentials are not likely to aggregate quickly as like charges repel one another. Negatively charged particles are therefore more stable due to their repulsion. Aggregation can be encouraged if a negative zeta potential was reduced to a more positive value (Weinberg et al., 2011). The changes in zeta potential observed during the bioassays could therefore be related to the different ionic strengths of the exposure media used for fish and invertebrates (Cai et al., 2008; Schrand et al., 2010). A change in aggregation (see Figure 4.2) may further be caused by a change in pH as a result of conductivity changes (Handy et al., 2011). The increased ionic strength of the exposure medium compared to the dispersion medium resulted in an increase in negative charge and subsequent increase in pH. The increased negative charge thus allows for more binding sites for the positively charged molecules on the surface of nanoparticle (Cai et al., 2008). Therefore at negative zeta potentials, the potential for aggregation decreases while aggregation increase as the zeta potential is increased to a more positive value (Weinberg et al., 2011). These results are in agreement with (Griffitt et al., 2008) who found that there was some degree of aggregation in moderately-hard freshwater for Ag, Cu, Al, Ni and both NPs and soluble salts as well as TiO₂ NPs.
5.1.2 Particle size - hydrodynamic size and surface morphology

Particle size has been found to have an effect on the stability of a solution. This stability can be changed by manipulating the particles on the surface of the molecule and the particle size has been found to influence the stability ratio of NPs (Karakoti et al., 2006). The hydrodynamic size is related to the zeta potential thus as size increases, the stability of the solution increases, and less agglomeration occurs (Alkilany & Murphy, 2010). For the GNP stock solution (500 mg/l) both the zeta potential and the hydrodynamic size remained very constant. The constant size may be attributed to the unstable state with resultant low agglomeration that the stock solution was in (i.e. zeta potential of -33 mV) since colloidal gold solutions are classified as being stable at either zeta potentials greater than +30 mV or less than -30 mV (Pongsuchart et al., 2012). However when transferred to the moderately-hard and hard exposure media, the hydrodynamic size changed from 1553 ± 408 to 292.2 ± 76.0 for hard exposure medium and 1078 ± 525.8 to 76.4 ± 32.1 for moderately-hard medium. The hard D. magna media demonstrated a small decrease in size, which shows a destabilization of particles but the zeta potential decreases indicating that limited agglomeration occurred. For the moderately hard reconstituted water there was an increase in size (stability) coupled to a greater decrease in the zeta potential.

Transmission electron microscope (TEM) micrographs are used to not only for visual imaging of NPs but also physical characterization of shape, size, and aggregation. The TEM images demonstrated well-defined spherical particle and showed that particles made with the citrate reduction and stabilization method were slightly larger than the defined nanoparticle size range (Figure 4.1 and 4.2). Gold nanoparticles in stock solution were mostly represented as single particles ranging in size of 18 nm to 37 nm. Following the dilution of the stock solution with reconstituted water the particles were found to agglomerate and particle size increased. The agglomeration is attributed to increased ionic strength (Farkas et al., 2010).

5.2 ACUTE TOXICITY

5.2.1 Gold nanoparticles

The results in this study show that GNPs is toxic to T. sparmannii P. reticulata and D. pulex at very high concentrations resulting in LC$_{50}$ values of 12.08, 52.57 and 75.31 mg/l. Due to low mortalities that were recorded for the other species, no LC$_{50}$s could be determined. For the purposes of this study LC$_{15}$ values were calculated for D. pulex, D. magna, D. rerio, P.
reticulata and T. sparrmanii (Table 4.10) to allow for the comparison of the relative toxicity. There were definite changes in behaviour and motility observed in each of the species that displayed higher mortalities during the experiments. The organisms swam erratically and often in a spiral pattern. The fish species became lethargic and were found stationary, but still ventilating, on the bottom of the test chamber. The LC<sub>50</sub> values for D. magna in studies on GNPs done by Li et al. (2010) were ≈70 mg/l. In this study the LC<sub>50</sub>s for D. pulex, i.e. 75.31 mg/l, were slightly higher but in general agreement with the results of Li et al. (2010). The GNPs seem to adhere to the appendages and ingested by the daphnids (Figure 5.1) thus potentially influencing their swimming behaviour. These observations are in agreement with studies conducted on carbon nanotubes and TiO<sub>2</sub> in D. magna (Klaine et al., 2008).

Some of the causes for daphnia mortality could be attributed to impaired natural malting cycles, which could shorten the daphnids lifespan and the inability of nutritional absorption from the gut due to the collection of GNP in the gut (Li et al., 2010; Klaine et al., 2008). The no- to very low toxicity observed for the fish species in this study is in accordance with the results of previous studies using GNPs with different capping agents (Asharani et al., 2011; Browning et al., 2009; Farkas et al., 2010; Furgeson et al., 2009). However, Klaine et al. (2008) found that exposure of D. rerio to Cu nanoparticles resulted in a 48-h LC<sub>50</sub> of 1.5 mg/l.

Other studies on metal-based NPs also indicated that these materials do exhibit acute toxicity, e.g. Ni NPs of 30, 60, and 100 nm had the same or less toxicity than soluble Ni in zebra fish (Ispas et al., 2009). Fifteen to 35 nm GNPs were nontoxic to mice (Asharani et al., 2011; Chen et al., 2008; Schrand et al., 2010). CNPs of 17 µm were nontoxic to mice (Schrand et al., 2010). No obvious acute toxicity was observed for CuO NPs to Cyprinus carpio and CeO<sub>2</sub> NPs showed no obvious toxicity to zebra fish embryos at 200 mg/l (Zhao et al., 2011). These results indicate that certain metal-based NPs such as Ag, fullerenes C60 in D. magna zinc oxide in zebra embryo (Krysanov et al., 2010) and D. magna (Heinlaan et al., 2008) and CuO NPs in human lung tissue (Zhao et al., 2011) are indeed toxic. The toxicity of these NPs was attributed to the dissolution of the free metal ion from the particle (Bilberg et al., 2010; Griffitt et al., 2008). Farkas et al. (2010) proposed that ionic Au<sup>3+</sup> (i.e. chloroaauric acid) is absorbed faster into rainbow trout hepatocytes than GNPs. The very low toxicity of the GNPs could therefore be related to the lack of dissolution of the Au ion from the particle.
Exposure to chloroauric acid resulted in acute toxicity in all the species tested. The LC$_{50}$s of *D. pulex*, *D. magna* and *L. aeneus* were very low i.e. below 0.93 mg/l, indicating that these species were the most sensitive. *Poecilia reticulata* was the least sensitive with a LC$_{50}$ of 20.58 mg/l thus proving that *P. reticulata* was the least sensitive species to ionic gold. The only species that had a similar vulnerability were *O. mossambicus* and *T. sparrmanii* with an LC$_{50}$ around 11 mg/l. It can be observed in these results that the invertebrates were more sensitive to gold ions than the fish species. Studies by Li *et al.* (2010) showed that daphnids had toxicity at 2 mg/l in chloroauric acid, which was lower than the LC$_{50}$s recorded for daphnids in this study. The toxicity results from this study compared to toxicity data reported by Nam *et al.* (2014) illustrate that the LC$_{50}$s for invertebrates are much lower in this study (<0.2 mg/l) than in the literature (0.64 mg/l). *Danio rerio*, *O. mossambicus* and *T. sparrmanii* have LC$_{50}$s of 4.85, 11.3 and 10.78 mg/l which is similar to *Thymallus arcticus* and *Oncorhynchus mykiss* with LC$_{50}$s of 4.1 and 10.7 mg/l (Nam *et al.*, 2014).

### 5.3 Species Sensitivity Distributions

Several methods can be used to calculate an SSD such as log-normal, log-logistic or Burr Type III (Ansara-Ross, 2010; Palmer *et al.*, 2004a; Wheeler *et al.*, 2002) and bootstrapping regression (Wepener *et al.*, 2006) methods. Species Sensitivity Distributions can also be derived from the numerous results of toxicity test from a toxicity database (Palmer *et al.*, 2004a). Ansara-Ross (2010) used the Burr Type III method to derive SSDs and confidence limits based on local and international data from numerous databases for pesticides (deltamethrin, dichlorvos and endosulfan). Browne (2005) extrapolated data from different
toxicological databases to determine the SSDs for NaCl and Na₂SO₄. Brix et al., (2001) estimated the SSDs for chronic distribution of copper from an acute distribution using a chemical specific acute–chronic ratio (ACR); this is calculated by “dividing a species’ acute LC₅₀ by the geometric mean of the chronic no-observed-effect concentration and lowest-observed-effect concentration”. The protective boundaries are derived from the SSDs automatically. Browne, (2005) suggested that “an important component for deriving SSDs is having large sets of data with which to work”. In this study the CADDIS_SSD US EPA software was used to ascertain numerous levels of protective concentrations HC₅₀s and related confidence levels. The median (HC₅₀) endpoints for GNPs and chloroaauric acid were acquired from the distribution models constructed with a collection of species. The CADDIS_SSD distribution fits the most frequent distribution, the log-probit, to toxicity data (US EPA, 2012).

Nam et al. (2014) used the reliability method of Australia and New Zealand to determine the SSD and HC₅₀ values for Au³⁺. Three methods are used to derive the Australian and New Zealand trigger guidelines which are based on the HC₅₀ levels. These methods are calculated from a number of variables; namely by using SSDs of chronic NOEC data, by division of acute LC₅₀ data from a 5 % SSD with ACR values to calculate the concentration, and by dividing the lowest chronic NOEC or LC₅₀ data with the assessment factor (AF) to determine the concentrations.

The HC₅₀(50) values for chloroaauric acid (Au ³⁺) from this study (Table 4.9) show that invertebrates from this study (0.039 mg/l) were more sensitive than fish (6.633 mg/l). When comparing the HC₅₀ (5) and HC₅₀ (50) values of the data for all species from this study with the international data shows that the local species are more sensitive (LC₅₀s between 0.015 and 1.834 mg/l) than the international species (LC₅₀s between 0.33 and 4.46 mg/l). There is an expected increase in sensitivity for both HC₅₀ (5) and HC₅₀ (50) when combining the local and international data. While the combined data showed similar sensitivities to invertebrates and fish, the local species was negligibly more sensitive for both groups of species. The hazardous concentrations with a 50 % certainty (HC₅₀, 50) for the local and combined local and international data were 1.834 and 2.442 mg/l respectively. Thus far there have not been any studies on NPs that have made use of SSDs to determine the protective values. The results of LC₅₀ or EC₅₀ on NPs are values that are often collated in review papers (Handy et al., 2011) and integrated using nonlinear regression techniques (Farkas et al., 2010).
Nam et al., (2014) proved that a SSD approach can be used to determine guideline values for Au$^{3+}$, this is verified by the information collected in this paper as HC$_5$ and HC$_{50}$ values were determined with the use of a SSD method. The HC$_5$ and HC$_{50}$ results for ionic gold of this paper (0.015mg/l and 1.834mg/l) are similar but much lower than the literature (Nam et al., 2014) values (0.33mg/l and 4.46mg/l), which can be attributed to the different species used. The information in Nam et al., (2014) is only on ionic gols and there is no information on the SSD of GNP. However, when comparing the HCp for the combined data of the ionic gold and GNPs it can be determined that GNP are less toxic than ionic gold with HC$_5$ and HC$_{50}$ values of 0.04mg/l and 2.24mg/l for ionic gold and 8.22 and 42.78mg/l for GNPs. This data illustrates the hazardous concentrations of GNP are much higher than ionic gold. From the information collected in this study and Nam et al., (2014) it can be said that SSD can be used to determine water quality guidelines for both ionic gold and GNPs using invertebrates and Chordata as indicators. However there is a major gap in information and more data needs to be collected before toxicity guidelines maybe constructed.
CHAPTER 6: CONCLUSION
6.1 Conclusion

The purpose of this study was to utilize acute toxicity tests to establish if GNP and ionic gold are toxic to indigenous and standard toxicity testing species. Moreover, it was aimed to determine if these species would be good representatives to compare the relative sensitivity to the two forms of gold. The attained results present new data on the toxicity of gold nanoparticles and ionic gold. The high sensitivities of *D. pulex, D. magna* and *L. aeneus* make them good test subjects for monitoring and other ecotoxicological analysis of ionic gold. To put the toxicity of an ionic gold into perspective potassium permanganate was used as the reference toxicant. Potassium permanganate is toxic in water at 0.18 mg/l, the concentrations of ionic gold in each species show a much higher concentration therefore ionic gold is more toxic compared to potassium permanganate.

Thus the hypothesis that GNPs are more toxic than ionic gold was rejected. Since the GNP have no to low toxicity, the toxicity data from this study were interpreted based on LC\textsubscript{15} values and resulted in HC\textsubscript{50} values of 40.05 mg/l and 395.51 mg/l for invertebrates and fish respectively. The HC\textsubscript{50} results of the ionic gold calculated from the LC\textsubscript{50} values show that the invertebrates and fish were 0.039 mg/l and 6.633 mg/l respectively.

The second hypothesis, that there is no difference in the toxicity of GNPs among the species tested is not accepted. The differences between species are presented in Tables 4.8 and 4.9. Single-species acute toxicity tests do not incorporate the interactions that environmental factors may have on toxicants. However the application of SSDs provides a better interpretation of acute toxicity data than the single species tests. The results obtained from this study show that the use of acute toxicity tests with indigenous species can be used in enhancing the ecological risk assessment guide for NPs, as well as being beneficial in including other contaminants such as NPs in South African water quality guidelines.

6.2 Recommendations

Toxicity of GNPs was only shown for three species indicating that under the conditions selected for this study rendered them non-toxic. For the purposes of this study the toxicity of
GNPs with a citrate capping was tested. The capping agent plays a major role in the characteristics of nanoparticles and the chemistry of the capping agent should be understood before being able to understand the properties of nanoparticles themselves (Ju-Nam & Lead, 2008). In light of this, extensive research of concentrations and capping agents of nanoparticles should be carried out before one can come close to understanding nanoparticles and their behaviour. The current assumption that toxicity is expressed due to a dose-response relationship needs to be explored further.


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