UNIVERSITY *of the*
WESTERN CAPE

Potamanautes warreni biomarker assays to monitor silver nanomaterial contaminants in aquatic environments

By

Chavon Rene Walters
MSc (Environmental Sciences)

Submitted in fulfilment of the requirements for the degree

DOCTOR OF PHILOSOPHY

in the

Department of Medical Biosciences

Faculty of Science

University of the Western Cape

February 2016

Supervisors: **Prof Edmund Pool**
Dr Vernon Somerset

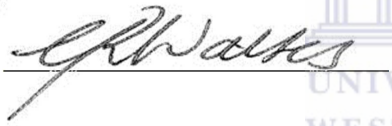
Final Copy

Declaration

I declare that:

Potamanautes warreni biomarker assays to monitor silver nanomaterial contaminants in aquatic environments is my own work, that is has not been submitted for any degree or examination in any other university, and that all the sources I have used or quoted have been indicated and acknowledged by complete references.

Chavon R. Walters

Signature: 

Date: February 2016

Summary

There has been extensive growth in nanoscale technology in the last few decades to such a degree that nanomaterials (NMs) have become a constituent in a wide range of manufactured commercial and domestic products. This surge has resulted in uncertainties regarding their environmental impact, due to the significant increases in the amount of NMs released into the environment (Dowling *et al*., 2004) through intentional and unintentional releases. Like many other toxins, the aquatic environment is particularly vulnerable as it acts as a sink for nanoparticles (NPs) (Scown *et al*., 2010). The escalating growth of NMs has not advanced without efforts to understand its properties. Despite the dramatic advances in both the production and application of NMs, very little is known regarding their interaction with and effects on environmental and human health. Given the lack in scientific knowledge, particularly under various environmental conditions, it is often difficult to accurately assess the potential exposure pathways to ecological receptors.

Of all NMs, silver nanoparticles (AgNPs) are the most widely used NPs, present in several consumer products mainly because of their anti-bacterial properties. It is estimated that the annual production exceeds 1000 tons/year (Piccinno *et al*., 2012). The increase uses of AgNPs in consumer products (*e.g.* textiles, cosmetics and personal hygiene), household appliances (*e.g.* washing machines and vacuum cleaners) and medical equipment have led to their increase release into the environment, thereby posing an environmental risk and human health concern. Silver NPs are known to induce the production of Reactive Oxygen Species (ROS) (Ahamed *et al*., 2010; Levard *et al*., 2012; Piao *et al*., 2011). Also since AgNPs are oxidized to ionic Ag (Ag^+), it is still unclear whether the effects of ROS can be attributed to Ag^+ release or to the AgNP itself (Fabrega *et al*., 2009; Miao *et al*., 2009).

The behaviour of AgNPs is collectively influenced by inherent (nanoparticle size, shape, surface area, surface charge, crystal structure, coating, solubility/dissolution) and environmental factors (temperature, pH, ionic strength, salinity, organic matter). Climate change predictions indicate that the frequency, intensity and duration of extreme natural events (such as temperature elevations) will increase in the future (IPCC, 2001; IPCC, 2007). Global warming and climate change could increase atmospheric temperatures by 2.4 – 6.4 °C (IPCC, 2001; IPCC, 2007). The main feature associated with global climate change is the anticipation of wetter winters (*i.e.* increased flood events) and drier, warmer summers (*i.e.* extreme temperatures). These changes are likely to affect the inputs of contaminants into the environment as well as affect their behaviour, fate and transport, and toxicity in aquatic

environments. It is known that the current temperature predictions in climate change scenarios could directly affect aquatic ecosystem communities (Carpenter *et al*., 1992), since temperature is also regarded as an important abiotic factor influencing growth and production of primary producers (*i.e.* algae, macrophytes *etc*.), and may also affect species distribution. For example, Liu *et al*. (2010) reported higher dissolution rates of AgNPs with increased temperature. Similarly, sudden hydrographic activity like high flood conditions may cause resuspension and redistribution of sediments.

Few studies have linked the foreseeable climate change with contaminant release and ecosystem impacts. Similarly, few studies have analyzed the behaviour of NMs in the environment considering these predicted changes in mean temperatures. This thesis focuses on the effects of AgNPs on oxidative stress responses in the Cape River crab *Potamonautes perlatus*. The present work was undertaken to interpret the biological effects of AgNPs (< 100 nm) on *P. perlatus*, as well as to assess its effects under different environmental conditions. To understand the uptake, accumulation and biological effects of AgNPs, freshwater microcosms were produced to mimic a typical aquatic environment and temperature manipulated microcosms to which a commercially-available AgNP powder was added. Nanoparticles were characterized in the dry state and in suspension under different environmental conditions. Dissolution of total Ag was measured by inductively coupled plasma mass spectrometry (ICP-OES). Nanoparticle toxicity was assessed by measuring mortality and biomarkers of oxidative stress (CYP450, SOD, CAT, GST) evaluated in crab tissues. The overall results demonstrated that: (1) AgNPs may be transformed in both size and state under variable environmental conditions. The formation of smaller aggregates at higher temperatures suggests higher toxicity, (2) the release of free metal ions from NPs and NPs aggregates contribute to a higher toxicity towards aquatic organisms, (3) oxidative stress is a significant mechanism of AgNP toxicity and consequently enzymatic activation/ inhibition with increasing AgNP concentration and temperatures, (4) oxidative stress responses to AgNPs particles were significantly modulated by temperature stress in *P. perlatus*, (5) mortality was observed from day 2 with maximum mortality achieved at day 7, (6) enzymes involved in detoxification, *i.e*. CYP450, has functional significance in the haemocytes, (7) *P. perlatus* has proved to be a significant target for AgNP exposure and, furthermore, has proved to be a suitable species to assess the ecotoxicity of AgNP in the aquatic environment, (8) antioxidant enzymes activities (are valuable tools to assess the oxidative status of crab tissues co-exposed to AgNPs and temperature. Furthermore, the results obtained in this study contributed to the understanding of the behaviour,

bioavailability, uptake and toxicity of AgNPs under variable temperatures. Recommendations for future work are given in Chapter 6.

References

Ahamed, M., Posgai, R., Gorey, T.J., Nielsen, M., Hussain, S.M., Rowe, J.J., 2010. Silver nanoparticles induced heat shock protein 70, oxidative stress and apoptosis in *Drosophila melanogaster*. Toxicol. Appl. Pharmacol. 242, 263-269.

Carpenter, S.R., Fisher, S.G., Grimm, N.B., and Kitchell, J.F., 1992. Global Change and Freshwater Ecosystems. Ann. Rev. Ecol. Syst. 23, 119–139.

Dowling, A.P., 2004. Development of nanotechnologies. Mat. Today. 7, 30–35.

Fabrega, J., Fawcett, S.R., Renshaw, J.C., Lead, J.R., 2009. Silver nanoparticle impact on bacterial growth: Effect of pH, concentration, and organic matter. Environ. Sci. Technol. 43, 7285–7290.

Fabrega, J.; Luoma, S.N.; Tyler, C.R.; Galloway, T.S.; Lea, J.R., 2011, Silver nanoparticles: behaviour and effects in the aquatic environment. Environ. Int. 37, 517–531.

IPCC (Intergovernmental Panel for Climate Change), 2001. Third assessment report of the working group I. In: Houghton, J.T., *et al*. (Eds.), The Science of Climate Change. Cambridge University Press, Cambridge, United Kingdom and New York, NY, USA.

IPCC (Intergovernmental Panel for Climate Change), 2007. Climate change 2007: the physical basis – summary for policy makers. In: Solomon, S., D. Qin, M. Manning, Z. Chen, M. Marquis, K.B. Averyt, M. Tignor and H.L. Miller (eds.). Fourth Assessment Report of the Intergovernmental Panel on Climate Change. Cambridge University Press, Cambridge, United Kingdom and New York, NY, USA.

Levard, C., Hotze, E.M., Lowry, G.V. and Brown, G.E., 2012. Environmental Transformations of Silver Nanoparticles: Impact on Stability and Toxicity. Environ. Sci. Technol. 46, 6900–6914.

Liu, H.P., Chen, F.Y., Gopalakrishnan, S., Qiao, K., Bo, J., Wang, K.J., 2010. Antioxidant enzymes from the crab *Scylla paramamosain*: gene cloning and gene/protein expression profiles against LPS challenge. Fish Shellfish Immunol. 28, 862–871.

Miao, A.J., Schwehr, K.A., Xu. C., Zhang, S.J. and Luo, Z.P., 2009. The algal toxicity of silver engineered nanoparticles and detoxification by exopolymeric substances. Environ. Poll. 157, 3034–3041.

Piao, M.J., Kang, K.A., Lee, I.K., Kim, H.S., Kim, S., Choi, J.Y., Choi, J., Hyun, J.W., 2011. Silver nanoparticles induce oxidative cell damage in human liver cells through inhibition of reduced glutathione and induction of mitochondria-involved apoptosis. Toxicol. Lett. 201, 92-100.

Piccinno, F., Gottschalk, F., Seeger, S., Nowack, B., 2012. Industrial production quantities and uses of ten engineered nanomaterials in Europe and the world. J. Nanoparticle Res. 14, 1109-1119.

Scown, T.M., Santos, E., Johnston, B.D., Gaiser, B., Baalousha, M., Mitov, S., Lead, J.R., Stone, V., Fernandes, T.F., Jepson, M., van Aerle, R., Tyler, C.R., 2010. Effects of Aqueous exposure to silver nanoparticles of different sizes in rainbow trout. Toxicol. Sci. 115(2), 521–534.

Dedication

This dissertation is dedicated to my family - **my husband, my son, my parents** - for their tremendous love and support.

Acknowledgement

I wish to thank all the many people that helped me during the course of my PhD research. Without their support none of this would have been possible:

- I want to thank my supervisor, **Prof. Edmund Pool (UWC)** for giving me the opportunity to undertake this PhD research under his supervision. His guidance and knowledge has assisted me throughout this research.
- My co-supervisor, **Dr Vernon Somerset (CSIR)** for his scientific advisement, research guidance and encouragement throughout my PhD. I'm delighted having him as a colleague and mentor, and I have learned a lot in these few years.
- I am indebted to **Dr Paul Cheng** for his guidance and support to carry out my research. I am grateful to him for imparting his knowledge and for his suggestions during the final part of my research, his kind help for providing financial support and the necessary infrastructure to carry out the research work.
- I wish to thank my lab mate **Dewald Schoeman** for his assistance for the most part of this research. We had some crazy weeks dissecting crabs, processing tissues and reading samples. #spanwerk
- I am grateful to **Christoff Truter** and **Charlton van der Horst** for always being willing to assist me with crab collection.
- I am grateful to **Sebastian Brown, Charney Anderson** and other members of the **CAS team in Stellenbosch** for assisting with the metal analyses.
- I gratefully acknowledge the financial support from the **NRF Thuthuka Fund** and other support granted by the **CSIR**.
- I also thank my **family** for their love and support. I could never have received an advanced education without the assistance that **my parents** have provided to me throughout my life. Also, **my husband Garth and son Hayden**, who have been a continual source of strength and motivation to me. Special thanks to my husband for enduring the highs and lows.
- I wish to thank the anonymous **examiners** for their helpful comments on this work.

Acronyms and Abbreviations

Ag	Silver
Ag^+	Ionic silver
AgNP	Silver nanoparticle
ANOVA	Analysis of variance
BET	Brunauer, Emmet and Teller
BSA	Bovine serum albumin
CAT	Catalase
CYP450	Cytochrome P450
DNA	Deoxyribonucleic acid
DO	Dissolved oxygen
ENM	Engineered nanomaterial
EDX	Energy-Dispersive X-ray
GPX	Glutathione peroxidase
GR	Glutathione reductase
GSH	Glutathione
GSSG	Glutathione disulphide
GST	Glutathione s-transferase
H_2O	Water
H_2O_2	Hydrogen peroxide
ICP-MS	Inductively coupled plasma mass spectrometry
ICP-OES	Inductively coupled optical emission spectrometry
LPO	Lipid peroxide
NADP+	Nicotinamide adenine dinucleotide phosphate
NADPH	Nicotinamide adenine dinucleotide phosphate reduced
NM	Nanomaterial
NP	Nanoparticle
$\bullet O_2^-$	Superoxide anion
O_2	Oxygen
•OH	Hydroxyl radicals
OECD	Organization for Economic Co-operation and Development
OM	Organic matter
PXRD	Powder x-ray diffraction

ROS	Reactive oxygen species
SEM	Scanning electron microscopy
SOD	Superoxide dismutase
TEM	Transmission electron microscopy
US EPA	United States Environmental Protection Agency

List of Publications and Presentations

Publications

- Walters, C., Pool, E. and Somerset, V., 2014. Ecotoxicity of silver nanomaterials in the aquatic environment: A review of literature and gaps in nano-toxicological research, Journal of Environmental Science and Health, Part A: Toxic/Hazardous Substances and Environmental Engineering, 49:13, 1588-1601, DOI: 10.1080/10934529.2014.938536.
- Walters, C., Pool, E. and Somerset, V., 2013. Aggregation and dissolution of silver nanoparticles in a laboratory-based freshwater microcosm under simulated environmental conditions, Toxicological & Environmental Chemistry, DOI: 10.1080/02772248.2014.904141.
- Walters, C., Pool, E. and Somerset, V., 2015. Effect of temperature on oxidative stress and enzymatic activity in the tissues of *Potamonautes perlatus* following exposure to silver nanoparticles. Accepted by Journal of Toxicology and Environmental Health, Part A: Current Issues, DOI: 10.1080/15287394.2015.1106357.
- Walters, C., Cheng, P., Anderson, C., Pool, E. and Somerset, V., 2015. Combined silver nanoparticles and temperature effects in the Cape River crab *Potamonautes perlatus* - interactions between chemical and climate stressors. Submitted to Aquatic Toxicology.
- Somerset, V., Van der Horst, C., Silwana, B., Walters, C. and Iwuoha, E., 2015. Biomonitoring and evaluation of metal concentrations in sediment and crab samples from the North-West Province of South Africa. Water Air Soil Pollut. 226, 43.

Book Chapters

- Walters, C., Pool, E., and Somerset, V. (2015) Nanotoxicity in Aquatic Invertebrates (2015) In: Larramendy, M.L. and Soloneski, S. (eds) Invertebrates - Experimental Models In Toxicity Screening. Intech, Croatia, ISBN 978-953-51-4625-4.

Book Chapters in Preparation

- Walters, C., Pool, E., and Somerset, V. (2016) The freshwater crab *Potamonautes perlatus* (Malacostraca, Potamonautidae) as model in nanotoxicity studies In:

Larramendy, M.L. (eds) Non-Conventional Animal Models in Ecotoxicological and Genotoxicological Studies. Royal Society of Chemistry, Cambridge, UK.

Oral and Poster Presentations at Conferences

- Walters, C., Pool, E. and Somerset, V., 2013. Physico-chemical and morphological characterization of silver nanoparticles – an ecosystem perspective 19th Biennial International Symposium for Toxicity Assessment (ISTA), Cape Town, 21-26 February 2013.
- Walters, C., Pool, E. and Somerset, V., 2013. Effect of changing environmental conditions on silver nanoparticle aggregation and dissolution. SETAC Europe 23st Annual Meeting held in Glasgow, Scotland 12-16 May 2013.
- Walters, C., Somerset, V. and Pool, E., 2014. Antioxidant responses in the freshwater crab (*Potamonautes perlatus*) exposed to silver nanoparticles. 17th International Conference on Heavy metals in the Environment (ICHMET), Guiyang, China 22-26 September, 2014.
- Walters, C., Somerset, V. and Pool, E., 2015. Effects of nano-stressors on oxidative stress in crustaceans: enzyme activity in the tissues of *Potamonautes perlatus*. SETAC Europe 25th Annual Meeting held in Barcelona, Spain, 3-7 May 2015.
- Walters, C., Somerset, V. and Pool, E, 2015. **Abstract accepted** to SETAC Africa. Meeting to be held in Langebaan, South Africa (October 2015).
- Walters, C., Somerset, V. and Pool, E, 2016. **Abstract accepted** to 11th International Symposium on Ecohydraulics. Meeting to be held in Australia (February 2016).
- Walters, C., Pool, E. and Somerset, V. 2016. **Abstract submitted** to SETAC Europe. Meeting to be held in Nantes, France (May 2016).

Table of Contents

List of Figures

List of Tables

List of Equations

Permissions List for the Use of Copyrighted Materials

Figure 2.3-A: Reprinted from Water Research, 44(20), Choi, O., Yu, C-P., Fernandez, E. and Hu. Z, Interactions of nanosilver with Escherichia coli cells in planktonic and biofilm cultures, Pages 9, Copyright (2010), with permission from Elsevier.

Figure 2.3-B: Reprinted from Journal of Cluster Science, 16(1), Glaspell, G.P., Surface enhanced raman spectroscopy using silver nanoparticles: the effects of particle size and halide ions on aggregation, Pages No., Copyright (2005), with permission from Springer.

Figure 2.3-C: Reprinted from Ecotoxicology and Environmental Safety, 78, Pham, C.H., Yi, J. and Gu, M.B., Biomarker gene response in male Medaka (*Oryzias latipes*) chronically exposed to silver nanoparticle, Pages 7., Copyright (2012), with permission from Elsevier.

Figure 2.3-D: Reprinted from Chemical Physics Letters, 511(4-6), Saini, R.K., Srivastava, A.K., Gupta, P.K. and Das, K., pH dependent reversible aggregation of Chitosan and glycol-Chitosan stabilized silver nanoparticles, Pages 5, Copyright (2011), with permission from Elsevier.

Figure 2.4: Reprinted from Journal of Nanobiotechnology, 10(14), Asghari, S., Johari, S.A., Lee, J.H., Kim, Y.S., Jeon, Y.B., Choi, H.J., Moon, M.C. and Yu, I.J., Toxicity of various silver nanoparticles compared to silver ions in *Daphnia magna*, Pages 11, Copyright (2014), with permission from Springer.

Figure 2.5: Reprinted from ACS Nano, 1(2), Lee, K.J., Nallathamby, P.D., Browining, L.M., Osgood, C.J. and Xu, X-H.N., In vivo imaging of transport and biocompatibility of single silver nanoparticles in early development of zebrafish embryos, Pages 11, Copyright (2007), with permission from American Chemical Society.

CHAPTER 1: INTRODUCTION, THESIS STRUCTURE AND OBJECTIVES

1. Introduction

Of all the nanomaterials (NMs), silver nanoparticles (AgNPs) represent the largest and fastest growing category of all NMs (approximately 23%) (www.nanotechproject.org), with 8.00 – 799 tons/year produced worldwide (Piccinno *et al.* (2012). When AgNP is discarded, it can enter the environment as aggregates and soluble ions, which can be highly toxic to aquatic organisms. The dissolution of AgNPs is a significant process determining AgNPs effects in the aquatic environment and its organisms. Although environmental concentrations of AgNPs have not been determined, it is estimated that more than 15% of Ag released into waters will come from plastics and textiles containing AgNPs (Blaser *et al.*, 2008). In addition, it is predicted that concentrations of AgNPs in natural waters range from 0.03 to 500 ng/L (Luoma, 2008). A fundamental question is whether AgNPs remains in the particle phase in the environment following dissolution, or whether it poses an additional risk.

In aquatic ecosystems, the cumulative effects of various environmental stressors that act on similar pathways (such as oxidative stress) can confound the assessment responses to NPs. NMs are affected by abiotic factors such as organic matter (OM), solution ionic strength, pH, temperature, concentrations of ligands and other environmental variables which alter their chemistry and influence their fate in the environment. There is a general consensus that climate change is a global challenge for the 21st century. Global warming and climate change could increase atmospheric temperatures by 2.4 – 6.4 °C (IPCC, 2007). The main feature associated with global climate change is the anticipation of wetter winters and drier, warmer summers. These changes are likely to affect the inputs of contaminants into the environment as well as affect their behaviour, fate and transport in aquatic environments. It is known that the current temperature predictions in climate change scenarios could directly affect aquatic ecosystem communities (Carpenter *et al.*, 1992), since temperature is also regarded as an important abiotic factor affecting physiological functions of aquatic organisms. For example, Liu *et al.* (2010) reported higher dissolution rates of AgNPs with increased temperature. Similarly, sudden hydrographic activity like high flood conditions may cause resuspension and redistribution of sediments. Temperature is also known to have an effect on biological responses. Studies have shown that elevated temperatures could enhance the negative impacts

of pollutants in bivalves, particularly as the temperatures approach the upper tolerance limits (Sokolova and Lannig, 2008).

Few studies have linked the foreseeable climate change with contaminant release and ecosystem impacts. Although several studies have analyzed the behaviour of NMs in the environment, understanding of NP effects on aquatic organisms is hindered by the scarcity of studies of the effects in the environmentally relevant context of multiple stress exposures (Falfushynska *et al.*, 2015). This thesis focuses on the combined effects of AgNPs and temperature on oxidative stress biomarkers in the freshwater crab *P. perlatus* using conventional biomarkers of oxidative stress (SOD, CAT, GST *etc*). Patterns of distribution and modes of action of AgNPs are also assessed.

1.1.Oxidative stress

Oxidative stress is defined as an imbalance between the production of reactive oxygen species (ROS) and the cells' ability to reduce ROS, which may be as a result an increased ROS production, a decrease in the cell's defence mechanisms, or a combination of both. Disturbances in the normal redox state of cells may cause toxic effects through the production of peroxides and free radicals that in turn damage cells, including proteins, lipids, and DNA. Because certain reactive oxidative species act as cellular messengers in redox signalling, oxidative stress may lead to disruptions in normal mechanisms of cellular signalling. ROS refers to oxygen free radicals, partially reduced intermediates of the four electron reduction of oxygen to water, *i.e.* superoxide anions ($\bullet O_2^-$), hydroxyl radicals (•OH) and the non-radical active species hydrogen peroxide (H_2O_2). Aerobic organisms, which derive their energy from the reduction of oxygen, are particularly susceptible to the damaging actions of the small quantities of $\bullet O_2^-$, •OH and H_2O_2 that form during the metabolism of oxygen (Chitra and Sajitha, 2014).

Biomarkers of oxidative stress can offer an early warning sign for exposure to xenobiotics. Biomarkers such as enzyme activity are widely used for environmental monitoring. Measurements in this category range from markers related to redox status (*e.g.* superoxide dismutase (SOD activity), reproduction-associated proteins (*e.g.* vitellogenin), and stress response pathways (*e.g.* antioxidant responses and heat shock protein) (Falfushynska *et al.*, 2015). Figure 1.1 represents a schematic of the major oxidative pathways. A brief description of the oxidative stress biomarkers used in this study follows in section 1.2.

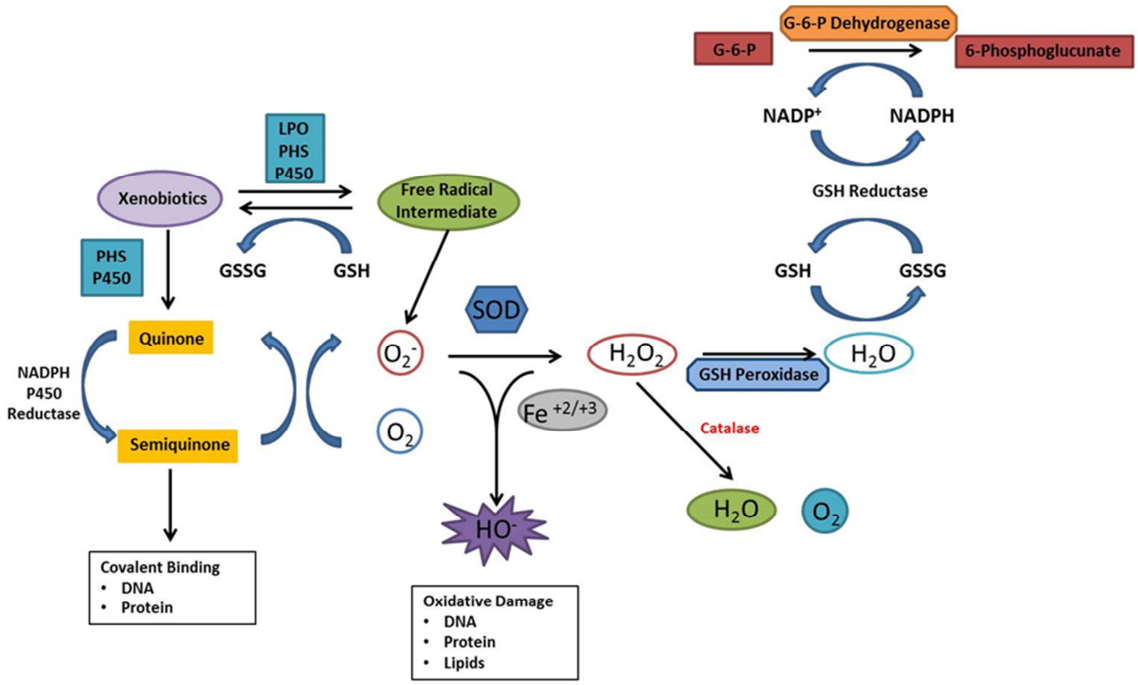


Figure 1.1: Schematic diagram of oxidative stress (adapted from www.sigmaaldrich.com).

1.2. Antioxidant defence system

A number of defence mechanisms have evolved to provide a balance between production and removal of ROS. Cells have a variety of elaborate defence mechanisms to restore the harmful effects of ROS. The removal of foreign substances (xenobiotics) from cells is catalyzed by several enzymes, particularly Phase I and Phase II enzymes. Phase I enzymes initiate the detoxification process by chemically transforming lipid soluble compounds into water soluble compounds in preparation for Phase II detoxification (Bucheli and Fent, 1995; Equation 1.1). These include the cytochrome P450 (CYP450) enzymes which are responsible for most Phase I reactions. CYP450 are typically found in the membranes of the endoplasmic reticulum (microsomes) within liver cells (hepatocytes). Activity of Phase I enzymes can typically lead to an increase in ROS production. Antioxidant enzymes facilitate the removal of these resulting ROS molecules and reactive chemical intermediates. The action of CYP enzymes results in the production of O_2 which consequently can be metabolized by SOD to hydrogen peroxide (H_2O_2) and oxygen (O_2) (Equation 1.2) which can in turn be reduced to water (H_2O) and O_2 by CAT (Equation 1.3) or glutathione peroxidase (GPx) (Equation 1.4) (Almroth, 2010).

$$H + O_2 + 2e^- + 2H^+ \rightarrow OH + H_2O$$

Equation 1.1: The cytochrome P450 (CYP450) function.

$$2O_2^- + 2H^+ \rightarrow H_2O_2 + O_2$$

Equation 1.2: The superoxide dismutase (SOD) function.

$$2H_2O_2 \rightarrow 2H_2O + O_2$$

Equation 1.3: The catalase (CAT) function.

$$2GSH + H_2O_2 \rightarrow GSSG + 2H_2O$$

Equation 1.4: The glutathione peroxidase (GPx) function.

Non-enzymatic antioxidants also play a role in detoxification. The tripeptide Glutathione exists as reduced glutathione (GSH) and oxidized glutathione (GSSG). Reduced glutathione (GSH) is a major tissue antioxidant that provides reducing equivalents for the GPx catalyzed reduction of lipid hydroperoxides to their corresponding alcohols and H_2O_2 to H_2O. When cells are exposed to increased levels of oxidative stress, GSSG accumulates and the GSSH:GSH ratio increases. This increased ratio of GSSG-to-GSH is indicative of oxidative stress. The reaction catalyzed by glutathione peroxidase requires GSH as substrate and is determined by the ratio of GSSG:GSH. This ratio is an indication of the redox state of cells (Kil and Park, 2006) and is important to ROS detoxification.

2. Uptake and accumulation of silver nanoparticles

Once introduce into aquatic ecosystems, the fate and transport of AgNPs and its uptake by aquatic biota depend on several factors. NP properties (such as size, shape and coatings), and water chemistry (such as dissolved organic carbon, ionic strength, pH, temperature) will largely influence the extent to which these particles will either remain in suspension, partition to dissolved organic carbon in the water column, form aggregates, and adsorb to suspended particles (USEPA, 2012).

In aquatic organisms, the major routes of entry are via ingestion or direct passage across the gill and other external surface epithelia. Recent studies with *Daphnia magna* have indicated that AgNPs may be internalised by these routes (Asghari *et al.*, 2012). At the cellular level, internalisation of NP occurs via endocytosis. Mechanisms of cellular uptake of NPs are

described in Figure 1.2. Three main mechanisms are responsible for NP uptake: phagocytosis, macropinocytosis and receptor-mediated endocytosis (Lorenz *et al.*, 2006). During phagocytosis (a specific form of endocytosis) particles are taken up the invagination of the plasma membrane. Jayaseelan *et al.*, (2014) showed internalization of nickel NPs in Mozambique tilapia *(Oreochromis mossambicus)*, demonstrating the feasibility of uptake via this route. Macropinocytosis involves the internalization of a larger area of membrane. Other forms of endocytosis include clathrin- and receptor-mediated endocytosis. Nanoparticles can also enter cells by diffusion or transport through the cell membrane, resulting in particles located freely in the cytoplasm (Moore, 2006).

The accumulation of NPs by aquatic organisms is dependent on both the uptake and the elimination (detoxification) of the NP out of the organism (Zhao and Wang, 2010). Processes which regulate the bioaccumulation (and bioavailability) of AgNPs include: the concentration of the AgNP, the physico-chemical properties the AgNP, the characteristics of the environment such as abiotic factors, the route of exposure, the biology and functional ecology of the organism involved, and exposure duration (Fabrega *et al.*, 2011).

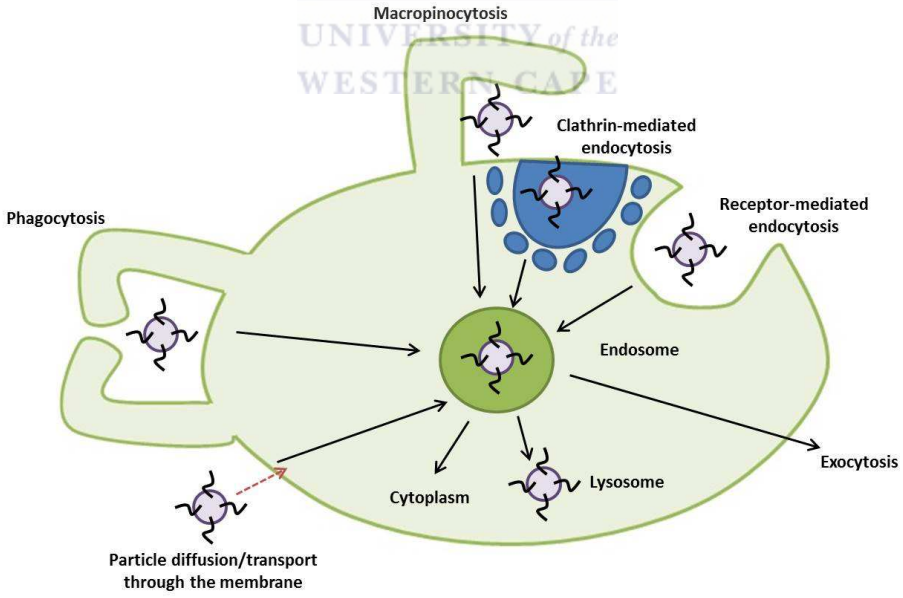


Figure 1.2: Mechanisms of cellular uptake of NPs (adapted from Wimpenny *et al.*, 2012).

3. Crustaceans and exposure routes in ecotoxicology

Potamonautes is a genus of freshwater crabs in the family Potamonautidae. They represent a fairly common species with the widest distribution in sub-Saharan Africa, and are widespread under boulders in the middle and lower reaches of rivers and other freshwater water courses. As with other crustaceans, they have a segmented body with a rigid exoskeleton and jointed limbs, and an open vascular system in which numerous haemocytes freely circulate in haemolymph. The colour of the Cape River crab *P. perlatus* can vary from dark brown to mottled green. Freshwater crabs typically have nine pairs of gills which lie in the two branchial chambers of the carapace (Cumberlidge, 1999). The digestive system is basically composed of a foregut, midgut and hindgut. The foregut is comprised of a mouth, oesophagus and stomach, while the midgut is composed of an anterior and posterior caecum and midgut gland (hepatopancreas). The hindgut is a simple straight tube, which finishes at the anus. The reproductive system is very simple consisting of paired gonads that open onto the ventral surface of the trunk (Cumberlidge, 1999).

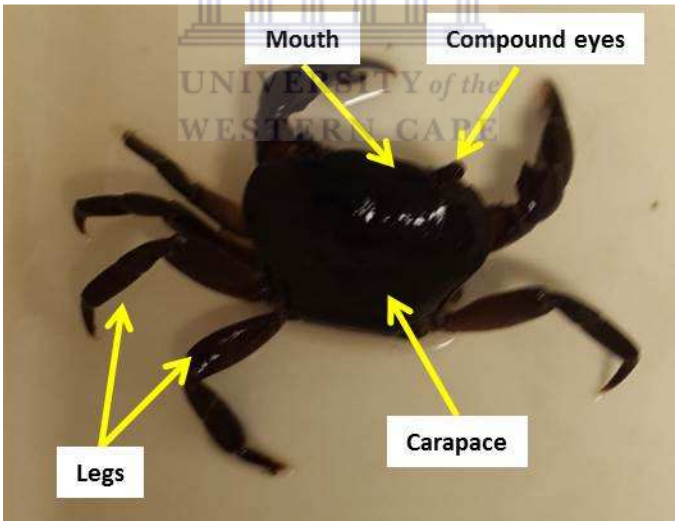


Figure 1.3: Dorsal view of *Potamonautes perlatus* representative of all crab samples collected and used in this study.

Crustaceans show a high sensitivity to environmental stressors (Qin *et al.*, 2012) and are therefore found to be useful bioindicators for monitoring the pollution state in aquatic environments (Issartel *et al.*, 2010). Contaminated ecosystems induce deleterious effects on aquatic organisms. In crustaceans, the exposure routes are mainly via ingestion and

adsorption to surface epithelia such as the gills. As an example, de Freitas Rebelo *et al.* (2000) reported histopathological effects in the gills (disruption of pilaster cells and collapse of gill lamellae) of the estuarine crab *Chasmagnathus granulate* following exposure to ammonia. In addition, NPs that are taken up via ingestion through the digestive tract may accumulate in the hepatopancreas (Lee, 2001). Nanoparticles may also diffuse into the circulating haemolymph and settle in a target organ.

4. Aims and Objectives

The use of NMs in consumer products and their potential environmental and human health risks are of increasing concern. As nanotechnologies and products increase, so nano-products entering the aquatic ecosystems and other water sources too will increase, thereby increasing the potential threat to aquatic organisms. With this said, the objective of this work was to investigate biomarkers in the Cape River crab *Potamonautes perlatus* exposed to AgNPs taking into account the effects of increasing temperatures associated with climate change.

- **Aim 1:** The initial aim of this project was to investigate the behaviour, aggregation and sedimentation of AgNPs in a freshwater microcosm under ambient conditions. (Chapter 3).
- **Aim 2:** Secondly, this work aimed to investigate the behaviour, aggregation and sedimentation of AgNPs in a freshwater microcosm under different temperature regimes. This aim will assess the effects of increased temperatures associated with changing climatic conditions. (Chapter 3).
- **Aim 3:** Thirdly, this thesis will evaluate the toxicological and biochemical effects of AgNPs in *P. perlatus* and the potential modulation of these effects by temperature. (Chapter 4 and 5).

The potential implications and effects of nanotechnology and NMs on environmental and human health is an important issue of global concern. The focus of the proposed research is to investigate the effects of AgNPs when exposed to simulated climate changes, thus mimicking the conditions experienced naturally in the environmental during potentially extreme conditions. Research areas which this proposal aims to address include NP fate and transport, bioavailability and ecotoxicology (or nano-ecotoxicology). Towards this end, the research proposed herein will take on the following major objectives:

- To investigate the behaviour, fate and transport, and bioavailability of NMs in a freshwater microcosm under ambient conditions (Chapter 3).
- To investigate the behaviour, fate and transport, and bioavailability of NMs in a freshwater microcosm when exposed to variable climate change drivers (Chapter 3).
- To determine the antioxidant defence responses of *P. perlatus* to AgNPs and simulated climate change drivers (Chapters 4 and 5).

The possible research question this proposed research study could address includes:

- What are the behaviour, fate and transport of AgNP under variable environmental conditions?
- What are the appropriate biomarkers which can be used to evaluate the potential nano-ecotoxicological effects of AgNPs?
- What are the potential toxicological effects of AgNPs to *P. perlatus*?
- Can temperature modulate the effects of AgNPs stress responses of *P. perlatus*?

The ecotoxicity of commonly-used metal NPs (*i.e.* AgNPs) was chosen as the test NMs principally due to its widespread industrial and commercial application in their bulk and nanoparticle forms. Limited data is available on the behaviour of AgNPs in the aquatic environment, particularly under extreme environmental conditions. As such, this approach will facilitate the understanding of AgNPs and its properties that lead to toxicity under variable temperature conditions, and thereby, also enhance the domain of environmental biomarkers. The outcome will thus enable researchers to predict the toxicological effects of AgNPs with the intent of guiding its development, application and regulation. This will be important when considering measures for exposure control and environmental remediation of AgNPs. Furthermore, this study seeks to contribute towards the safe, responsible and sustainable exploitation of nanotechnology capabilities by quantifying the potential risks that are associated with them.

5. Thesis structure

The main focus of this thesis was to fill the gap in nanotoxicity by studying the effects of a commercially available metal-NPs used in a several industrial applications and consumer products, AgNPs, using the Cape River crab *Potamonautes perlatus* as a bioindicator species.

In addition, the effects of co-exposure to temperature are also assessed. Toxicity, Ag uptake and bioaccumulation in tissues of *P. perlatus*, together with their biochemical effects through the use of conventional biomarkers are investigated. To achieve this, the thesis has four main chapters, three of which are manuscripts already published (Chapters 2, 3 and 4) and one which has been submittted to a peer reviewed journal (Chapter 5). The dissertation thus contains six chapters including an introduction (Chapter 1), literature review (Chapter 2), and conclusions and recommendations for further work (Chapter 6). Experimental work, data analysis and write-up were undertaken by the student. Contributions from supervisors included corrections and editing. Other individual's contributions are acknowledged at the end of each chapter.

Details for each chapter are given below:

Chapter 1: Introduction, thesis structure and objectives

This first chapter presents an introduction to nanomaterials, freshwater crabs, oxidative stress, biomarkers and the antioxidant defence system. This chapter also describes the structure of the thesis and what each chapter entails. The aims and objectives of this study are also highlighted.

Chapter 2: Ecotoxicity of silver nanomaterials in the aquatic environment: A review of literature and gaps in nano-toxicological research

This second chapter reviews the available research pertaining to nanomaterials and specifically, AgNPs in the aquatic environment (in plants, aquatic invertebrates and fish), and the use of crabs in biomarkers studies. The chapter concludes with providing recommendations on future nano-toxicological studies using invertebrates. This chapter was published in Journal of Environmental Science and Health, Part A: Toxic/Hazardous Substances and Environmental Engineering (2014) volume 49: 13 by C. R. Walters, with co-authors E. J. Pool and V. S. Somerset.

Chapter 3: Aggregation and dissolution of silver nanoparticles in a laboratory-based freshwater microcosm under simulated environmental conditions

This chapter investigates the effects of AgNPs in its natural state (dry powder form) and in solution under different environmental conditions. It uses a method to determine whether

AgNPs would form aggregates, or behave as isolated particles, or dissolve when in aqueous media and under different environmental conditions. This chapter was published in Toxicological & Environmental Chemistry volume 95: 10 by C. R. Walters, with co-authors E. J. Pool and V. S. Somerset.

Chapter 4: Effect of temperature on oxidative stress parameters and enzyme activity in the tissues of *Potamonautes perlatus* following exposure to silver nanoparticles

This chapter is aimed as assessing the toxicity of AgNPs in *P. perlatus* and to determine whether co-exposure to common environmental stressors (*i.e*. elevated temperature) modulates cellular responses to and toxicity of AgNPs. The identification of target organs of AgNPs toxicity was assessed. Antioxidant enzyme responses (superoxide dismutase (SOD) and catalase (CAT)), and the non-enzymatic antioxidant (glutathione S-transferase (GST)) were assessed in the gills and hepatopancreas of crabs exposed to concentrations (10 and 100 µg/mL) of AgNPs for seven days. This chapter has been accepted for publication by Journal of Toxicology and Environmental Health, Part A: Current Issues. Article DOI: 10.1080/15287394.2015.1106357.

Chapter 5: Combined silver nanoparticles and temperature effects in the Cape River crab Potamonautes perlatus - interactions between chemical and climate stressors

With the intent to elucidate the mechanisms of uptake, target tissues and toxicity of AgNPs in *P. perlatus*, assays using environmental realistic concentrations of AgNPs were conducted. The influence of AgNPs and temperature stress in toxicity and oxidative stress responses (CYP450, SOD, CAT and GST) were investigated in the gills, hepatopancreas, haemolymph, haemocytes and muscles. This Chapter has been submitted to Journal of Toxicology and Environmental Health, Part A: Current Issues.

Chapter 6: Conclusions and recommendations

This chapter highlights the main findings of the research undertaken, and also gives recommendations for future work.

6. References

Almroth, B.C., 2010. Oxidative Damage in Fish Used as Biomarkers in Field and Laboratory Studies (Ph.D.thesis).University of Gothernburg, (74p.)

Asghari, S., Johari, S.A., Lee, J.H., Kim, Y.S., Jeon, Y.B., Choi, H.J., Moon, M.C. and Yu, I.J., 2012. Toxicity of various silver nanoparticles compared to silver ions on Daphnia magna. J. Nanobiotech. 10, 14–34.

Blaser, S.A., Scheringer, M., MacLeod, M.; Hungerbühler, K., 2008. Estimation of cumulative aquatic exposure and risk due to silver: Contribution of nano–functionalized plastics and textiles. Sci. Tot. Environ. 390, 396–409.

Bucheli, T.D. and Fent, K., 1995. Induction of cytochrome P450 as a biomarker for environmental contamination in aquatic ecosystems. Crit. Rev. Environ. Sci. Technol. 25, 201-268.

Carpenter, S.R., Fisher, S.G., Grimm, N.B and Kitchell, J.F., 1992. Global change and freshwater ecosystems. Annu. Rev. Ecol. Syst. 23, 119–139.

Chitra, K.C. and Sajitha, R., 2014. Effect of bispenol-a on the antioxidant defense system and its impact on the activity of succinate dehydrogenase in the gill of freshwater fish, *Oreochromis mossambicus*. J. Cell Tiss. Res. 14(2), 4219-4226.

Cumberlidge, N., 1999. The freshwater crabs of West Africa. Family Potamanautidae. IRD, 382p.

De Freitas Rebelo, M., Rodriguez, E.M., Santos, E.A. and Ansaldo, M., 2000. Histopathological changes in gills of the estuarine crab *Chasmagnathus granulata* (Crustacea-Decapoda) following acute exposure to ammonia. Comp. Biochem. Physiol. C. 125, 157–164.

Fabrega, J., Luoma, S.N., Tyler, C.R., Galloway, T.S. and Lead, J.R., 2011. Silver nanoparticles: behaviour and effects in the aquatic environment. Environ. Int. 37, 517-513.

Falfushynska, H., Gnatyshyna, L., Yurchak, I., Sokolova, I. and Stoliar, O., 2015. The effects of zinc nanooxide on cellular stress responses of the freshwater mussels *Unio tumidus* are modulated by elevated temperature and organic pollutants. Aquat. Toxicol. 162, 82-93.

IPCC (Intergovernmental Panel on Climate Change), 2007. Climate change 2007: the physical science basis. In: Solomon S, Qin D, Manning M, Chen Z and others (eds)

Contribution of Working Group I to the 4th assessment report of the Intergovernmental Panel on Climate Change. Cambridge University Press, Cambridge

Issartel, J., Boulo, V., Wallon, S., Geffard, O., Charmantier, G., 2010. Cellular and molecular osmoregulatory responses to cadmium exposure in *Gammarus fossarum* (Crustacea, Amphipoda). Chemosphere. 81, 701–710.

Jayaseelan, C., Rahuman, A.A., Ramkumar, R., Perumal, P., Rajakumar, G., Kirthi, A.V., Santhoshkumar, T. and Marimuthu, S., 2014. Effect of sub-acute exposure to nickel nanoparticles on oxidative stress and histopathological changes in Mozambique tilapia, *Oreochromis mossambicus*. Ecotoxicol. Environ. Saf. 107, 220–228.

Kil, I.S. and Park, J-W., 2005. Regulation of mitochondrial NADP+-dependent isocitrate dehydrogenase activity by glutathionylation. J. Bio. Chem. 280, 10846-10854.

Lee, R.F., 2001. Bioavailability, biotransofrmtion and fate of organic contaminants in estuarine animals. In: Coastal and Estuarine Risk Assessment, Eds. Newman, M.C., Roberts, M.H. Jr and Hale, R.C. Lewis Publishers, CRC Press, p368

Liu, H.P., Chen, F.Y., Gopalakrishnan, S., Qiao, K., Bo, J. and Wang, K.-J., 2010. Antioxidant enzymes from the crab *Scylla paramamosain*: gene cloning and gene/protein expression profiles against LPS challenge. Fish Shellfish Immunol. 28, 862–871.

Lorenz, M.R., Holzapfel, V., Musyanovych, A., Nothelfer, K., Walther, P. and Frank, H., 2006. Uptake of functionalized, fluorescent-labeled polymeric particles in different cell lines and stem cells. Biomaterials. 27, 2820–8.

Luoma, S.N., 2008. Silver Nanotechnologies and the Environment: Old Problems or New Challenges. Project on Emerging Nanotechnologies. Publication 15. Woodrow Wilson International Centre for Scholars and PEW Charitable Trusts, Washington, DC.

Moore, M.N., 2006. Do nanoparticles present ecotoxicological risks for the health of the aquatic environment? Environ. Int. 32, 967–976.

Piccinno, F., Gottschalk, F., Seeger, S., Nowack, B., 2012. Industrial production quantities and uses of ten engineered nanomaterials in Europe and the world. J. Nanopart. Res. 14, 1109-1119.

Qin, Q., Qin, S., Wang, L. and Lei, W., 2012. Immune repsonses and ultrastructural changes of hemocytes in freshwater crab *Sinopotamon henanense* exposed to elevated cadmium. Aquat. Toxicol. 106-107, 140-146.

Sokolova, I.M., Lannig, G., 2008. Interactive effects of metal pollution and temperature on metabolism in aquatic ectotherms: implications of global climate change. Clim. Res. 37, 181–201.

USEPA (United States Environmental Protection Agency), 2012. Nanomaterial Case Study: Nanoscale Silver in Disinfectant Spray. EPA/600/R-10/081F.

Wimpenny, I., Markides, H. and El Haj, A.J., 2012. Orthopaedic applications of nanoparticle-based stem cell therapies. Stem Cell Research & Therapy, 3:13

Zhao, C.M., Wang, W.X., 2010. Biokinetic uptake and efflux of silver nanoparticles in *Daphnia magna*. Environ. Sci. Technol. 44, 7699-7704.

CHAPTER 2: NANOMATERIALS IN THE AQUATIC ENVIRONMENT - A REVIEW OF LITERATURE AND GAPS IN NANO-TOXICOLOGICAL RESEARCH

This chapter was published as an article in Journal of Environmental Science & Health, Part A: Toxic/Hazardous Substances and Environmental Engineering on August 2014 copyright Taylor & Francis, available online: http://www.tandfonline.com/ Article DOI: 10.1080/10934529.2014.938536

Abstract

There has been extensive growth in nanoscale technology in the last few decades to such a degree that nanomaterials (NMs) have become a constituent in a wide range of commercial and domestic products. With NMs already in use in several consumer products, concerns have emerged regarding their potential adverse environmental impacts. Although research has been undertaken in order to minimise the gaps in our understanding of NMs in the environment, little is known about their bioavailability and toxicity in the aquatic environment. Nano-toxicology is defined as the study of the toxicity of nanomaterials (Klaine *et al*., 2012). Nano-toxicology studies remain poorly and unevenly distributed. To date, most of the research undertaken has been restricted to a narrow range of test species such as daphnids. Crabs are bio-indicators that can be used for toxicological research on NMs since they occupy a significant position in the aquatic food chain. In addition, they are often used in conventional ecotoxicological studies due to their high sensitivity to environmental stressors and are abundantly available. Because they are benthic organisms they are prone to contaminant uptake and bioaccumulation. To our knowledge the crab has never been used in nano-toxicological studies. In this context, an extensive review on published scientific literature on the ecotoxicity of silver NPs (AgNPs) on aquatic organisms was conducted. Some of the most common biomarkers used in ecotoxicological studies are described. Emphasis is placed on the use of biomarker responses in crabs as monitoring tools, as well as on its limitations. Additionally, the gaps in nano-toxicological research and recommendations for future research initiatives are addressed.

Keywords: Biomarkers; crabs; ecotoxicity; nanomaterials; *Potamonautes perlatus*; silver nanoparticles.

1. Introduction

The advancements of nanotechnology in the last few decades have seen NMs become a constituent in a wide range of manufactured commercial and domestic products. Nanoparticles have unique properties, (such as a high specific surface area and mobility); however, those unique properties could potentially lead to unanticipated environmental health hazards.

Nanomaterials are currently applied to several commercially available products. Between 2005 and 2010, the engineered NMs (ENMs) list increased linearly by over 520%, with more than 1300 products registered (Figure 2.1). Similarly, reported revenues for nanotechnology were approximately US $ 1545 million in 2009, and is expected to increase to approximately US $ 5335 million by 2015 (Peralta-Videa *et al*., 2011). An online inventory of nanotechnology-based consumer products lists silver NPs (AgNPs) as the largest group, making up over 55 % of all NPs produced worldwide (Figure 2.2). Silver NPs are widely used in several consumer products including personal care products, laundry additives, home appliances, paints and textiles (Maynard *et al*., 2006). As such it is likely that AgNPs will be released into the aquatic environment, where it will be a source of Ag exerting toxic effects to aquatic organisms.

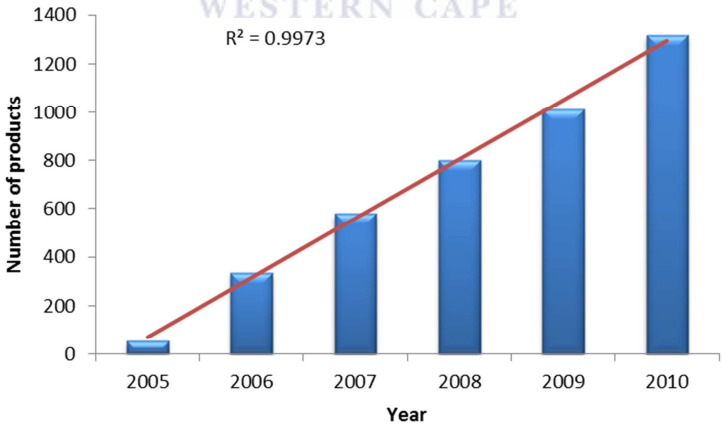


Figure 2.1: Nanomaterial growth trend 2005–2010 (Project on Emerging Nanotechnologies).

Aquatic ecosystems are progressively coming under pressure, largely due to the presence of anthropogenic contaminants posing health hazards to inhabitant organisms. Nanomaterials are introduced into the aquatic environment through several sources, such as solid, liquid and atmospheric emissions from industrial activity, runoff from domestic sources, and accidental

spillages. Although some studies have reported on the transport and fate of NMs in aquatic ecosystems (Klaine *et al*., 2008), the effects of NMs in the environment under different conditions are not well understood. In aquatic systems, NPs form colloidal suspensions that aggregate. In the aquatic environment NMs are generally associated with sediments Klaine *et al*., 2008). Consequently, NPs may be available for ingestion by aquatic organisms or direct aqueous uptake. As such, the mobility, bioavailability and toxicity of AgNPs in aquatic ecosystems are governed by colloidal stability (Romer *et al*., 2011).

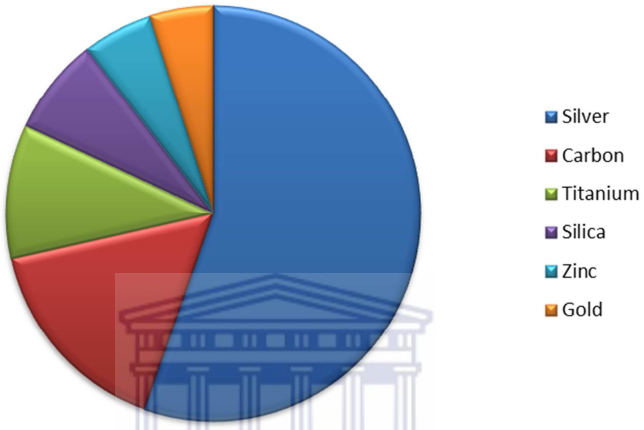


Figure 2.2: Percentage of products associated with a specific material (Project on Emerging Nanotechnologies) <www.nanotechproject.org> accessed 4 July 2012.

The assessment of NMs in the aquatic environment has received considerable attention, particularly since the water cycle is ultimately at the receiving end of runoff and wastewater from both domestic and industrial use. In addition, aquatic organisms are inevitably the recipients of most contaminants released into the environment (Farre *et al*., 2009; Peralta-Videa *et al*., 2011). Despite the recent acquired knowledge on NMs, little is known about the modes of biological uptake, bioaccumulation and biomagnification in aquatic organisms.

Nano-toxicology studies remain poorly and unevenly distributed. Most toxicological studies have largely focussed on the use of aquatic invertebrates as test species. Invertebrates are composed of a large and diverse group of animals. However, of the 1000 different species, Daphnia magna is the most common test species used in conventional and nano-toxicological studies. Although other crustaceans such as crabs have been used in conventional toxicological studies, only few studies have investigated the adverse effects posed by NMs, specifically biomarker responses, to these compounds. Crabs are benthic

organisms and are prone to contaminant uptake and bioaccumulation. For these reasons, crabs represent model species that can be used to evaluate the toxicological effects posed by NMs.

The use of biomarkers in nano-toxicological studies as early warning indicators of risks to ecosystems and humans has increased in recent years. As with conventional contaminants, biomarkers used in nano-toxicological studies are useful as they assess uptake, bioavailability and adverse effects of NMs in the aquatic environment. The formation of reactive oxygen species (ROS) by metallic NPs may lead to oxidative stress responses and inactivity of enzymes, mutations and cell death (Elia *et al*., 2003; Oberholster *et al*., 2011). Biomarkers, such as glutathione peroxidase (GPx), glutathione (GSH), superoxide dismutase (SOD) have been used to trace the processes of and monitor antioxidant defence systems (Li *et al*., 2008). Genotoxicity biomarkers in aquatic invertebrates, measuring the genotoxic effects of NMs, have proved useful tools for monitoring aquatic toxicity due to NPs (Landsiedel *et al*., 2009; Park, *et al*., 2010).

Although nanotechnology has promise in several applications, its products are considered to be potentially toxic when released into the environment. Despite the significant increases in their concentrations in the environment due largely to anthropogenic activities, the current information available on the potential environmental risks posed by AgNPs remains limited. As such, there is a requirement for research to understand and anticipate the implications of NMs in aquatic ecosystems so as to mitigate environmental exposure.

The purpose of this paper is to review published scientific literature on the behaviour of AgNPs in the environment. This study also clarifies, specifically, the existing ecotoxicological data in respect to AgNPs, their ecotoxicity in the aquatic environment, and the potential routes of uptake of AgNPs by aquatic organisms. A third objective of this paper is to review the available literature pertaining to the use of biomarkers in crabs, specifically *Potamonautes perlatus*. Additionally, we highlight the gaps in nano-toxicological research and the use of crabs in nano-toxicology.

2. Toxicity of AgNPs

The literature on the ecotoxicology of NMs is still an emerging field, although there have been several recent reviews (Oberdorster *et al*., 2006; Baun *et al*., 2008; Handy *et al*., 2008; Fabrega *et al*., 2011). Metal NPs, specifically, have received increasing interest due to their extensive use in several applications. As mentioned, of all the metal NPs, AgNPs constitute

the largest group of NPs produced worldwide. It is estimated that, globally, the production of silver-based NMs is at about 500 t/a (Mueller and Nowack, 2008), and is predicted to increase progressively over the next few years. Silver NPs are rapidly being exploited in consumer products, largely due to their antibacterial properties (http:/www.nanotechnology.org/). The release of AgNPs into the environment is therefore inevitable, yet little is known about the environmental effects of exposure to AgNPs.

In ecotoxicological assessments, it is essential to understand the physico-chemical properties of NPs governing their toxicity. Physico-chemical properties, such as particle size and surface area, are important characteristics affecting NM bioavailability and toxicity (Nel *et al*., 2006). As particle size decreases, its surface area increases allowing for a greater proportion of its atoms or molecules to be displayed on the surface rather than the interior of the material. Once released into the environment, NPs form colloidal suspensions that aggregate (Velzeboer *et al*., 2008), which consequently affects their functional properties and likelihood of uptake into living organisms (Royal Commission, 2008).

Metal NMs are able to dissolve, aggregate or remain suspended as single particles in aqueous solutions (Stebounova *et al*., 2011). However, NPs weakly bound together could potentially disaggregate (reversal of the aggregation), thereby providing smaller sized particles with larger surface areas. Aggregation (and disaggregation) processes regulate NM speciation, transport, fate and bioavailability, particle concentration and toxicity (O'Meila, 1980). The aggregation (and disaggregation) state is influenced by a combination of several factors including, organic matter (OM), colloidal clay, ionic strength, pH and surface charge. Therefore, the physico-chemical characterization of NPs under different conditions is important to understanding their behaviour and effect in the environment.

Aggregated NPs are less mobile and may be taken up by filter-feeders and sediment-dwelling organisms, and could potentially result in biomagnification in the food chain. It is generally assumed that aggregation reduces NPs toxicity (Royal Commission, 2008). The fate and toxicity of NMs in aquatic ecosystems is, therefore, largely dependent on the inherent characteristics of NM, namely: particle size, particle coating and aggregation. This was supported by Choi *et al*. (2010) who investigated the aggregation behaviour of AgNPs (Figure 2.3), and reported an increase in average particle size of up to a factor of 40. Aggregation potential was also measured by Romer *et al*. (2011) who reported a reduction in aggregation following dilution of AgNP medium. Similar observations are also reported by others (Glaspell *et al*., 2005; Pham *et al*., 2012; Saini *et al*., 2012). Also, it is generally

assumed that NP aggregation is more enhanced in marine waters than freshwater, due to the low ionic strength of freshwaters (Batley and McLaughlin, 2010).

Prior to the interests in nano-ecotoxicity, Ag ions (Ag^{+}) were generally regarded as the most toxic form of Ag in the aquatic environment (Liau *et al*., 1997; Ratte, 1999) while Ag was generally considered relatively nontoxic to humans (Fabrega *et al*., 2011). The properties of Ag+ favour their uptake via cell membrane ion transport (Luoma, 2008), and it is, therefore, bioconcentrated in aquatic organisms. At the nanoscale (diameter > 1 nm < 100 nm) (Wiench *et al*., 2009) Ag is toxic even at low concentrations (Croteau *et al*., 2011). Silver NPs are introduced into the environmental via several sources, including synthesis and manufacturing, emissions from industrial and domestic activities, and disposal/recycling (Kohler *et al*., 2008).

The bioavailability of AgNPs is vital in determining its toxicity (Croteau *et al*., 2011). Although there is no evidence symptomatic of a direct threat of AgNPs to humans through use of AgNP-containing consumer products, the release of AgNPs into the environment is likely to persist and bioaccumulate (Fabrega *et al*., 2011). In aquatic environments, the assimilation of AgNPs in organisms' body burdens is either through aqueous absorption or dietary uptake (Zhao and Wang, 2010). For example, Baun *et al*. (2008) observed that NPs may adhere to the walls of algae which may in turn be ingested by filter-feeders, thus transferring to higher trophic levels. Similarly, Zhao and Wang (2010) illustrated directly aqueous NP uptake. In addition to uptake, the net bioaccumulation is dependent on the elimination of AgNPs out of the organism. As such, the biokinetic factors are important for calculating NP bioavailability.

The fate and transport of NPs in sediments are poorly studied. In sediments, NPs might undergo aggregation or sedimentation, making them available for bioaccumulation by aquatic organisms, thereby entering the aquatic food chain. Therefore, as with conventional contaminants, sediment is regarded as an important sink for NPs.

The research regarding the ecotoxicity of NPs is still emerging, and gaps still exist in our knowledge of this area. However, the sections below attempt to summarise the available literature pertaining to the toxicology of AgNPs in the aquatic environment, and provides a baseline for concerns regarding the impacts and risks associated with AgNPs to aquatic organisms and ecosystems.

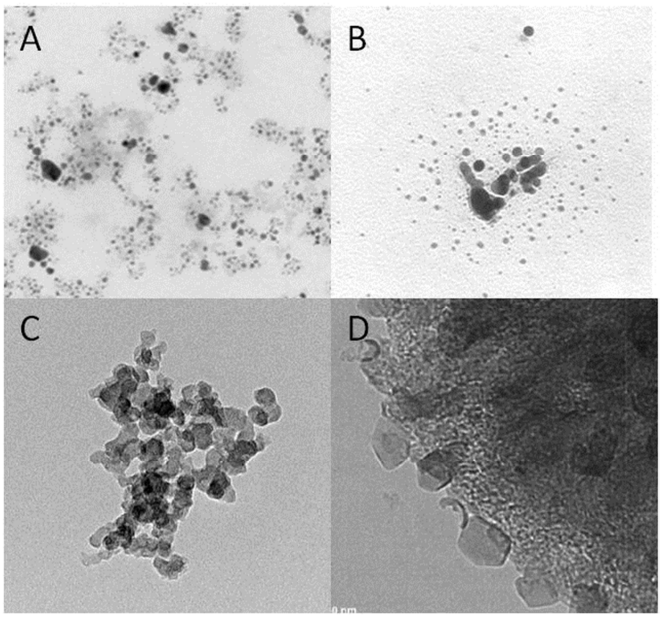


Figure 2.3: Typical examples of nanoparticle aggregation (A: Choi *et al.* (2010); B: Glaspell *et al.* (2005); C: Pham *et al.* (2012); D: Saini *et al.* (2012)).

3. Effects of AgNPs on Aquatic Organisms

3.1. Aquatic plants

Aquatic plants are located at the base on the aquatic food chain, and therefore form the basic nourishment in the aquatic environment. Aquatic plants, such as algae, are known to be sensitive test species for metal and metal oxide NM exposure studies (Aschberger *et al*., 2012). As such, any destructive effects to these primary producers could potentially cause irreversible ecosystem impairment. Few studies have investigated the effects of AgNPs in aquatic plants (Navarro *et al*., 2008; Miao *et al*., 2009; Miao *et al*., 2010; Gubbins *et al*., 2011; Oukarroum *et al*., 2012) (Table 2.1). It is known that NMs are relatively more toxic than larger particles. This was supported by Navarro *et al*. (2008) studying the toxicity of both AgNP and bulk $AgNO_3$ on the algae *Chlamydomonas reinhardtii*. Although similar EC50 values were reported after 1 and 2 hr $AgNO_3$ exposure, AgNPs were relatively more toxic. Additionally, the results demonstrated the significant role of Ag^+ in AgNP toxicity. In aqueous suspensions Ag has high mobility and can, therefore, be easily transported to the larger aquatic environment (Blaser *et al*., 2008). Studies have reported conflicting results for AgNP *vs*. Ag^+ toxicity. For AgNPs, Lok *et al*. (2006) reported biological effects at concentrations up to 1000 times lower than Ag^+, while Griffit *et al*. (2009) demonstrated the enhanced toxicity of metallic NPs. This was also supported by Gubbins *et al*. (2011) who,

studying the phytotoxicity of AgNPs on Lemnar minor using modified OECD methods (OECD 221 guideline), observed plant growth inhibition at 5 µg/L AgNP concentration, whereas Ag^{+} caused greatest toxicity at concentrations of 40 µg/L. Such variability in toxicity could be attributed to several factors, such as differential uptake (Yeo and Pak, 2008) and particle dissolution (Chae *et al*., 2009). Miao *et al*. (2009) reported that the toxicity of AgNPs was mainly due to the release of Ag^{+}.

Algae species vary widely in their response to different contaminants. Oukarroum *et al*. (2012) employed ROS formation and lipid peroxidation (LPO) biomarkers to assess the toxic effects of AgNPs in the freshwater microalgae Chlorella vulgaris and marine microalgae *Dunaliella tertiolecta*. When compared to the control, the authors reported a 7-fold and 25-fold increase in ROS formation for *C. vulgaris* and *D. tertiolecta*, respectively. In terms of LPO, a 4-fold and 15-fold increase for *C. vulgaris* and *D. tertiolecta*, respectively, when compared to the control. The discrepancy in these results could be explained by the fact that *D. tertiolecta* lacks a cell wall, thereby classifying it more sensitive to AgNP toxicity than *C. vulgaris*. Miao *et al*. (2010) measured toxicity in *Ochromonas danica*, and reported a significant uptake of AgNPs and increase in Ag concentrations following addition of GSH.

Table 2.1: A non-exhaustive summary of the toxic effects of AgNPs to aquatic plants.

Test species	NP Size (nm)	NP concentration	Major findings	Reference
Lemnar minor	20; 100	0, 5, 10, 20, 40,	Plant growth inhibition at 5 mg/L AgNPs concentration	Gubbins *et al*. (2011)
Chlamydomonas reinhardtii	10–20	1 g/L	Similar EC_{50} values reported; AgNP was more toxic; Toxicity of Ag NPs mediated by Ag ions released from Ag NP in contact with cell	Navarro *et al*. (2008)
Chlorella vulgaris; Dunaliella tertiolecta	50	0-10 mg/L	7-fold and 25-fold increase in ROS formation for *C. vulgaris* and *D. tertiolecta*, respectively; for LPO a 4-fold and 15-fold increase in *for C. vulgaris* and *D. tertiolecta*	Oukarroum *et al*. (2012)
Ochromonas danica	1–10	27.8–278.1 nM	Significant uptake of AgNPs; increase in Ag concentrations following addition of GSH	Miao *et al*. (2010)
Chlamydomonas reinhardtii	10–200	10–100 000 nM	Toxicity was higher for $AgNO_3$ than for AgNP (in terms of EC_{50}), when compared as a function of the AgC toxicity of AgNP was much higher than that of $AgNO_3$	Navarro *et al*. (2008)
Thalassiosira weissflogii	60–70	0.02–0.0002 nM	Release of Ag^+ from AgNPs reduced cell growth, photosynthesis and chlorophyll production	Miao *et al*. (2009)

3.2.Aquatic invertebrates

Extensive research exists which investigates the toxicity of AgNPs in aquatic invertebrates (Table 2.2). In aquatic organisms, uptake of NPs generally occurs across the gills and other epithelial surfaces (Scown *et al*., 2010). Once taken up, either from the water column or sediment, NPs are known to cause cell damage by disrupting cell membrane integrity and may cause severe damage by ROS (Klaine *et al*., 2008).

Of all the aquatic invertebrates, *D. magna* is the most common test species used in ecotoxicological studies and several international guidelines (*e.g.* OECD, ISO and EPA) for using this species as bio-indicators. This is largely due to their trophic position, feeding habits and sensitivity. Daphnids are planktonic filter-feeders with combs of setae which act as a mesh filtering large volumes of water and particles (around 0.4 – 40 μm range) (Geller *et al*., 1981) from their surroundings. As such, daphnids are considered to be of special ecological relevance.

In order to assess the uptake and bioaccumulation of NPs by aquatic organisms it is important to understand the characteristics (such as particle size and solubility) of NMs. Nanoparticles are able to penetrate the semi-permeable membranes of some aquatic organisms, forming aggregates around the exoskeleton of aquatic organisms (Baun *et al*., 2008), and inducing physical effects and loss of mobility. Uptake of NPs by *D. magna* has also been shown by light microscope imaging, further illustrating the ease of penetration (Figure 2.4) (Asghari *et al*., 2012). Romer *et al*. (2011) employed OECD toxicity tests (OECD 202 and 211 guidelines) on *D. magna*. The results reported enhanced aggregation which resulted in changes in exposure levels.

Nanomaterials differ from their bulk counterparts in several ways, including high surface/volume ratio. As mentioned, Ag^{+} is toxic in the aquatic environment (Liau *et al*., 1997; Ratte, 1999), and their uptake is strongly reliant on Ag speciation (Navarro *et al*., 2008). Gaiser *et al*. (2012) investigated the biological effects of AgNPs and CeO_2 on D. magna. Their results reported that AgNPs were generally more toxic that CeO_2, and further supported the increased toxicity of NPs relative to their bulk particles. In an earlier study, Gaiser *et al*. (2011) assessed survival and molting in *D. magna* in both acute and chronic tests. Similarly, the results reported significant toxicity of AgNPs compared to that of CeO_2 NPs, and further supported the destructive effects of AgNPs on aquatic organisms. These findings were in contrast to others (Li *et al*., 2010). The conflicting results suggest that physical characteristics of the NMs (such as particle size and solubility) may be accountable for the inconsistencies.

Table 2.2: A non-exhaustive summary of the toxic effects of AgNPs to aquatic invertebrates.

Test species	NP size (nm)	NP concentration	Major Findings	Reference
Daphnia magna	35	0 - 10 mg/L	AgNP were generally more toxic that CeO_2	Gaiser *et al*. (2012)
Daphnia magna	35	0 – 10 mg/L	Significant toxicity of AgNP compared to that of CeO_2 NP	Gaiser *et al*. (2011)
Daphnia magna	20	250, 400, 500 µg/L	Uptake efflux rate lower for AgNPs than for Ag^+; assimilation efficiency higher for AgNP than Ag^+	Zhao and Wang (2010)
Lymnaea stagnalis	17 ± 5	0.6 – 87; 1 – 72 nM	Faster uptake rates were reported for Ag^+ than for AgNPs for both aqueous and dietary exposure routes	Croteau *et al*. (2011)
Daphnia magna	20	2 – 500 µg/L	>70% of AgNPs were accumulated through ingestion	Zhao and Wang (2010)
Caenorhabditis elegans	100	0.05, 0.1, 0.5 mg/L	Significant reduction in reproduction	Roh *et al*. (2009)
Crassostrea virginica	15	1.6 - 0.0016; 0.16 µg/L	Normal embryonic development was significantly impaired	Ringwood *et al*. (2010)
Daphnia magna	100	0 – 50 µg/L	DNA strand breaks were increased following exposure	Park and Choi (2010)
Daphnia magna	15.83	0.001 – 0.32 mg/L	AgNPs were ingested by *D. magna* and accumulated under the carapace; caused abnormal swimming by the *D. magna*.	Asghari *et al*. (2012)
Daphnia magna	7; 10; 20	2.2 mg/L	Loss of mobility and fecundity	Romer *et al*. (2011)
Chironomus tentans	50 - 400	5 - 5000 µg/kg	Percentage survival and growth length inhibition; catalase and peroxidase enzyme activity showed that toxicant stress of the NMs	Oberholster *et al*. (2011)

As previously mentioned, biokinetic factors (such as uptake rate constant, assimilation efficiency) are important for calculating NP bioavailability. Zhao and Wang (2010) employed a radiotracer methodology to measure the biokinetics of AgNPs in *D. magna*, and reported that uptake rates and efflux rate were relatively lower for AgNPs when compared to Ag^{+}, while assimilation efficiency was higher for AgNP than Ag^{+}. These results suggest the difficulty in eradicating AgNPs.

In toxicity tests with other aquatic invertebrates, Croteau *et al*. (2011) investigated the bioaccumulation dynamics in the snail *Lymnaea stagnalis* following both aqueous and dietary exposure to AgNPs and Ag^{+}. *L. stagnalis* efficiently accumulated Ag from sources. Faster uptake rates were reported for Ag+ than for AgNPs for both exposure routes, but more so for waterborne uptake, suggesting enhanced particle aggregation and consequent reduced dietary uptake. However, in an earlier study, Zhao and Wang *et al*. (2010) reported >70% of AgNPs were accumulated through ingestion. This observation emphasizes the significance of the transport of NPs along the aquatic food chain.

Since sediments are ultimately the repository of anthropogenic contaminants (including NMs) it proves advantageous to include benthic organisms in toxicological studies. However, such toxicological data on benthic organisms are limited. Using survival, growth and reproduction as the ecotoxicological endpoints, Roh *et al*. (2009) investigated the effects of AgNPs in the nematode *Caenorhabditis elegans*. The most dramatic effects were observed for reproduction, which was significantly reduced. Species of the benthic invertebrate genus Chironomus, including *Chironomus riparius* and *Chironomus tentans*, have been used for both acute and chronic testing. Oberholster *et al*. (2011) used *C. tentans* as a test species to determine the effects of a suite of NMs, and reported that the percentage growth length of *C. tentans* was significantly reduced when compared to the reference treatment, and further declined with increasing concentrations of each NM over a 10 day exposure period.

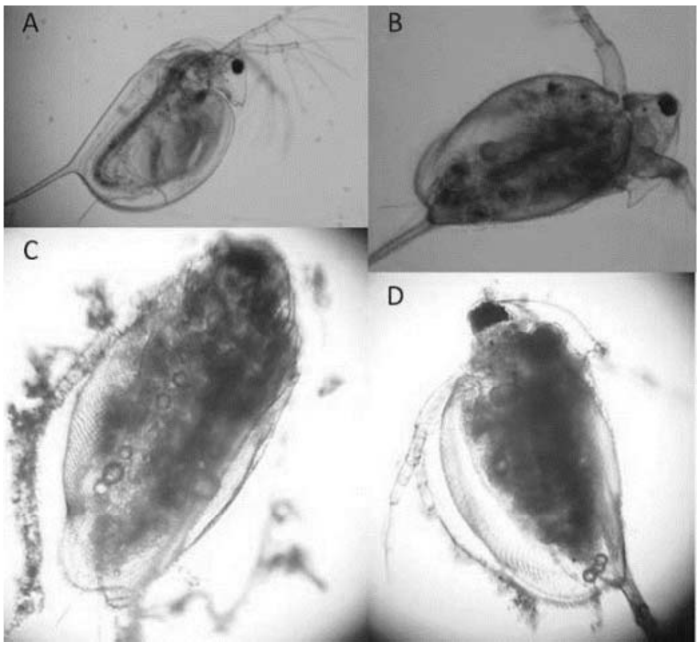


Figure 2.4: Light microscope images of daphnia exposed to AgNPs. A: control, B: live daphnia with pigmentation (circles), C and D: bubbles visible under the carapace; nanoparticles visible on the antennae and body surface (adapted from Asghari *et al*., 2012).

Toxic effects of AgNPs on reproduction and development have been reported (Ringwood *et al*., 2010). There is a general consensus that toxicants may cause detrimental effects such as impaired embryonic development and physiological functions. Recent studies have shown that AgNPs can have significant impacts on embryonic development even at low levels (Ringwood *et al*., 2010). Ringwood *et al*. (2010) characterized the toxicity of AgNPs on the embryonic development of oysters, *Crassostrea virginica*, following exposure at various concentrations (*i.e.* 0.0016, 0.016, 0.16 and 1.60 µg/L) and observed that normal embryonic development was significantly impaired.

The genotoxic potential of NMs depends on several factors including the test material used, exposure route and endpoint measured (Johnston *et al*., 2009). Few ecotoxicological studies have investigated genotoxic endpoints of NMs in aquatic organisms. Park and Choi (2010) employed the comet assay to evaluate whether AgNPs induced any genetic toxicity in D. magna. The results proved that DNA strand breaks were increased following exposure to 1 and 1.5 µg/L AgNPs and Ag^{+}. As expected, the degree of DNA strand breaks was more significant than for AgNPs than for Ag^{+}.

3.3. Fish

Fish are regarded as good sentinel species of environmental stress, as they are sensitive to a wide range of contaminants. In addition, their position in the aquatic food chain not only offers an indication of the ecosystem health of lower trophic levels, but also gives an indication of their safety for human consumption. The potential routes of NMs uptake in fish include their absorption via the gill epithelia, gut epithelia (through dietary exposure), and skin (Handy *et al*., 2008).

Reported ecotoxicological assessments of NPs for fish are limited (Lee *et al*., 2007; Chae *et al*., 2009; Yeo and Pak, 2008; Scown *et al*., 2010; Griffit *et al*., 2012; Pham *et al*., 2012; Table 2.3). The vast majority of fish nano-toxicological studies published are acute studies, while fewer papers report on chronic studies (Aschberger *et al*., 2012). In sheepshead minnow *Cyprinodon variergatus*, chronic exposure to low levels of AgNPs resulted in significant thickening of gill epithelia tissues and significantly altered gene expression profiles in both juveniles and adults (Griffit *et al*., 2012). In another study, chronic toxicity tests of AgNPs in Medaka (*Oryzias latipes*) were investigated by Pham *et al*. (2012). They reported significant induction of metallothionein (MT) and glutathione S-transferase (GST) genes in the livers of test species exposed to 1 µg/L, while heat shock proteins (HSP) was suppressed following a 28-d exposure period.

The results concluded that AgNPs increase metal detoxification, oxidative and inflammatory stress, and stimulated immune responses.In an earlier study Yeo and Pak (2008) investigated changes in the expression of stress-related biomarkers (MT, HSP, GST), and reported that AgNPs caused cellular and DNA damage, as well as carcinogenic and oxidative stress, while Ag^{+} caused lower overall stress responses. Endpoints such as mortality, development and growth have also been investigated. Early-life stages in fish are most sensitive to environmental disturbances (Weis and Weis, 1989). This was supported by Lee *et al*. (2007) who investigated the transport of AgNPs in zebrafish embryo *Danio rerio* using in vivo imaging and its effects on early embryonic development. The results showed an increase in mortalities and abnormalities in early life stages (Figure 2.5), as well as mortalities with increasing NP concentration. Nanoparticle size is known to affect toxicity. Scown *et al*. (2010) reported size dependant uptake of AgNPs (10 – 35 nm) and associated oxidative stress in the gills of *D. rerio*.

Table 2.3: A non-exhaustive summary of the toxic effects of AgNPs to fish.

Test species	NP size (nm)	NP concentration	Major Findings	Reference
Cyprinodon variergatus	20-30	10 µ g/L	Significant thickening of epithelia gill tissues and significantly altered gene expression profiles in both juveniles and adults	Griffit *et al*.(2012)
Oryzias latipes	23.5	1 – 25 µg/L	Significant induction of MT and GST genes in the liver; suppression of HSP	Pham *et al*. (2012)
Zebrafish embryo	11.6	0.08 nM	Increase in zebrafish mortalities and abnormalities in early life stages and mortalities	Lee *et al*. (2007)
Perca fluviatilis	30–40	63, 129, 300 µg /L	Impairment of the tolerance to hypoxia; internal hypoxia during low water oxygen tensions	Bilberg *et al*. (2010
Oryzias latipes	49.6	1; 25 µg/L	Cellular and DNA damage, carcinogenic and oxidative stresses	Chae *et al*. (2009)
Zebrafish	10-20	0.4; 4 ppm	Defects in fin regeneration and penetration into organelles and cell nucleus	Yeo and Pak (2008)
Oncorhynchus mykiss	10, 35, 600-1600	10; 100 µg/L	Size dependent uptake AgNPs concentrated in gills and liver; Increase of oxidative stress in gills	Scown *et al*. (2010)

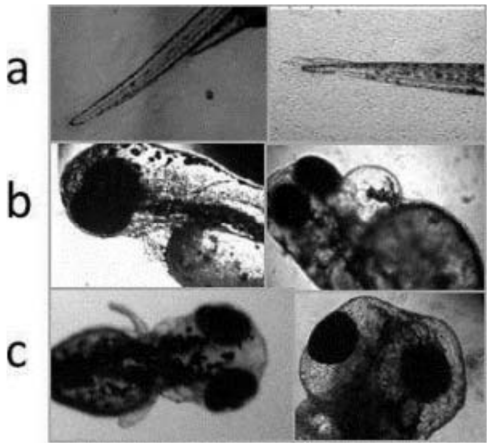


Figure 2.5: Optical images of normally developed (left) and deformed (right) D. rerio. A: tail/spinal cord, B: cardiac; C: head (Adapted from Lee *et al.* (2007)).

4. Recommendations for Future Research in Invertebrate Nano-ecotoxicology

This review has outlined the current available knowledge on AgNP toxicity as a potential problem for environmental health, and highlighted the gaps in the research. Based on the current literature review the sections below propose recommendations for the development of nano-toxicology, by highlighting the significance of crabs as model test organisms.

Nano-toxicology studies remain poorly and unevenly distributed, in spite of increased environmental concern. In nano-toxicology, invertebrate-based studies employ daphnids and other cladocerans as test organisms (Cattaneo *et al.*, 2009), making them convenient test species for ecotoxicological studies. Crabs occupy a significant position in the aquatic food chain. They are often used in conventional ecotoxicological studies due to their high sensitivity to environmental stressors, and are abundantly available. Because they are benthic organisms, they are prone to contaminant uptake, biomagnification and bioaccumulation. However, to our knowledge the crab biomarkers have never been used in nano-toxicological studies. And only limited studies for specific endpoints of toxicity for AgNPs have been conducted. Consequently, crabs represent model organisms for nano-toxicological research, and highlight the potential approaches that would promote the advancement of future nano-toxicolgical studies to follow.

The distributions of contaminants within target organs are largely unknown (Elumalai *et al.*, 2007; Pereira *et al.*, 2009). In crabs, contaminants are known to be sequestered in the hepatopancreas, gills and other tissues. As such, it is of great interest to investigate the nano-toxicity and potential biomarkers of toxicity in crabs. In the sections below, the applications

of biomarkers in conventional ecotoxicology in crabs are discussed. This serves to endorse the standpoint of investigating potential crab biomarkers in nano-toxicological studies.

5. Biomarkers in Crabs Exposed to Environmental Contaminants

There is a growing perception that the use of chemical data is insufficient to reliably assess the potential risks of contaminants in the aquatic environment. Exposure to environmental stressors can result in biochemical, physiological and histological alterations. As such, investigating the biological effects of contaminants has become a major focus of aquatic research, particularly since the environment is continuously being loaded with contaminants released by anthropogenic activities.

Biomarkers, such as enzyme activity or protein-based measurements, are common practice in conventional ecotoxicological studies, and are used as early warning monitoring tools to signal the onset of contaminant exposure in aquatic organisms. The intention of most biomarker studies is to identify and quantify the degree of exposure, as well as the biological effects of the contaminant. The World Health Organization (WHO) classifies biomarkers into three categories, namely: biomarkers of exposure, effect or susceptibility (Figures 2.6 and 2.7) (WHO, 2001). Biomarkers of effect measure both "early" and clinical effects. Biomarkers of exposure measure contaminant concentrations in specific compartments/tissues/organs relative to external or internal exposure; and can be used to confirm and assess the exposure of individuals to a particular substance (Van der Oost *et al*., 2003). Biomarkers of susceptibility measure a specific response of the organisms following exposure to a specific contaminant. Biomarkers of effect will form the focus of this study, since a measurable biochemical and/or physiological effect will be measured within tissues of *P. perlatus*.

Biomarkers provide tools for assessing uptake, bioavailability and harmful effects of NMs in the aquatic environment, and their usefulness have been employed by several authors (Pinho *et al*., 2005; Maria *et al*., 2009; Pereira *et al*., 2009; Lavarias *et al*., 2011). Metallic NPs (including AgNPs) are known to generate oxyradicals causing cytotoxicity by creating ROS. The generation of ROS may damage cellular lipids, carbohydrates, proteins and DNA leading to oxidative stress responses, inactivation of enzymes, mutations and cell death (Elia *et al*., 2003; Oberholster *et al*., 2011).

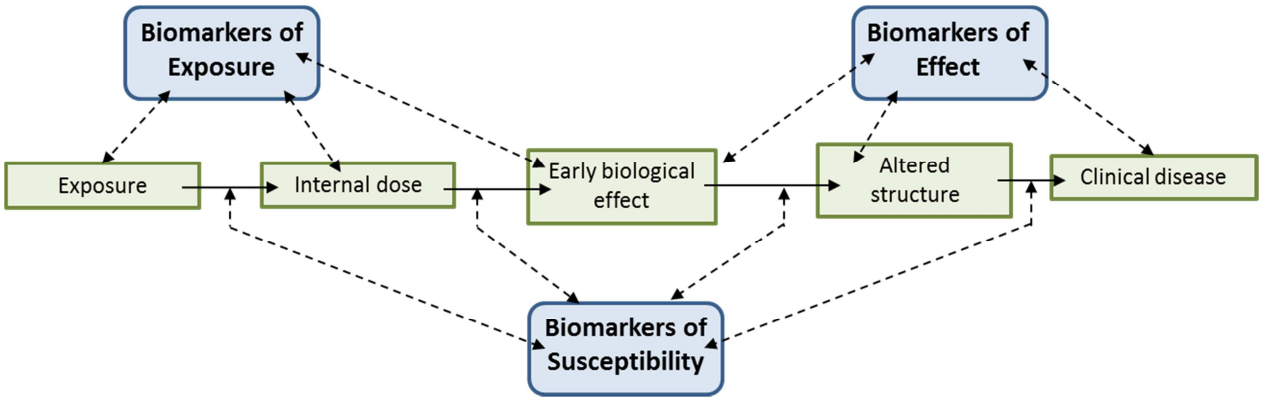


Figure 2.6: The three categories of biomarkers (biomarkers of exposure, biomarkers of effect and biomarkers of susceptibility).

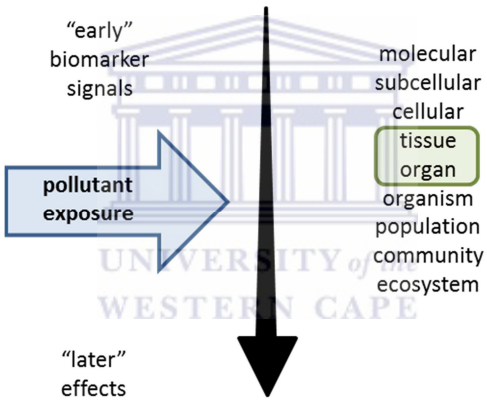


Figure 2.7: Schematic representation of the sequential order of response to pollutant stress within biological system.

6. Biomarkers used in conventional ecotoxicological studies involving crabs

Ecotoxicity studies based on biomarkers have already been developed using crabs (Table 2.4). The sections below report on scientific literature on the most common biomarkers frequently used in conventional ecotoxicological studies involving crabs.

For several reasons, crabs have been used as biomarkers in conventional ecotoxicological studies to estimate exposure in aquatic organisms. Their ecological importance, widespread distribution, high availability, sensitivity to environmental toxicants, and high capability of bioaccumulation make them suitable as test organisms in biomonitoring studies. In freshwater, marine and estuarine crabs, the hepatopancreas, haemolymph and gills are the

target tissues used in biomarker studies. The hepatopancreas is responsible for metabolism and detoxification (Saravana Bhavan *et al*., 2001) and is the key site of heavy metal accumulation (Gibson and Barker, 1979). Haemolymph is a fluid in the circulatory system similar to the blood in vertebrates, and is therefore responsible for the transfer of pollutants into other organs. In the gills, oxygen consumption is reduced in the presence of toxins, therefore osmoregulatory functions in crustaceans are disturbed (Ghate and Mulherkar, 1979).

Reactive oxidative species (ROS) are molecules which are known to cause oxidative damage to protein, lipids and DNA (Luqing *et al*., 2001), following environmental stress where ROS levels are usually elevated. This state is referred to oxidative stress. Environmental contamination is known to enhance ROS and antioxidant imbalance. The principal antioxidant enzymes for assessing oxidative stress and protecting against cellular oxidative damage include: catalase (CAT), superoxide dismutase (SOD) and glutathione peroxidase (GPx). Catalases (CAT) are hematin-containing enzymes which facilitate the removal of hydrogen peroxide (H_2O_2) from organisms (Van der Oost *et al*., 2003) and are also associated with the metabolism of fatty acids (Hugget *et al*., 1992; Stegeman *et al*., 1992). Glutathione peroxidase catalyses the reduction of hydroperoxides using glutathione (GSH) and protects cells against oxidative damage. Superoxide dismutases (SOD) are antioxidant enzymes which catalyze the dismutation of superoxide into oxygen (O2) and H_2O_2. Peroxide can be destroyed by CAT or GPx reactions. Enzymes (such glutathione S-transferase (GST) and lactate dehydrogenase (LDH)) are widely used as environmental biomarkers, as they play a vital physiological role. [60] Glutathione S-transferase (GST) is involved in intracellular transport and offers defence against oxidative damage and peroxidative products of DNA and lipids (Van der Oost *et al*., 2003).

Table 2.4: A non-exhaustive summary of biomarker studies involving crabs.

Species	Toxin	Parameter / Biomarker	Major findings	Reference
Macrobrachium borellii	hydrocarbons	CAT, GST, LPO, SOD	Antioxidant defences were significantly affected	Lavarias *et al*. (2011)
Carcinus maenas	Ni,Cu,Cd	CAT,GPx, GST	Females were more vulnerable to peroxidative damage compared to males. Males showed decreased EROD activity	Pereira *et al*. (2009)
Charybdis japonica	Cd	MT, SOD, CAT, GPx, DNA strand breaks	MT induced after 3 days; dose–response relation between MT and Cd; time–response relation in hepatopancreas; gill was more sensitive to Cd than hepatopancreas; hepatopancreas was the main detoxification tissue	Pan and Zhang, 2006
Carcinus maenas	Metals, PAHs		High gills lipid peroxidation; hepatopancreas DNA integrity decreased in male crabs, female and male *C. maenas* antioxidant defences and damage biomarkers were sensitive to the mixture of contaminants	Maria *et al*., 2009
Charybdis japonica	Cu, Pb, Cd	Genotoxicity (comet assay; DNA alkaline unwinding assay)	levels of DNA damage in gills were higher than those in hepatopancreas in the same experimental group	Liqing *et al*., 2011
Chasmagnathus granulata	Cu, Zn, Cd, Pb	Survival curves and LC50	First zoeae were more sensitive than young crabs to acute exposure to metals, young crabs were considered potentially dangerous agents of transference to the associated trophic chain because of their relatively elevated resistance and their capacity to bioaccumulate heavy metals in their tissues	Ferrer *et al*. (2006)
Scylla serrata	As	ACP, ALP	inhibition of activity of ACP and ALP; dose dependent	Saha *et al*. (2009)

			decrease in the activities of ACP and ALP	
Callinectes sapidus	Cu	Acute toxicity and in vivo accumulation tests	Acute dissolved copper toxicity was higher at 2 ppt than at 30 ppt; copper flux into the gills is higher than into other tissues analysed.	Martins *et al*. (2011)
Fundulus heteroclitus	Cu; salinity	Physiology	maximal dissolved Cu concentration at 10 ppt was 973 µg/l and the highest mortality was 33 ± 3%; Na+ gradients are key parameters influencing relative sensitivity to Cu;	Grosell *et al*., 2007).
Litopenaeus vannamei	pH	Immune responses, SOD	Increase in pH resulted in significant decreases in phenoloxidase (PO) activity, respiratory burst, phagocytic activity, SOD activity and total haemocyte count (THC)	Li and Cheng (2008)
Callinectes sapidus	TBT	vivo effects of long-term exposure	Respiration rates were significantly decreased; hydroxylation of [14C]testosterone by hepatopancreas microsomes increased significantly	Oberdorster *et al*., 1998
Carcinus maenas	Metallothioneins	Defence and damage biomarkers signals	Gills and hepatopancreas glutathione-S-transferase were reduced; Metallothioneins induction occurred; High gills lipid peroxidation; hepatopancreas DNA integrity decreased	Maria *et al*., 2002;

Antioxidant responses and oxidative stress were investigated in the hepatopancreas of the estuarine crab *Chasmagnathus granulates* following oral microcystin administration (Pinho *et al*., 2005) The antioxidant enzyme activities of CAT, GST and SOD were measured. The results reported higher hepatopancreas CAT activity in crabs exposed to the highest microcystin doses and higher GST activity in those exposed to lower doses. However, a lack of SOD response was observed. Other authors such as Lavarias *et al*. (2011) reported that freshwater prawns exposed to hydrocarbons showed significant increases in CAT, SOD and

GST activities in hepatopancreas and CAT activity in gills. In an earlier study, Pereira *et al*. 2009) investigated the susceptibility of crab hepatopancreas to oxidative stress, reporting increased activity of CAT, GPx and GST in female crabs and GPx and GST in male crabs; suggesting that these crabs suffered from pro-oxidant stress. The effects of contaminants under different environmental conditions (pH, temperature *etc*.) are prominent. Season-related fluctuations in hepatic and gill GST and CAT activity have been reported by Lavarias *et al*. (2011) however SOD activity did not show significant differences among seasons. Other studies have shown the relative toxicity of contaminants in crab tissues to be dose- and time- dependent (Ching *et al*., 2001; Pan and Zhang, 2006; Maria *et al*., 2009).

Heavy metals released from anthropogenic activities, such as industrial and mining discharges, enter aquatic ecosystems and become toxic to aquatic organisms. The bioaccumulation of heavy metals promotes the formation of ROS which have the potential to generate oxidative stress within cells (Luqing *et al*., 2011). Ferrer *et al*. (2006) performed 96h acute toxicity test with first zoeae and young crabs of *Chasmagnathus granulate*, following exposure to Cd, Cu, Pb and Zn, as well as mixtures of Cd/Cu and Cd/Zn. The toxicity of Cd presented the highest acute toxicity for both life cycle stages, and followed the order: Cd > Zn > Cu > Pb. Non-enzymatic proteins (such as MT) are known for their metal-binding capacity, and therefore play a vital role in the homeostatic control on essential metals. The toxicological effects of essential and non-essential metals can be countered by regulating the internal metal concentrations by MTs (Roesijadi, 1992).

Other commonly used biomarkers of oxidative stress are those which reflect oxidative changes to lipids. Lipid peroxidation (LPO) defined as the oxidative deterioration of lipids which decompose to form complex, reactive by-products. Metals, such as Cu, Cd, Ni and Pb have been implicated in LPO. Pereira *et al*. (Pereira *et al*., 2009) reported significant increases of LPO in the female shore crab *Carcinus maenas*. In contrast, Maria *et al*. (2009) reported reduced LPO levels in gills and hepatopancreas of female *C. maenas*.

Alkaline phosphatase (ALP) is a metalloenzyme which catalyzes the non-specific hydrolysis of phosphate monoesters (McComb *et al*., 1979) and transphosphorylation. Acid phosphatase (ACP) is a hydrolytic lysosomal biomarker whose functions are distorted during stress (Rajalakshmi and Mohandas, 2005). Phosphatases are involved in the molting physiology of crustaceans (Vijayavel and Balasubramanian, 2006). Saha *et al*. (2009) investigated the effects of both ALP and ACP in the haemocytes of *Scylla serrata* following exposure to arsenic (As). Maximum inhibition activity was achieved at 0.008 uM mg/ min and 0.016 uM/min for ALP and ACP, respectively, after 15 day 3ppm sodium arsenite exposure.

Environmental stress factors (such as pH, salinity, hypoxia) are known to affect the homeostatic and metabolic balances (Zhou *et al*., 2009), resulting in physiological alterations (Martins *et al*., 2011). Martins *et al*. (2011) performed in vivo and in vitro toxicity tests on the blue crab *Callinectes sapidus* following 96h exposure to Cu under different salinity regimes. Following in vivo exposure, the authors reported that acute waterborne toxicity was approximately 10-fold lower for salinity 2 ppt than for salinity 30 ppt. This is in accordance to other studies, who reported lower toxicity of Cu to both crustaceans and fish at higher salinities (Grosell *et al*., 2007). Li and Chen (2008) reported that variations in environmental stressors (such as pH) could also be acutely toxic to crustaceans, resulting in reductions in growth and survival, and eventually death. Hypoxia is also known to have adverse effects on aquatic organisms ultimately resulting in oxidative injury and mortality (Ying and Xiong, 2010). Other factors, such as temperature are also known to promote metabolic processes and increase ROS production (Lapresta-Fernandez *et al*., 2012).

Stress response measurements, such as, heat shock protein (HSP) are involved in the protection and repair of the cell against stress and harmful conditions (Sander, 1993). Stress-protein response is one of the most important cellular mechanisms to prevent and repair the adverse effects of environmental stresses (Feige *et al*., 1996). Aquatic organisms respond to environmental stresses by increasing cellular concentrations of stress proteins (Iwama *et al*., 1998). Heat shock protein induction was used as a biomarker of stress to several contaminants, including tributyltin (TBT) (Oberdorster *et al*., 1998). Long-term in vivo exposure and induction of HSP showed significant increases in hydroxylation of [14C] testosterone by hepatopancreas microsomes and a reduction in P450 enzyme activity.

Genotoxicology is defined as the study of contaminant-induced changes in the genetic material of an organism (Van der Oost *et al*., 2003). DNA damage may lead to mutations, strand breaks, altered bases (Shugart, 2009), carcinogenesis, teratogenesis and genotoxic disease syndrome (Kurelec, 1993). DNA damage can be used as a potential biomarker of contamination in aquatic organisms (Maria *et al*., 2009; Gravato *et al*., 2005; Bolognesi *et al*., 2004). Comet assay and DNA alkaline unwinding assay were conducted on the hepatopancreas, hemocytes and gills of the marine crabs Charybdis japonica in order to assess the genotoxicity of heavy metal ions (Cu^{2+}, Pb^{2+}, and Cd^{2+}) (Luqing *et al*., 2001). The results showed dose-time relationships suggesting a significant increase in DNA single strand breaks when compared to the control set.

7. Conclusions

Although still in its infancy, nanotechnologies and nanomaterials have attracted tremendous attention in recent researches. The potential for ecological toxicity associated with NMs is a growing area of research. The use of NMs in consumer products and their potential environmental and human health risk are of increasing concern. As nanotechnologies and products increase, nano-products entering the aquatic ecosystems and other water sources too will increase, thereby increasing the potential threat to aquatic organisms. In the present review, several studies in both conventional toxicology and nano-toxicological studies were cited. The use of stress-related biomarkers particularly in crabs was also highlighted. With the existing information available, the current research gaps were identified. The ever-increasing use of NMs and the usefulness of crabs in conventional ecotoxicological studies have increased their benefits for use in nano-toxicological research. It is therefore recommended that biomarkers in *P. perlatus* be applied to elucidate the nano-toxicological effects of AgNPs.

8. Acknowledgments

This work was funded by the National Research Foundation (NRF) (Thuthuka Grant) and the Council for Scientific and Industrial Research (CSIR).

9. References

Aschberger, K., Micheletti, C., Sokull-Kluttgen, B., Christensen, F.M., 2012. Analysis of currently available data for characterising the risk of engineered nanomaterials to the environment and human health - Lessons learned from four case studies. Environ. Int. 37, 1143–1156.

Asghari, S., Johari, S.A., Lee, J.H., Kim, Y.S., Jeon, Y.B., Choi, H.J., Moon, M.C. and Yu, I.J., 2012. Toxicity of various silver nanoparticles compared to silver ions on Daphnia magna. J. Nanobiotech. 10, 14–34.

Batley, G.E. and McLaughlin, M.J., 2010. Fate of manufactured nanomaterials in the Australian environment, CSIRO Niche Manufacturing Flagship Report. Department of the Environment, Water, Heritage and the Arts, Australia.

Baun, A., Hartmann, N.B., Grieger, K. and Kusk, K.O., 2008. Ecotoxicity of engineered nanoparticles to aquatic invertebrates: a brief review and recommendations for future toxicity testing. Ecotoxicol. 17, 387–395.

Blaser, S.A., Scheringer, M., MacLeod, M. and Hungerb€uhler, K., 2008. Estimation of cumulative aquatic exposure and risk due to silver: contribution of nano-functionalized plastics and textiles. Sci. Tot. Environ. 90, 396–409.

Bolognesi, C., Frenzilli, G., Lasagna, C., Perrone, E. and Roggieri, P., 2004. Genotoxicity biomarkers in *Mytilus galloprovincialis*: Wild versus caged mussels. Mutation Res. 552, 153–162.

Cattaneo, A.G., Gornati, R., Chiriva-Internati, M. and Bernardini, G., 2009. Ecotoxicology of nanomaterials: the role of invertebrate testing. ISJ. 6, 78–97.

Chae, Y.J., Pham, C.H., Lee, J., Bae, E., Yi, J. and Gu, M.B., 2009. Evaluation of the toxic impact of silver nanoparticles on Japanese medaka (*Oryzias latipes*). Aquat. Toxicol. 94, 320–327.

Ching, E.W.K., Siu, W.H.L., Lam, P.K.S., Xu, L., Zhang, Y.Y. and Richardson, B.J., 2001. DNA adduct formation and DNA strand breaks in green-lipped mussels (*Perna viridis*) exposed to benzo[a]pyrene: dose- and time-dependent relationships. Mar. Poll. Bull. 42, 603–610.

Choi, O., Yu, C.-P., Fernandez, G.E. and Hu, Z., 2010. Interactions of nanosilver with *Escherichia coli* cells in planktonic and biofilm cultures. Water Res. 44, 6095–6103.

Croteau, M-N., Misra, S.K., Luoma, S.N. and Valzami-Jones, E., 2011. Silver bioaccumulation dynamics in a freshwater invertebrate after aqueous and dietary exposures to nanosized and ionic Ag. Environ. Sci. Technol. 45, 6600–6607.

Elia, C., Galarini, R., Taticchi, M.I., Dorr, A.J. and Mantilacci, L., 2003. Antioxidant responses and bioaccumulation in *Icttalurus melas* under mercury exposure. Ecotoxicol. Environ. Saf. 55, 162–167.

Elumalai, M., Antunes, C. and Guilhermino, J., 2007. Enzymatic biomarkers in the crab *Carcinus maenas* from the Minho River estuary (NW Portugal) exposed to zinc and mercury. Chemosphere. 66, 1249–1255.

Fabrega, J., Luoma, S.N., Tyler, C.R., Galloway, T.S. and Lea, J.R., 2011. Silver nanoparticles: behaviour and effects in the aquatic environment. Environ. Int. 37, 517–531.

Farre, M., Gajda-Schrantz, K., Kantiani, L. and Barcelo, D., 2009. Ecotoxicity and analysis of nanomaterials in the aquatic environment. Anal. Bioanal. Chem. 393, 81–95.

Feige, U., Morimoto, R.I., Yahara, I. and Polla, B.S., 1996. Stress-inducible cellular responses. Basel, Switzerland: Birkhauser Verlag, 512.

Ferrer, L., Andrade, S., Asteasuain, R.; and Marcovecchio, J., 2006. Acute toxicities of four metals on the early life stages of the crab *Chasmagnathus granulata* from Bahia Blanca estuary, Argentina. Ecotoxicol. Environ. Saf. 65, 209–217.

Gaiser, B.K., Fernandes, T.F., Jepson, M.A., Lead, J.R., Tyler, C. R., Baalousha, M., Biswas, A., Britton, G.J., Cole, P.A., Johnston, B.D., Ju-Nam, Y., Rosenkranz, P., Scown, T.M. and Stone, B.D., 2012. Interspecies comparisons on the uptake and toxicity of silver and cerium dioxide nanoparticles. Environ. Toxicol. Chem. 31(1), 144–154.

Gaiser, B.K., Biswa, A., Rosenkranz, P., Jepson, M.A., Lead, J.R., Stone, V., Tyler, C.R. and Fernandes, T.F., 2011. Effects of silver and cerium dioxide micro- and nano-sized particles on *Daphina magna*. J. Environ. Mon. 13, 1227–1235.

Geller, W. and Muller, H., 1981. The filtration apparatus of cladocera: Filter mesh-sizes and their implications on food selectivity. Oecologia. 49, 316–321.

Ghate, H.V. and Mulherkar, L., 1979. Histological changes in the gills of two freshwater prawn species exposed to copper sulphate. Indian J. Exp. Biol. 17, 838–840.

Gibson, R. and Barker, P.L., 1979. The decapoda hepatopancreas. Ocean. Mar. Biol. Ann. Rev. 17, 285–346.

Glaspell, G.P., Zuo, C. and Jagodzinski, P.W., 2005. Surface enhanced raman spectroscopy using silver nanoparticles: The effects of particle size and halide ions on aggregation. J. Cluster Sci. 16, 39–51.

Gravato, C., Oliveira, M. and Santos, M.A., 2005. Oxidative stress and genotoxic responses to resin acids in Mediterranean mussels. Ecotoxicol. Environ. Saf. 61, 221–229.

Griffitt, R.J., Hyndman, K., Denslow, N.D. and Barber, D.S., 2009. Comparison of molecular and histological changes in zebrafish gills exposed to metallic nanoparticles. Toxicol. Sci. 107, 404–415.

Griffit, R.J., Brown-Peterson, N.J., Savin, D.A., Manning, C.S., Boube, I., Ryan, R.A. and Brouwer, M., 2012. Effects of chronic nanoparticulate silver exposure to adult and juvenile sheepshead minnows (*Cyprinodon variegatus*). Environ. Toxicol. Chem. 31(1), 160–167.

Grosell, M., Blanchard, J., Brix, K.V. and Gerdes, R., 2007. Physiology is pivotal for interactions between salinity and acute copper toxicity to fish and invertebrates. Aquat. Toxicol. 84, 162–172.

Gubbins, E.J., Batty, L.C. and Lead, J.R., 2011. Phytotoxicity of silver nanoparticles to *Lemna minor L*. Environ. Poll. 159, 1551–1559.

Handy, R.D., Henry, T.B., Scown, T.M., Johnston, B.D. and Tyler, C. R., 2008. Manufactured nanoparticles: their uptake and effects on fish – a mechanistic analysis. Ecotoxicol. 17, 396–409.

Huggett, R.J., Kimerle, R.A., Mehrle-Jr., P.M. and Bergman, H.L., 1992. Biomarkers: biochemichal, physiological, and histological markers of anthropogenic stress, Lewis Publishers, Chelsea, MI.

Iwama, G.K., Thomas, P.T., Forsyth, R.B. and Vijayan, M.M., 1998. Heatshock protein expression in fish. Rev. Fish Biol. Fish. 8, 56.

Johnston, H.J., Hutchison, G.R., Christensen, F.M., Peters, S., Hankin, S. and Stone, V., 2009. Identification of the mechanisms that drive the toxicity of TiO_2 particulates: the contribution of physicochemical characteristics. Part. Fibre Toxicol. 6, 33–59.

Klaine, S.J., Alvarez, P.J.J., Batley, G.E., Fernandes, T.F., Handy, R.D. and Lyon, D.Y., 2008. Nanomaterials in the environment: behaviour, fate, bioavailability, and effects. Environ. Toxicol. Chem. 27, 1825–1851.

Klaine, S.J., Koelmans, A.A., Home, N., Carley, S., Handy, R.D., Kapustka, L., Nowack, B. and von der Kammer, F., 2012, Paradigms to assess the environmental impact of manufactured nanomaterials. Environ. Toxicol. Chem. 31(1), 3–12.

Kohler, A.R., Som, C., Helland, A. and Gottschalk, F., 2008. Studying the potential release of carbon nanotubes throughout the application life cycle. J. Cleaner Prod. 16, 927–937.

Kurelec, B., 1993. The genotoxic disease syndrome. Mar. Environ. Res. 35, 341–348.

Landsiedel, R., Kapp, M.D., Schulz, M., Wiench, K. and Oesch, F., 2009. Genotoxicity investigations on nanomaterials: Methods, preparation and characterization of test material, potential artifacts and limitations—Many questions, some answers. Mutation Res. 681, 241–258.

Lapresta-Fernandez, A., Fernandez, A. and Blasco, J., 2012. Nanoecotoxicity effects of engineered silver and gold nanoparticles in aquatic organisms. Trends Anal. Chem. 32, 40–59.

Lavarias, S., Heras, H., Pedrini, N., Tournier, H. and Ansaldo, M., 2011. Antioxidant response and oxidative stress levels in *Macrobrachium borellii* (Crustacea: Palaemonidae) exposed to the water-soluble fraction of petroleum. Comp. Biochem. Physiol. C. 153, 415–421.

Lee, K.J., Nallathamby, P.D., Browining, L.M., Osgood, C.J. and Xu, X-H.N., 2007. In vivo imaging of transport and biocompatibility of single silver nanoparticles in early development of zebrafish embryos. CS Nano, 2007, 1 (2), 133–143.

Li, H.C., Zhang, J., Wang, T., Luo, W., Zhou, Q. and Jiang, G., 2008. Elemental selenium particles at nano-size (Nano-Se) are more toxic to Medaka (*Oryzias latipes*) as a consequence of hyper-accumulation of selenium: a comparison with sodium selenite. Aquat. Toxicol. 89, 251–256.

Li, C.C. and Chen, J.C., 2008. The immune response of white shrimp *Litopenaeus vannamei* and its susceptibility to *Vibrio alginolyticus* under low and high pH stress. Fish Shellfish Immunol. 25(6), 701–709.

Li, J.J., Muralikrishnan, S., Ng, C.T., Yung, L.Y.L. and Bay, B.H., 2010. Nanoparticle-induced pulmonary toxicity. Experim. Biol. Med. 235, 1025–1033.

Liau, S.Y., Read, D.C., Pugh, W.J., Furr, J.R. and Russel, A.D., 1997. Interaction of silver nitrate with readily identifiable groups: relationship to the antibacterial action of silver ions. Lett. Appl. Microbiol. 25, 279–283.

Lok, C.N., Ho, C.M., Chen, R., He, Q.Y., Yu, W.Y., Sun, H.Z., Tam, P.K.H., Chiu, J.F. and Che, C.M., 2006. Proteomic analysis of the mode of antibacterial action of silver nanoparticles. Journal of Proteome Research, 5: 916–924.

Luoma, S., 2008. Silver Nanotechnologies and the Environment, Woodrow Wilson International Center for Scholars. Washington, DC, USA, 72.

Luqing, P., Na, L., Hongxia, Z., Jing, W. and Jingjing, M., 2001. Effects of heavy metal ions (Cu^{2+}, Pb^{2+} and Cd^{2+}) on DNA damage of the gills, hemocytes and hepatopancreas of marine crab, *Charybdis japonica*. J. Oc. Univ. China (Ocean Coast Sea Res.). 10(2), 177–184.

Maria, V.L., Santos, M.A. and Bebianno, M.J., 2009. Contaminant effects in shore crabs (*Carcinus maenas*) from Ria Formosa Lagoon. Comp. Biochem. Physiol. C, 150, 196–208.

Martins, C.D.G., Barcarolli, I.F., de Menezes, E.J., Giacomin, M. M., Wood, C.M. and Bianchini, A., 2011. Acute toxicity, accumulation and tissue distribution of copper in the blue crab *Callinectes sapidus* acclimated to different salinities: In vivo and in vitro studies. Aquat. Toxicol. 101, 88–99.

Maynard, A.D., Aitken, R.J., Butz, T., Colvin, V., Donaldson, K., Oberdorster, G., Philbert, M.A., Ryan, J., Seaton, A., Stone, V., Tinkle, S.S., Tran, L., Walker, N.J. and Warheit, D.B., 2006. Safe handling of nanotechnology. Nature. 444, 267–269.

McComb, R.B., Bower, G.N. and Posen, S., 1979. Alkaline phosphatase, Plenum Press, New York.

Miao, A.J., Schwehr, K.A., Xu. C., Zhang, S.J. and Luo, Z.P., 2009. The algal toxicity of silver engineered nanoparticles and detoxification by exopolymeric substances. Environ. Poll. 157, 3034–3041.

Miao, A-J., Luo, Z., Chen, C-S., Chin, W-C., Santschi, P.H. and Quigg, A., 2010. Intracellular uptake: a possible mechanism for silver engineered nanoparticle toxicity to a freshwater alga *Ochromonas danica*. PLoS ONE. 5(12), e15196.

Moore, M.N., 2006. Do nanoparticles present toxicological risks for the health of the aquatic environment? Environ. Int. 32, 967–976.

Mueller, N.C. and Nowack, B., 2008. Exposure modeling of engineered nanoparticles in the environment. Environ. Sci. Technol. 42, 4447–4453.

Navarro, E., Piccapietra, F., Wagner, B., Marconi, F., Kaegi, R. and Odzak, N., 2008. Toxicity of silver nanoparticles to *Chlamydomonas reinhardtii*. Environ. Sci. Technol. 42, 8959–8964.

Nel, A., Xia, T., Madler, L. and Li, T., 2006. Toxic potential of materials at the nano level. Science. 11, 622–627.

Oberdorster, E., Rittschof, D. and McClellan-Green, P., 1998. Induction of cytochrome P450 3A and heat shock protein by tributyltin in blue crab, *Callinectes sapidus*. Aquat. Toxicol. 41, 83–100.

Oberdorster, E., Zhu, S.Q., Blickley, T.M., Clellan-Green, P. and Haasch, M.L., 2006. Ecotoxicology of carbon-based engineered nanoparticles: Effects of fullerene (C-60) on aquatic organisms. Carbon. 4, 1112–1120.

Oberholster, P.J., Musee, N., Botha, A-M., Chelulu, P.K., Fock, W.W. and Ashton, P.J., 2011. Assessment of the effect of nanomaterials on sediment-dwelling invertebrate *Chironmus tentans* larvae. Ecotoxicol. Environ. Saf. 74, 416–423.

O'Melia, C.R., 1980. Aquasols — The behaviour of small particles in aquatic systems. Environ. Sci. Technol. 14, 1052–1060.

Oukarroum, A., Bras, S., Perreault, F. and Popovic, R., 2012. Inhibitory effects of silver nanoparticles in two green algae, *Chlorella vulgaris* and *Dunaliella tertiolecta*. Ecotoxicol. Environ. Saf. 78, 80–85.

Pan, L.Q. and Zhang, H.X., 2006. Metallothionein, antioxidant enzymes and DNA strand breaks as biomarkers of Cd exposure in a marine crab, *Charybdis japonica*. Comp. Biochem. Physiol. C. 144, 67–75.

Park, S-Y. and Choi, J., 2010. Geno- and ecotoxicity evaluation of silver nanoparticles in freshwater crustacean *Daphnia magna*. Environ. Eng. Res. 15(1), 23–27.

Peralta-Videaa, J.R., Zhaoa, L. and Lopez-Morenoc, M.L., 2011. Nanomaterials and the environment: a review for the biennium 2008-2010. J. Haz. Mat. 186, 1–15.

Pereira, P., de Pablo, H., Subida, M.D., Vale, C. and Pacheco, M., 2009. Biochemical responses of the shore crab (*Carcinus maenas*) in a eutrophic and metal-contaminated coastal system (Obidos lagoon, Portugal). Ecotoxicol. Environ. Saf. 72, 1471–1480.

Pham, C.J., Yi, J. and Gu, M.B., 2012. Biomarker gene response in male Medaka (*Oryzias latipes*) chronically exposed to silver nanoparticle. Ecotoxicol. Environ. Saf. 78, 239–245.

Pinho, G.L.L., da Rosa, C.M., Maciel, F.E., Bianchini, A., Yunes, J.S., Proenca, L.A.O. and Monserrat, J.M., 2005. Antioxidant responses and oxidative stress after microcystin exposure in the hepatopancreas of an estuarine crab species. Ecotoxicol. Environ. Saf. 61, 353–360.

Rajalakshmi, S. and Mohandas, A., 2005. Copper-induced changes in tissue enzyme activity in a freshwater mussel. Ecotoxicol. Environ. Saf. 62, 140–143.

Ratte, H.T., 1999. Bioaccumulation and toxicity of silver compounds: a review. Environ. Toxicol. Chem. 18, 89–108.

Ringwood, A.H., McCarthy, M., Bates, T.C. and Carroll, D.L., 2010. The effects of silver nanoparticles on oyster embryos. Mar. Environ. Res. 36, S49–S51.

Roesijadi, G., 1992. Metallothioneins in metal regulation and toxicity in aquatic animals. Aquat. Toxicol. 22(2), 81–114.

Roh, J-Y., Sim, S.J., Yi, J., Park, K., Chung, K.H., Ryu, D-Y. and Choi, J., 2009. Ecotoxicity of silver nanoparticles on the soil nematode *Caenorhabditis elegans* using functional ecotoxicogenomics. Environ. Sci. Technol. 43, 3933–3940.

Romer, I., White, T.A., Baalousha, M., Chipman, K., Viant, M. R. and Lead, J.R., 2011. Aggregation and dispersion of silver nanoparticles in exposure media for aquatic toxicity tests. J. Chromatogr. 1218(27), 4226–4233.

Royal Commission., 2008. 27th Report: Novel Materials in the Environment: The Case of Nanotechnology. Royal Commission on Environmental Pollution: London, 154.

Saha, S., Ray, M. and Ray, S., 2009. Activity of phosphatases in the hemocytes of estuarine edible mudcrab, *Scylla serrata* exposed to arsenic. J. Environ. Biol. 30(5), 655–658.

Saini, R.K., Srivastava, A.K., Gupta, P.K. and Das, K., 2012. pH dependent reversible aggregation of Chitosan and glycol-Chitosan stabilized silver nanoparticles. Chem. Phys. Lett. 511, 326–330.

Sanders, B.M., 1993. Stress proteins in aquatic organisms: an environmental perspective. Crit. Rev. Toxicol. 23(1), 49–75.

Saravana Bhavan, P. and Geraldine, P., 2001. Biochemical stress responses in tissues of the prawn *Macrobrachium malcolmsonii* on exposure to endosulfan. Pesticide Biochem. Physiol. 70, 27–41.

Scown, T.M., Santos, E., Johnston, B.D., Gaiser, B., Baalousha, M., Mitov, S., Lead, J.R., Stone, V., Fernandes, T.F., Jepson, M., van Aerle, R., Tyler, C.R., 2010. Effects of aqueous exposure to silver nanoparticles of different sizes in rainbow trout. Toxicol. Sci. 115(2), 521–534.

Shugart, L.R., 2000. DNA damage as a biomarker of exposure. Ecotoxicol. 9, 329–340.

Stebounova, L.V., Guio, E., Grassian, V.H., 2011. Silver nanoparticles in simulated biological media: a study of aggregation, sedimentation, and dissolution. J. Nanopart. Res. 13, 233–244.

Stegeman, J.J., Brouwer, M., Richard, T.D.G., Forlin, L., Fowler, B.A., Sanders, B.M. and van Veld, P.A., 1992. Molecular responses to environmental contamination: enzyme and protein systems as indicators of chemical exposure and effect. In: Biomarkers: Biochemical, Physiological and Histological markers of Anthropogenic Stress, Huggett, R.J., Kimerly, R.A., Mehrle, P.M., Jr, Bergman, H.L., Eds., Lewis Publishers: Chelsea, MI, 235–335.

The Project on Emerging Nanotechnologies. Available at <http:/www.nanotechnology.org/> accessed 4 July 2012

Van der Oost, R., Beyer, J. and Vermeulen, N.P.E., 2003. Fish bioaccumulation and biomarkers in environmental risk assessment: a review. Environ. Toxicol. Pharm. 13, 57–149.

Velzeboer, I., Hendriks, A.J., Ragas, A.M.J. and van de Meent, D., 2008. Aquatic ecotoxicity tests of some nanomaterials. Nanomat. Environ. 27(9), 1942–1947.

Vijayavel, K.and Balasubramanian, M.P., 2006. Fluctuations of biochemical constituent and marker enzymes as a consequence of naphthalene toxicity in the edible estuarine crab, *Scylla serrata*. Ecotoxicol. Environ. Saf. 63, 141–147.

Weis, J.S. and Weis, P., 1989. Effects of environmental pollutants on early fish development. Rev. Aquat. Sci. 1, 45–73.

Wiench, K., Wohlleben, W., Hisgen, V., Radke, K. and Salinas, E., 2009. Acute and chronic effects of nano- and non-nano-scale TiO2 and ZnO particles on mobility and reproduction of the freshwater invertebrate *Daphnia magna*. Chemosphere. 76, 1356–1365.

WHO (World Health Organization), 2001. Biomarkers in Risk Assessment: Validity and Validation. Environmental Health Criteria 222, World Health Organization: Geneva, 238 pp.

Yeo, M.K. and Pak, S.W., 2008. Exposing zebrafish to silver nanoparticles during caudal fin regeneration disrupts caudal fin growth and p53 signaling. Mol. Cell. Toxicol. 4(3), 11–17.

Ying, W. and Xiong, Z.G., 2010. Oxidative stress and NAD(C) in ischemic brain injury: current advances and future perspectives. Curr. Med. Chem. 17, 2152–2158.

Zhao, C-M. and Wang, W-E., 2010. Biokinetic uptake and efflux of silver nanoparticles in Daphnia magna. Environ. Sci. Technol. 44, 7699–7704.

Zhou, J., Wang, W.-N., Wang, A.-L., He, W.-Y., Zhou, Q.-Y., Liu, Y. and Xu, J., 2009. Glutathione S-transferase in the white shrimp *Litopenaeus vannamei*: Characterization and regulation under pH stress. Comp. Biochem. Physiol. C. 150, 224–230.

CHAPTER 3: AGGREGATION AND DISSOLUTION OF SILVER NANOPARTICLES IN A LABORATORY-BASED FRESHWATER MICROCOSM UNDER SIMULATED ENVIRONMENTAL CONDITIONS

This chapter was published as an article in Toxicological & Environmental Chemistry on April 2014 copyright Taylor & Francis, available online: http://www.tandfonline.com/ Article DOI: 10.1080/02772248.2014.904141

Abstract

Silver nanoparticles (NP) are used in several applications, including their use as antimicrobial agents in textiles, personal care, and other domestic products. As such, there is a high potential for the release of silver nanoparticles (AgNP) in the aquatic environment. In aquatic ecosystems, nanomaterials are affected by abiotic factors, such as temperature, that alter their chemistry and influence their fate in the environment. Preliminary studies indicate that NP tend to form aggregates which are potentially more recalcitrant than unaggregated NP. These and other fate processes are largely dependent on both the characteristics of the NP and that of the environment. In this study, lab experiments were conducted to investigate the physicochemical properties and temperature solubility of AgNP (<100 nm) that may potentially influence the fate and behavior of AgNP in the aqueous environment. Results indicated that, under these tested conditions, AgNP may be transformed in size and thereby affect fate, bioavailability, and toxicity. In this study, a novel method was used to determine whether AgNP would form agglomerates, or behave as isolated particles, or dissolve when in aqueous media and under different environmental conditions. The new aspects evaluated in this study demonstrated that AgNP are transformed in both size and state under variable environmental conditions.

Keywords: Aggregation; characterization; dissolution; environmental changes; microcosms; nanomaterials; silver nanomaterials

1. Introduction

The unique properties of nanomaterials (NM) and their increasing use in several consumer products raised concerns regarding potential adverse effects on environmental systems. Of all NM, silver nanoparticles (AgNP) are the most widely used, making up approximately 55% of NP-containing consumer products (www.nanotechproject.org). Due to their antibacterial properties, AgNP are present in several consumer and domestic products. As a result, release of AgNP in the environment is expected to increase in the near future.

Freshwater ecosystems are susceptible to environmental contamination since they are at the receiving end of contaminants, particularly from runoff sources. Once released into the environment, AgNP are expected to form aggregates, which may be trapped or eliminated through sedimentation (Farre *et al*., 2009). Generally, aggregated NP are less mobile than individual NP (Nowack and Bucheli, 2007). However, NP are still taken up by aquatic organisms (Gagne *et al*., 2013). The aggregation process depends on several factors, including particle shape, particle size, particle surface area, and particle surface charge (Handy *et al*., 2008) Tiede *et al*., 2009), as well as physicochemical processes and the characteristics of the aquatic system (Farre *et al*., 2009). The dissolution of AgNP is also a significant process determining NP-mediated effects in the aquatic environment and organisms.

The state and behaviour of NP in the environment are dependent on environmental conditions, including temperature, pH, and ionic strength, in which NP occur. As such, characterization of both the physical and chemical properties of NM and that of the environment is necessary in order to predict the NM behaviour and potential effects on the environment. It is known that temperature directly affects aquatic ecosystem communities (Carpenter *et al*., 1992), since it is regarded as an important abiotic factor influencing the growth and production of primary producers such as algae and macrophytes, thereby also affecting species distribution. Liu and Hurt (2010) reported higher dissolution rates of AgNP with increased temperature. Similarly, sudden hydrographic activity like high flood conditions may produce resuspension and redistribution of sediments. Extreme flood events substantially enhance the potential toxicity of river waters (Byrne *et al*., 2009). These changes are likely to affect inputs of contaminants into the environment as well as affect behaviour, fate, and transport within aquatic environments. In addition, aquatic sediments, which act as sinks for contaminants, including NM, might be released during periods of extreme flood conditions (Weber *et al*., 2009).

In an effort to link environmental scenarios and behavioural responses of AgNP, this study focused on the behaviour of AgNP associated with: (1) increased water temperatures and (2) intensities of high-flow (flooding) events. Specifically, the primary objective of this study was to: (1) investigate whether AgNP are present as isolated particles or as aggregates in aqueous solutions under different environmental conditions and (2) determine whether these environments promote dissolution of the AgNP.

2. Experimental methodology

2.1. Materials and particle characterization

Silver nanopowder (CAT no. 7440-22-4) was purchased from Sigma-Aldrich, South Africa. Based on the manufacturer's specifications, AgNP were spherical particles with a particle size < 100 nm, specific surface area of 5 m^2/g, density of 10.5 g/cm^3, and purity of 99.5% based on trace metal analysis. Our own characterization of AgNP was carried out to confirm these specifications. An EVO ® MA15 scanning electron microscope (SEM) was used to characterize particle size where an AgNP suspension was pipetted on to the carbon surface of an SEM stub. An SEM with energy-dispersive X-ray (EDX) spectrometry capabilities was used to confirm the presence of Ag. Samples were identified with secondary electrons and/or secondary electron images, and compositions were quantified by EDX analysis using an Oxford Instruments® X-Max 20 mm^2 detector and Oxford INCA software. Transmission electron microscopy (TEM) characterization was performed to obtain the AgNP size and morphology. This was achieved by using a JEOL 1200-EX II electron microscope at an accelerating voltage of 120 kV. Samples were imaged with a MegaView Camera with Gatan Microscopic software with a resolution of 1376 x 1032, and two seconds exposure time. In brief, a suspension of AgNP was dispersed in ethanol, and subsequently deposited on to copper grids and air-dried. ImageJ software was used to generate a particle size distribution based on the TEM images. Brunauer–Emmet–Teller (BET) surface areas were determined using ASAP 2010 (Accelerated Surface Area and Porosimetry System; Micromeritics Instrument Corporation, GA, USA). In brief, samples were degassed overnight at 100 °C prior to analysis. Powder X-ray diffraction (PXRD), using a Panalytical X'pert Pro, was used to determine the crystalline nature of the AgNP. The PXRD pattern was collected between angles of 2Θ from 3° to 90°.

Dissolution of total Ag was measured in unfiltered aqueous media and in the sediment of each AgNP treatment using inductively coupled plasma optical emission spectrometry (ICP-OES) following sample digestion with concentrated HNO_3 under pH < 2 conditions. Digital photographs were used to observe NP settlement (*i.e.* sedimentation) on the surface of the sediment layer. On day 7, a subsample was taken from each treatment for morphological characterization using TEM. Table 3.1 is a summary of exposure conditions used for these experiments.

Table 3.1: Composition of microcosm for each treatment (T1–T4).

	Treatment			
Parameter	**T1**	**T2**	**T3**	**T4**
Water				
Volume (mL)	100	100	100	100
Temperature (°C)	15.4	28.4	32.4	15.4
Sediment				
Kaolin (g; %)	9.90; 30	9.90; 30	9.90; 30	9.90; 30
Quartz sand (g; %)	21.45; 65	21.45; 65	21.45; 65	21.45; 65
A-cellulose (g; %)	1.58; 4.8	1.58; 4.8	1.58; 4.8	1.58; 4.8
Calcium carbonate (g; %)	0.07; 0.2	0.07; 0.2	0.07; 0.2	0.07; 0.2
Nanoparticle				
Concentration (mg/mL)	1	1	1	1
Agitation	0	0	0	70

2.2. Microcosm preparation

This study involved four different microcosm treatments: (1) a laboratory-controlled microcosm kept at ambient temperature (*i.e.* 15.37 °C; T1), (2) a low-temperature regime kept at a contact temperature of 28.4 °C (T2), (3) a high-temperature regime kept at a constant temperature of 32.4 °C (T3), and (4) a high-flow regime (T4) representing

conditions of increased agitation due to flood events. The high-flow regime was achieved by placing the beaker in an orbital shaker at a speed of 70 revolutions per minute (rpm). Each treatment was performed in triplicate for statistical purposes.

Formulated sediment was used in all treatments and was composed of kaolin (30%), quartz sand (65%), a-cellulose (4.8%), and calcium carbonate (0.2%). The sediment composition was based on other freshwater microcosm studies undertaken by Clement *et al*. (2004) and Clement and Cadier (1998). Approximately, 33 g of formulated sediment was distributed into 250 ml beakers. Approximately, 100 ml deionized water was added to each beaker and allowed to saturate (Figure 3.1). Stock suspensions of AgNP were prepared by dispersing 1 mg dry AgNP into 1 ml deionized water. Mixtures were sonicated for 5 min in an ultrasonic bath in order to disrupt any possible aggregates. The 1 mg/mL AgNP suspension was added to each of the beakers and left to settle. Microcosmic exposure experiments lasted for seven days.

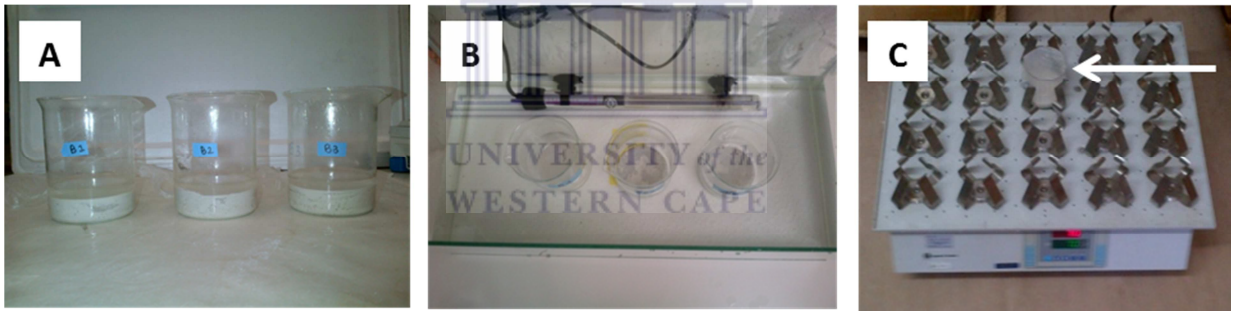


Figure 3.1: Microcosm setup containing formulated sediment, deionized water, and AgNP suspension for each treatment (A = T1, B = T2 and T3, C = T4).

The low-temperature (T2) and high-temperature (T3) regimes were achieved by placing the beakers in a 450 200 mm fish tank half filled with water and fitted with an aquarium heater set to achieve a temperature of 28.4 °C representing the low-temperature regime (*i.e.* T2) and 32.4 °C representing the high-temperature regime (*i.e.* T3). To mimic the high-flow regime (T4), beakers were placed in a Model OS-560D orbital shaker at a speed of 70 rpm for the entire exposure period of seven days. All treatments were subjected to an exposure period of seven days. The pH, temperature (°C), electrical conductivity (EC), dissolved oxygen (DO), and redox potential (Eh) were measured in triplicate in the aqueous medium prior to day 0 and at regular intervals throughout the exposure period. A Hanna HI 8424 portable meter was

used to measure temperature and Eh, a Hanna HI 991301 multimeter was used to measure pH and EC, while a Crison OXI 45P portable meter was used for DO measurements. On day 7, a subsample was taken for morphological characterization.

2.3.Statistical analysis

Statistical analysis was performed using Microsoft Excel and XLStat2015® statistical packages. A correlation matrix was constructed to assess potential collinearity among variables.

3. Results and discussion

3.1. Characterization of test waters

The pH, temperature (°C), EC, and DO were measured in triplicate in the aqueous medium of each treatment at regular intervals throughout the exposure period. These physicochemical compositions are shown in Figure 3.2. The pH values across all treatments were relatively similar with values ranging from 4.17 in T1 to 7.64 in T2. The average pH values across the 7-day exposure period were 5.63 ± 1.05, 7.15 ± 0.35, 6.13 ± 0.34, and 5.31 ± 1.36 in treatments T1–T4, respectively. Values for EC, DO, and Eh were in the range 200–760 µS/cm, 7.26–10.45 mg/L, and 63.8–321.5 mV, respectively.

Lowest pH values were recorded where temperatures were not manipulated, i.e. at T1 (mean pH = 5.63 ± 1.05) and T4 (mean pH = 5.31 ± 1.36), while the highest pH values were recorded in the T2 (mean pH = 7.15 ± 0.35) and T3 (mean pH = 6.12 ± 0.34) regimes. Similar observations were obtained for the average EC measurement, whereas the inverse observation was made for Eh and DO measurements.

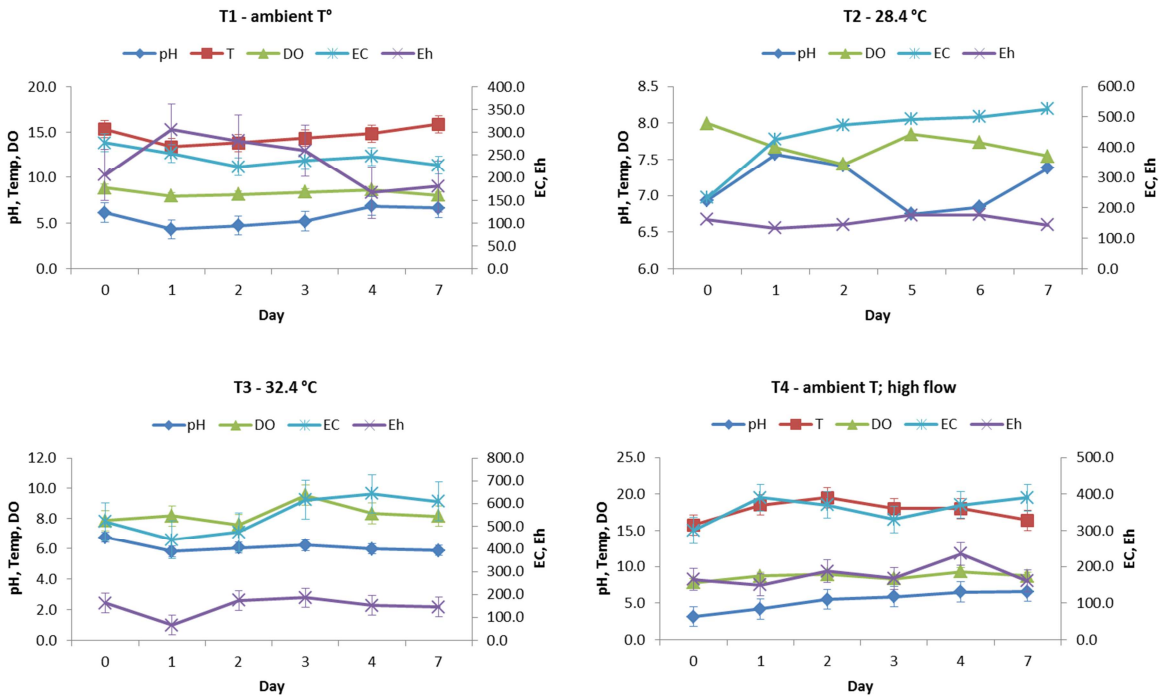


Figure 3.2: Average physicochemical parameters measured in each treatment (T1–T4) (temperature in °C, DO in mg/L, EC in mS/cm, and Eh in mV).

3.2. Characterization of AgNP

3.2.1. Initial particle characterization of commercially manufactured dry AgNP

The aggregation state of AgNP is an important property to evaluate since it impacts NP fate, transport, and toxicity (Levard *et al*., 2012). Scanning (SEM; Figure 3.3) and transmission (TEM; Figure 4) electron microscopy analyses were used to measure the particle size. SEM and TEM revealed that AgNP formed small, loosely packed aggregates not more than 100 nm in size. The primary and aggregate size of AgNP in the dry state is presented in the TEM image in Figure 3.4. The TEM image verified the spherical nature of AgNP, while the particle size distribution showed that the majority of particles measured 10 nm, with a small quantity of larger particles in the 50–100 nm range. EDX confirmed the presence of elemental Ag (Figure 3.3). The specific surface area of the AgNP was determined to be 7.533 $m^2/g \pm 0.0028$ (Figure 3.5). The PXRD pattern recorded for AgNP, collected between angles of 2Θ from 3° to 90°, is shown in Figure 3.6, and demonstrated the crystalline nature of the AgNP.

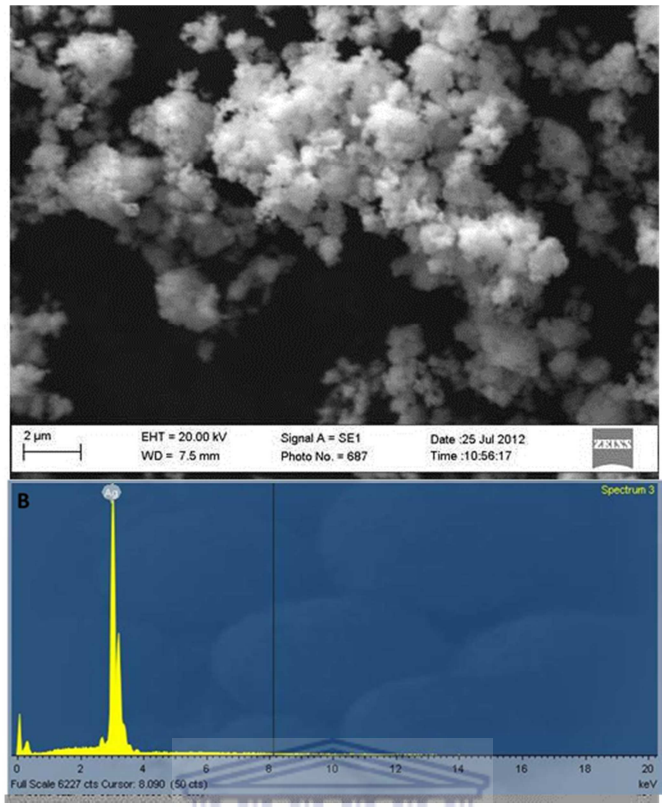


Figure 3.3: SEM image of AgNP showing spherical particles in the order of 100 nm (top) and its corresponding EDX spectrum showing elemental Ag composition (bottom).

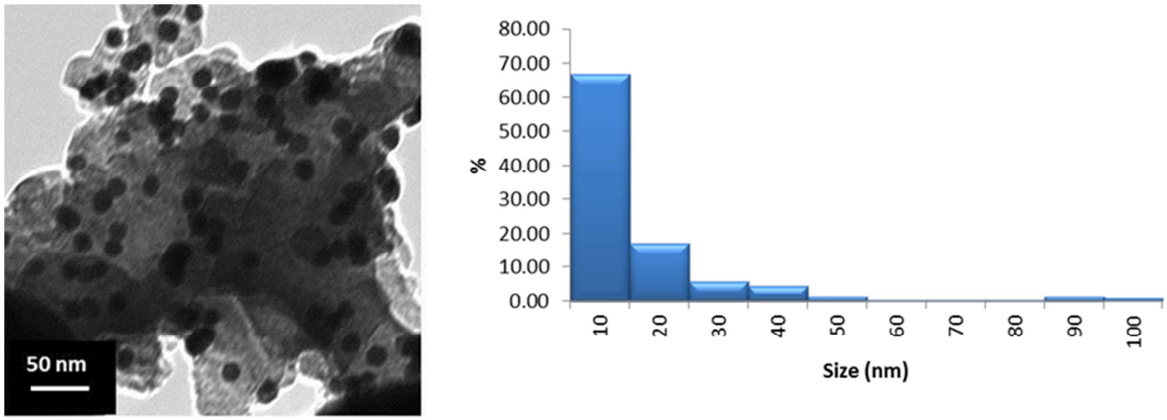


Figure 3.4: TEM image of AgNP (left) with the corresponding particle size distribution (n = 200) (right).

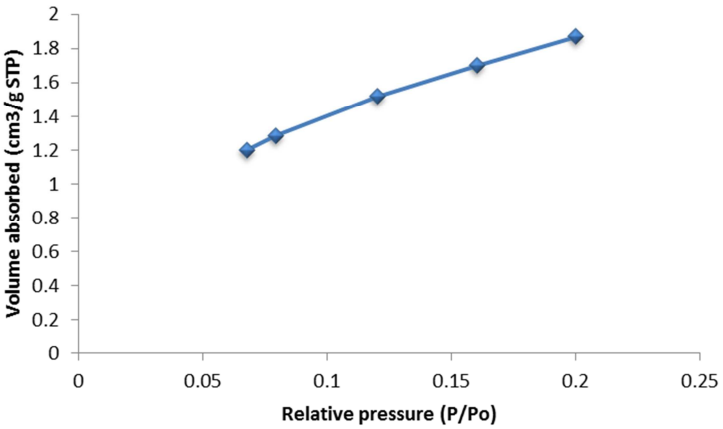


Figure 3.5: Results for the BET surface area of the AgNP.

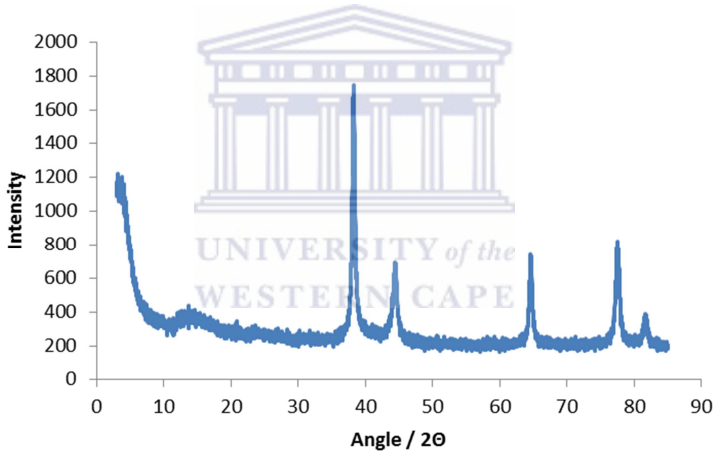


Figure 3.6: PXRD pattern of AgNP.

3.2.2. Characterization of aqueous AgNP suspensions

The morphology and particle size distribution of AgNP were determined for AgNP suspensions under ambient conditions (T1; average temperature = 14.6 °C). The TEM analysis and digital photographs taken daily are shown in Figures 3.7 and 3.8, respectively. Digital photographs indicated that aggregation of the primary AgNP in the aqueous phase was more pronounced from day 4. The TEM images indicated that the particle size of the dry AgNP increased when in suspension, with particles measuring >100 nm in size as evidenced by the particle distribution histogram (Figure 3.7).

The toxicity of Ag has been widely investigated by several authors (Ratte, 1999; Roh *et al*., 2009; Gagne *et al*., 2013), who reported adverse effects of Ag in aquatic organisms. The dissolution of AgNP in the aqueous phase under ambient conditions was investigated using ICP-OES. The bioavailability and toxicity of AgNP are affected by the rate of dissolution of the AgNP. A slightly higher total Ag was measured in the aqueous medium when compared to the sediment for the T1 treatment (0.53 mg vs. <0.44 mg, respectively; Table 3.2), which suggests that AgNP did not readily settle in sediment but remained suspended during this treatment. This is also supported by the digital photographs which showed a lack of pigmentation on the sedimentary layer.

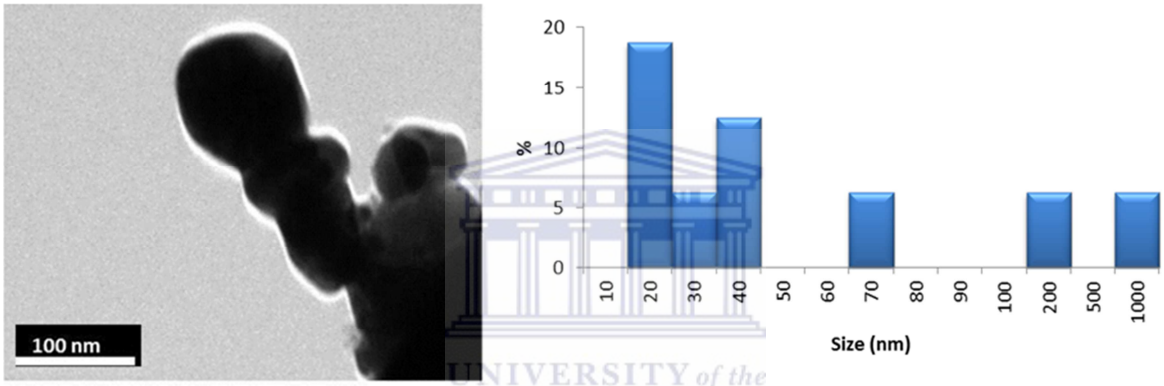


Figure 3.7: TEM image of AgNP suspension for control (T1) taken at 20,000 magnification and the associated histogram of particle distribution.

Figure 3.8: Digital photographs of AgNP suspension for control (T1) indicating increased aggregation between day 1 and day 4.

Table 3.2: Summary of analysis for Ag (mg) in AgNP in water and sediment for each treatment (T1–T4) at day 7.

	Treatment			
Medium	**T1**	**T2**	**T3**	**T4**
Ag in water (mg; %)	0.53; 53	0.047; 4.7	0.011; 1.1	0.003; 0.3
Ag in sediment (mg; %)	0.44; 44	0.82; 82	0.69; 69	0.62; 62
% recovery	97.0	86.7	70.1	65.3

3.2.3. Aggregation and dissolution behavior of AgNP in different environmental scenarios

Since NP display different aggregation states depending on the chemical environment, characterization in environmental media is important in toxicological experiments. Similarly, morphology is an important parameter dictating the fate of AgNP in the environment (Levard *et al*., 2012). TEM analysis was used to determine morphological characteristics and particle size distributions of AgNP suspensions under different environmental conditions. Images and digital photographs are shown in Figures 9 and 10, respectively. Figure 3.9A shows the TEM image from treatment T2. Results indicate that AgNP were present as larger aggregates. This implies that NP toxicity is largely reduced, since it is known that larger aggregates reduce bioavailability and toxicity (Navarro *et al*., 2008). From the TEM image for T3 (Figure 3.9B), minimal aggregation is visible as indicated by the arrows, while larger aggregates were visible in T4 (Figure 3.9C).

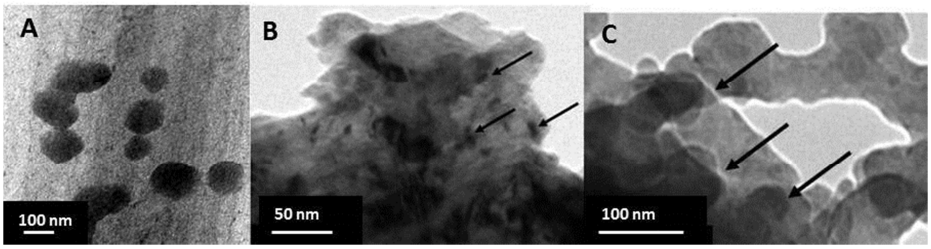


Figure 3.9: TEM images of low-temperature (T2; A), high-temperature (T3; B), and high-flow (T4; C) regimes.

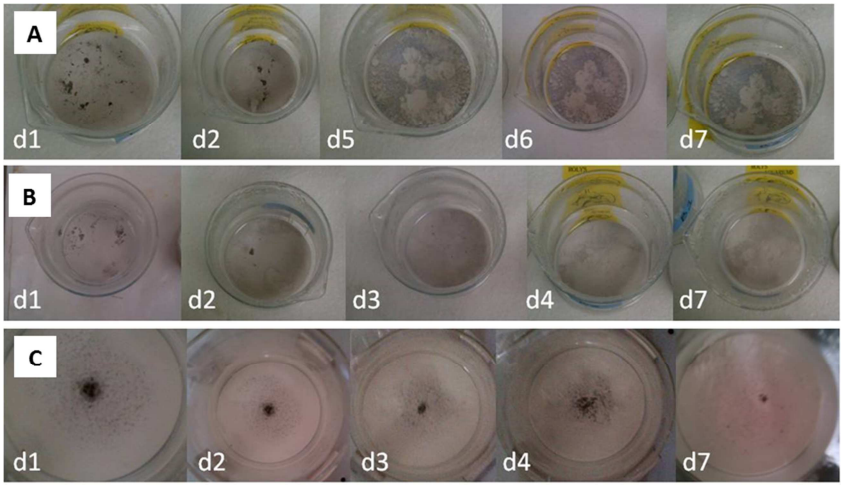


Figure 3.10: Digital photographs of AgNP suspensions under low-temperature (A), high-temperature (B), and high-flow (C) regimes.

Evident in the digital photographs of the temperature-manipulated treatments (*i.e.* T2 and T3; Figure 3.10) is the initial particle aggregation at the beginning of the exposure periods. The lack of obvious aggregation on the water surface from day 5 in T2 and day 3 in T3 likely reflects dissolution of AgNP at elevated temperatures, and possible sedimentation, since aggregation typically leads to sedimentation (Kent, 2011). This was supported by the pigmentation of sediments depicted on day 5 to day 7 in T2, and day 3 to day 7 in T3.

The dissolution of AgNP in aqueous media contributes to the concentration of Ag in the environment (Kent, 2011). The mass of total Ag in solution following an NP exposure was quantified using ICP-OES analysis. The results for treatments T2–T4 are reported in Table 2. It is generally accepted that dissolution occurs more rapidly at elevated temperatures (Liu and Hurt, 2010). This was supported when comparing the digital photographs of day 2 in treatments T2 and T3, whereas this trend was not observed in T1 and T4 (treatments where the temperature was not manipulated and was generally below 20 °C). The ICP-OES analyses of the aqueous and sedimentary media indicated that approximately 4.7% of the AgNP remained in solution and 82% settled in sediment. The amount of total Ag at the end of the seven-day exposure period for T2 and T3 was 0.047 and 0.011 mg, respectively. Total Ag concentrations were more prominent in the T2 regime compared to T3, differing from reports that concluded that increased temperatures favour dissolution of AgNP. In contrast, our results indicated that the highest total Ag concentrations were measured in the sediment of T3, suggesting that temperatures favour sedimentation. This is also supported by the correlation matrix in Tables 3.3 and 3.4, which indicates that aqueous total Ag concentrations

are correlated with temperature (likely associated with T2), as well as with Eh and EC, while sedimentary total Ag concentrations were strongly correlated with pH. Several studies attempted to link dissolution to pH, temperature, AgNP concentration, and NP size. Kittler *et al*. (2010) reported that dissolution of PVP-coated AgNP fluctuated from 5% at 5 °C to 50% at 25 °C to 90% at 37 °C. AgNP are expected to dissolve more rapidly at low pH due to increased dissolution rates (Liu and Hurt, 2010).

Table 3.3: Correlation between aqueous Ag concentrations versus physicochemical parameters.

	[Ag] mg/L	Eh	pH	T	DO	EC
[Ag] mg/L	1					
Eh	0.95	1				
pH	-0.34	-0.55	1			
T	-0.69	-0.88	0.72	1		
DO	0.02	0.22	-0.93	-0.42	1	
EC	-0.82	-0.94	0.49	0.95	-0.14	1

Table 3.4: Correlation between sedimentary Ag concentrations versus physicochemical parameters.

	[Ag] mg/kg	Eh	pH	T	DO	EC
Ag	1					
Eh	-0.56	1				
pH	0.91	-0.55	1			
T	0.54	-0.88	0.72	1		
DO	-0.86	0.22	-0.93	-0.42	1	
EC	0.37	-0.94	0.49	0.95	-0.14	1

In this study, sedimentary total Ag concentrations were strongly correlated with pH and DO (Table 3.4). Increased precipitation (and associated agitation induced by flood events), may enhance the toxicity of contaminants by altering water chemistry and metabolic rates of

contaminants (Schiedek *et al*., 2007). The TEM images obtained for primary AgNP suspensions in the flood regime (T4) show micrometer-size aggregates of varying densities (Figure 3.9C). The digital photographs taken during T4 confirm that the stirring action promoted almost immediate aggregation on the water surface and little dissolution. This was confirmed by the relatively low total Ag measured in aqueous media analyzed from this treatment (0.003 mg; Table 3.2). In contrast, sediments obtained from T4 had similar total Ag concentrations (0.69 mg in T3 vs. 0.65 mg in T4) to those reported for sediments obtained from T3, suggesting that both elevated temperature and agitation promoted the sedimentation of AgNP.

4. Conclusions

The purpose of this study was to contribute to the growing knowledge of potential environmental effects of AgNP, particularly considering variable environmental conditions. Since AgNP are the most widely used NM, their likelihood of entering water resources is significant. Changes in temperature and flood events may lead to altered water chemistry and metabolic rates of contaminants (Schiedek *et al*., 2007). This study attempted to answer relevant questions pertaining to fate, transport, and toxicity of AgNP, and the different environmental conditions under which AgNP aggregate. In addition, the dissolution of AgNP contributes to the concentrations of Ag in the aquatic environment.

The novelty of this research lies in a few key points. To our knowledge, most studies have dealt with a single parameter and have assessed NP behaviour in only one environmental medium, whereas our study investigated the effects of both variable temperature and flow regimes in Ag release in both aqueous and sedimentary media. The new aspects evaluated in this study showed that AgNP may be transformed in both size and state under variable environmental conditions. In this study, AgNP aggregation and dissolution were largely induced by temperature changes as well as the agitated influences associated with flood events. It is known that aggregated NPs tend to be less mobile than individual NP. NP aggregation was more enhanced particularly in T2 when compared to other treatments, as evidenced by the TEM results, suggesting that, although aggregated NPs tend to be less toxic, these particles are taken up by filter feeders and sediment-dwelling animals and may lead to possible biomagnification in the food chain (Farre *et al*., 2009). This study also observed that total Ag release and sedimentation are more enhanced at higher temperatures and agitation

promoted by flood events, which suggests AgNP present in the environment during increased temperatures and hydrographic events might present a more severe threat to aquatic organisms. In addition, our microcosms constitute a simulated scenario for short-term-exposures experiments.

5. Acknowledgments

This work was funded by the National Research Foundation (NRF) Thuthuka Grant. The following field personnel are also acknowledged: Mr Charlton van de Horst and Ms Bongiwe Silwana. The images presented were generated using instruments and services provided by the Central Analytical Facility (CAF) at the University of Stellenbosch (SUN). The authors also wish to thank the following for their work undertaken: Dr Vincent Smith (SUN) and Mrs Lize Engelbrecht (SUN).

6. References

Byrne, P., Reid, I. and Wood, P.J., 2009. Short-term fluctuations in heavy metal concentrations during flood events through abandoned metal mines with implications for aquatic ecology and mine water treatment. In Abstract of the International Mine Water Conference, 124–129. Pretoria, South Africa.

Carpenter, S.R., Fisher, S.G., Grimm, N.B and Kitchell, J.F., 1992. Global change and freshwater ecosystems. Ann. Rev. Ecol. Syst. 23, 119–139.

Clement, B., and Cadier, C., 1998. Development of a new laboratory freshwater/sediment microcosm test. Ecotoxicol. 7, 279–290.

Clement, B., Devaux, A., Perrodin, Y., Danjean, M. and Ghidini-Factus, M., 2004. Assessment of sediment ecotoxicity and genotoxicity in freshwater laboratory microcosms. Ecotoxicol. 12, 323–333.

Farre, M., Gajda-Schrantz, K., Kantiani, L. and Barcelo, D., 2009. Ecotoxicology and Analysis of nanomaterials in the aquatic environment. Anal. Bioanal. Chem. 393, 81–85.

Gagne, F., Auclair, J., Turcotte, P. and Gagnon, C., 2013. Sublethal effects of silver nanoparticles and dissolved silver in freshwater mussels. J. Toxicol. Environ. Health A, 76: 479–490.

Handy, R.D., Owen, R. and Valsami-Jones, E., 2008. The ecotoxicology of nanoparticles and nanomaterials: current status, knowledge gaps, challenges, and future needs. Ecotoxicol. 17, 315–325.

Kent, R.D., 2011. Controlled evaluation of silver nanoparticle dissolution using atomic force microscopy. (MSc thesis), Virginia Polytechnic Institute and State University.

Kittler, S., Greulich, C., Diendorf, J., Koller, M. and Epple, M., 2012. Toxicity of silver nanoparticle increases during storage because of slow dissolution under release of silver ions. Chem. Mat. 22, 4548–4554.

Levard, C., Hotze, E.M., Lowry, G.V., and Brown, G.E., 2012. Environmental transformations of silver nanoparticles: impact on stability and toxicity. Environ. Sci. Technol. 46, 6900–6914.

Liu, J., and Hurt, R.H., 2010. Ion release kinetics and particle persistence in aqueous nano-silver colloids. Environ. Sci. Technol. 44, 2169–2175.

Navarro, E., Piccapietra, F., Wagner, B., Marconi, F., Kaegi, R. and Odzak, N., 2008. Toxicity of silver nanoparticles to *Chlamydomonas reinhardtii*. Environ. Sci. Technol. 42, 8959–8964.

Nowack, B., and Bucheli, T.D.. 2007. Occurrence, behaviour and effects of nanoparticles in the environment. Environ. Poll. 150, 5–22.

Ratte, H.T., 1999. Bioaccumulation and toxicity of Ag compounds: A review. Environ. Toxicol. Chem. 18, 89–108.

Roh, J-Y., Sim, S.J., Yi, J., Park, K., Ryu, D-Y., and Choi, J., 2009. Ecotoxicity of silver nanoparticles on the soil nematode *Caenorhabditis elegans* using functional ecotoxicogenomics. Environ. Sci. Technol. 43, 3933–3940.

Schiedek, D., Sundelin, B., Readman, J.W., and Macdonald, R.W., 2007. Interactions between climate change and contaminants. Mar. Poll. Bull. 54, 1845–1856.

Tiede, K., Hassellov, M., Breitbarth, E., Chaudhry, Q. and Boxall, A.B., 2009. Considerations for environmental fate and ecotoxicity testing to support environmental risk assessments for engineered nanoparticles. J. Chromatogr. A. 1216, 503–509.

Weber, F-A., Voegelin, A. and Kretzschmar, R., 2009. Multi-media contaminant dynamics in temporarily flooded soil under sulphate limitation. Geochim. Cosmochim. Acta. 73, 5531–5527.

CHAPTER 4: EFFECT OF TEMPERATURE ON OXIDATIVE STRESS PARAMETERS AND ENZYME ACTIVITY IN THE TISSUES OF *POTAMONAUTES PERLATUS* FOLLOWING EXPOSURE TO SILVER NANOPARTICLES

This chapter has been accepted for publication by Journal of Toxicology and Environmental Health, Part A: Current Issues. Article DOI: 10.1080/15287394.2015.1106357

Abstract

Biomarkers of oxidative stress have been widely used in environmental assessments to evaluate the effects of exposure of aquatic organisms to contaminants from various anthropogenic sources. Silver nanoparticles (AgNPs), the most produced NPs worldwide and used in several consumer products, are known to cause oxidative stress in aquatic organisms. Similarly, temperature is also known to affect reactive oxygen species (ROS) by affecting the inputs of contaminants into the environment, as well as affecting their behaviour, fate and transport. Aquatic ecosystems are affected by both anthropogenic releases of contaminants and increased temperature. To test this hypothesis, the influence of AgNPs and temperature in the response to multiple biomarkers of oxidative stress were studied in the gills and hepatopancreas of the Cape River crab *Potamonautes perlatus*. Responses were evaluated through activities of antioxidant enzymes, *i.e*. superoxide dismutase (SOD), catalase (CAT), and the non-enzymatic antioxidant glutathione S-transferase (GST). The response of the oxidative stress biomarkers analysed was always higher in the hepatopancreas than in the gills. Elevated temperatures (28 °C) induced oxidative stress by increasing SOD, CAT and GST activities particularly at 100 µg/mL AgNPs. These data indicate that AgNP toxicity to *P. perlatus* is modulated by elevated temperatures, although this relationship is not linear as discussed later in this paper. Co-effects of AgNPs and temperature are reported for the first time in *P. perlatus*.

Keywords: Antioxidant enzyme activity; gills, hepatopancreas, oxidative stress; *Potamonautes perlatus*; silver nanoparticles, temperature

1. Introduction

The increasing use of nano-products exemplifies the need to clarify the environmental risks associated with the impacts of nanomaterials (NMs). Environmental pollutants (such as nanoparticles (NPs)) and changes in environmental conditions (such as temperature) can induce oxidative damage in aquatic organisms by altering antioxidant enzyme activity (Vinagre *et al*., 2014). Silver NPs are the most widely used NPs, present in several consumer products mainly because of their anti-bacterial properties. It is estimated that the annual production exceeds 1000 tons/year (Piccinno *et al*., 2012). Furthermore, it is estimated that more than 15% of Ag released into waters will come from plastics and textiles containing AgNPs (Blaser *et al*., 2008). The generation of reactive oxygen species (ROS) and its associated free radicals is known to cause oxidative stress and activation/inactivation of antioxidant defence system (Lapresta-Fernández *et al*., 2012). As such, there is an increasing risk of environmental contamination by AgNPs since exposure to AgNPs is known to induce the production of ROS (Ahamed *et al*., 2010; Hayashi *et al*., 2012; Levard *et al*., 2012; Piao *et al*., 2011). In terms of temperature, the current climate models indicate an increase in atmospheric temperatures by 2.4 – 6.4 °C (IPCC, 2007). It has been reported that fluctuating environmental temperatures can inflict stress leading to oxidative stress in organisms by the production of ROS and the inability to detoxify ROS (Halliwell, 1994; Ahmed *et al*., 2005). For instance, the formation of ROS during heat stress can occur as a direct consequence of hyperthermia (Abele *et al*., 2002; Freire *et al*., 2011; Lushchak, 2011) but also as a result of tissue re-oxygenation during recovery (Halliwell and Gutteridge, 1999). Previous observations on temperature effects have addressed several aquatic organisms: crabs (Novo *et al*., 2005), shrimps (Wang *et al*., 2006), and fish (Madeira *et al*., 2013). Similarly, the effects of exposure to AgNPs have also been documented for aquatic plants (Navarro *et al*., 2008; Gubbins *et al*., 2011), daphnia (Zhao and Wang, 2010; Gaiser *et al*., 2012), earthworms (Gomes *et al*., 2015), and fish (Griffit *et al*., 2012; Pham *et al*., 2012). So far these two important stresses on aquatic ecosystems have mainly been discussed independently and information on their combined responses is lacking. In order to fill this gap of knowledge, this study was undertaken to evaluate the cumulative effects of these stressors that act on similar pathways (such as toxicity, oxidative stress responses). The hypothesis behind the work was that exposure to temperature modulates toxicity of and cellular responses to AgNPs.

To cope with these and other stressors, aquatic organisms are able to modulate their physiological and biochemical metabolism through antioxidant defences, which consist mainly of antioxidant enzymes (such as superoxide dismutase (SOD), catalase (CAT), and glutathione S-transferase (GST)). These antioxidant enzymes reduce the damaging effects of hydrogen peroxide (H_2O_2) to prevent the production of hydroxyl radicals (HO•), the most damaging oxygen species (Halliwell and Gutteridge, 1999). These oxidative stress biomarkers have been widely used an "early-warning" signs of environmental stress. Antioxidant enzymes perform a pivotal function in maintaining redox homeostasis in tissues by initiating corresponding modulations in biochemical metabolism. As such, any modulation in such levels is used as a biomarker of oxidative stress in aquatic organisms to monitor stressors and pollutants. In aquatic organisms, redox state is achieved through the balance between the rate of ROS generation and the rate of its neutralization by the antioxidant capacity of the organism. As such, organisms experience oxidative stress when the rate of generation of ROS exceeds the rate of neutralization, either due to the increase generation of ROS or due to the decrease in antioxidant capacity (Halliwell and Gutteridge, 2007). Oxidative stress caused by an over-production of ROS can ultimately cause injury to cells.

The Cape River crab *Potamonautes perlatus* is a widely available species in South Africa's freshwater resources, but mostly common in the waters of the south-western region of the Western Cape (Snyman *et al*., 2002), and have previously been used in ecotoxicological studies (Reinecke *et al*., 2003). In this study, the effects of different AgNP concentrations (0, 10 and 100 µg/mL) and temperatures (18, 21 and 28 °C) on the biochemical effects in *P. perlatus* were evaluated for the first time. The experimental temperatures were chosen taking into account the predicted mean atmospheric and aquatic water temperatures (Bates *et al*., 2008). Climate change predictions indicate that the frequency, intensity and duration of thermal extremes will increase in the future (IPCC, 2001). The AgNP concentrations were chosen to reflect environmentally realistic concentrations. Although it is estimated that concentrations of AgNPs in natural waters range from 0.088 – 2.63 ng/L (Gottschalk *et al*., 2009) or 40 - 320 ng/L (Blaser *et al*., 2008), it is predicted that Ag-nanotechnologies could increase to 1216 tons by 2020 (Massarsky, 2014), which would subsequently also lead to an increased release of AgNPs in the environment. The study will for the first time evaluate the combined effects of AgNPs and temperature on oxidative stress responses in *P. perlatus*.

2. Experimental methodology

2.1.Preparation and characterisation of NPs

Details of AgNP preparation and characterization have been previously reported (Walters *et al*., 2013) and include: TEM, SEM, EDX, PXRD and BET. Procedures for each characterization technique are detailed in Walters *et al*. (2013). Total protein concentrations were expressed as protein per mg wet weight of tissue.

2.2.Experimental setup

Adult *P. perlatus* (averaging 50 ± 5 mm in length and 75 ±10 g in body weight) were randomly sampled from an uncontaminated section of the Eerste River (Stellenbosch, South Africa) during spring 2014 using handmade traps comprising of a fishing rod fitted with a mesh net containing bait. They were taken to the laboratory and kept in water at 21±2 °C, and a natural photoperiod for three days to acclimatise before experiments. Following the acclimatization period, crabs were divided into nine experimental treatments of three temperature-dependant regimes (*i.e*. 18 °C, 21 °C (control temperature), 28 °C) and each temperature-dependant regime consisted of three AgNP-dependant regimes (*i.e*. 0 µg/mL (control AgNP group), 10 µg/mL, and 100 µg/mL). The number of individuals per regime was six (nine regimes with six crabs each = 54 crabs in total). Crabs were kept unfed during the acclimatization and exposure periods. Crabs were exposed for seven days. Ethical clearance was obtained and ethical animal care guidelines were followed.

2.3.Collection and preparation of biological tissues

At the end of the exposure period crabs were cryoanaethesized and tissues (gills and hepatopancreas) were excised from each crab sample. Ethical animal care guidelines were followed. Samples were then homogenized (Omni-Ruptor 400 (Omni Internation Inc.) with a 30 % 12 second pulse cycle after addition of 800 µL 1:20 a protease inhibitor cocktail (Sigma Aldrich, MO, USA) prepared with a phosphate buffer solution (PBS). Samples were then centrifuged for 2 minutes at 13 000 rpm at 4 °C (Universal 32R, Hettich Zentrigugen, Germany). This protease inhibitor cocktail contained 4-(2 aminoethyl)benzenesulfonyl fluoride (AEBSF), pepstatin A, E-64, bestatin, leupeptin, and aprotinin, and was supplied as a ready to use solution in DMSO. Supernatants were collected and used for protein and enzyme determinations.

2.4.Protein content determination

Protein concentration in samples was determined according to Bradford (1976) using bovine serum albumin (Bio-rad Laboratories, USA) as standard.

2.5.Enzymatic assays

Biochemical analyses were determined spectrophotometrically (FLUOstar Omega, BMG Labtech, Ortenberg, Germany) using commercially-available assay kits, *i.e*. SOD (Sigma Aldrich, MO, USA), CAT (Arbor Assays, MI, USA), and GST (Sigma Adlrich, MO, USA). On the day of the assay, samples were thawed on ice. The SOD assay is based on the ability of SOD to inhibit superoxide radical-dependent reactions using the SOD activity assay kit. The SOD assay was used according to the manufacturer's instructions and the absorbance was read at 450 nm using a microplate reader (FLUOstar Omega, BMG Labtech, Ortenberg, Germany). Final SOD activity levels were expressed U SOD/mg protein. CAT activity was assessed by measuring the absorbance of 200 mM H_2O_2 at 520 nm. The enzyme activity is expressed as U CAT/mg of protein. GST activity was determined following the manufacturer's instructions. The test is performed by the addition of Dulbecco's Phosphate Buffered Saline (DPBS) solution, 200 mM L-Glutathione and 100 mM -Chloro-2,4-dinitrobenzene (CNDB). The absorbance change was performed at 340 nm. Results are reported as GST specific activity/mg of protein.

2.6.Statistical analysis

Statistical analysis was performed using Microsoft Excel and XLStat2015® statistical packages. Comparisons with more groups were performed by one-way analysis of variance (ANOVA) followed by Tukey's (HSD) and Dunnett (two sided) tests. Significant differences were established at $p < 0.05$. Principal Component Analysis (PCA; Microsoft Excel and XLStat2015®) was employed to detect variables that significantly contributed to differences in the activity of the investigated enzymes between the AgNP concentrations and temperature regimes.

3. Results and Discussion

3.1.Nanoparticle characteristics

Analytical characterizations of the AgNPs were performed to confirm the supplier's specifications, and were previously reported (Walters *et al.*, 2013). The physical and chemical properties of the particular AgNP are summarized in Table 4.1. The dry AgNP used were spherical in shape (mean diameter = 7.5329 ± 0.0028) and not strongly aggregated. The size of the dry AgNPs reported by the manufacturer (< 100nm) is in agreement with the size obtained by TEM results. Larger sized particles were obtained for AgNPs suspensions, attributable to the propensity of particles to aggregate in solution. The PXRD pattern of the AgNP is shown in Figure 4.1. The intensity and position of the diffraction peaks are in good agreement with the reported values and no peaks of impurities were found in PXRD pattern. These findings were also supported by other studies that used AgNP (Park and Choi, 2010; Scown *et al.*, 2010).

Table 4.1: Summary of physical and chemical properties of AgNPs (Walters *et al.*, 2013).

Chemical structure	Shape	Size distribution (nm)	Surface area (m^2g^2)	Chemical composition	Purity
Dry AgNP	Spherical	10-100[1]	7.5329 ± 0.0028[2]	Ag[3]	99.5[4]
AgNP suspension	Aggregates	>100[1]	-	Ag[3]	-

[1] By TEM, SEM (this study)
[2] By BET (this study)
[3] By EDX (this study)
[4] By supplier (Sigma-Aldrich)

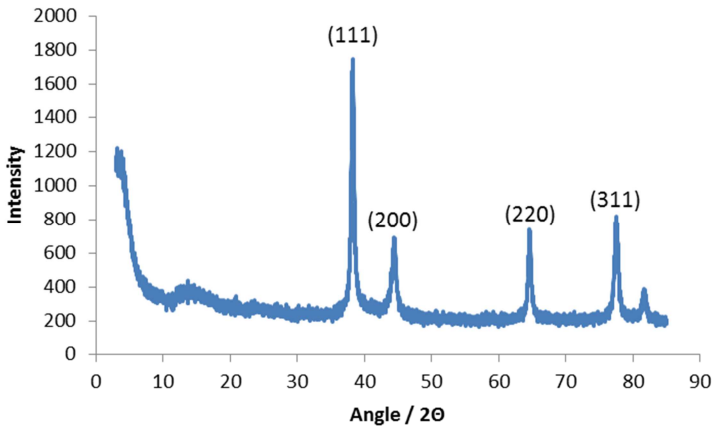


Figure 4.1: XRD pattern of Ag NPs (Walters *et al.*, 2013).

3.2.Oxidative stress and antioxidant responses to AgNPs at 21 °C

Oxidative stress is well documented as a common mechanism for cell damage when exposed to stressors (Massarsky *et al.*, 2014; Falfushynska *et al.*, 2015). Nanoparticles may induce oxidative stress thus leading to the generation of free radicals and alterations in antioxidant enzyme activities. To prevent impairment due to oxidative stress, antioxidant defence is provided by enzymes including SOD, CAT and GST. These enzymes constitute the major defence system against ROS (Sies, 1993). Total protein content was determined in the gills and hepatopancreas of crabs exposed to three AgNP-dependant regimes (*i.e.* 0 µg/mL (control), 10 µg/mL and 100 µg/mL) at 21 °C. Protein content decreased in both tissues at higher AgNP concentrations. Analyses of antioxidant levels in *P. perlatus* gills and hepatopancreas were performed in order to examine changes in response to AgNP exposure at 21°C. Collectively, the SOD–CAT system provides the first defence against oxidative toxicity at a cellular level. SOD is responsible for catalysing the dismutation of the superoxide radical O_2^- to O_2 and H_2O_2. SOD is particularly sensitive to the stress rendered by pollutants and can be induced by mild oxidative stress as a compensatory response, and can therefore be used as an oxidative stress signal for the early warning of environmental pollution. CAT is a key enzyme in the antioxidant defence system responsible for the detoxification and conversion of the resulting free radical H_2O_2 catalyzed by SOD to water and oxygen. The depletion of SOD activity is used as an indication of free radical scavenging ability, showing that the antioxidant defence system is overwhelmed by ROS (Vander *et al.*, 2003). Activity of SOD was inhibited to levels below that of the control for both AgNP concentrations in the gills (13% and 2% lower in the 10 µg/mL and 100 µg/mL AgNP

groups, respectively), but induced in the hepatopancreas (51% and 13% higher in the 10 µg/mL and 100 µg/mL AgNP groups, respectively) (Figure 4.2). In the gills, CAT activity was induced following exposure to 10 µg/mL (9%) but inhibited at 100 µg/mL (24%). In the hepatopancreas CAT activities were inhibited at both AgNP concentrations (9% and 29% lower when compared to the control). CAT induction provides an indication of the overproduction of peroxides, which is indicative of the presence of redox-active compounds. Noticeably, CAT activities were largely inhibited at 100 µg/mL (Figure 4.2). Antioxidant enzymes (such as SOD and CAT) can be induced at mild oxidative stress as a compensatory response. However, an over-production of ROS can overwhelm the detoxifying and antioxidant mechanisms leading to significant oxidative damage and a loss of the compensatory mechanisms, thereby suppressing the activities of the antioxidant enzymes (Zhang *et al*., 2004). This could likely explain the inactivation of CAT at both AgNP concentrations and the slight induction of SOD activities in both AgNP concentrations. A similar response was previously observed by Zhu *et al*. (2011). In addition, Ag^{+} (dissolved from the AgNPs) is known to interact with thiol–groups found in antioxidants that when disrupted can result in the inactivation of enzymes such as SOD and CAT, which may also lead to excess ROS production and significant oxidant stress (Bar–Ilan *et al*., 2009; Lapresta–Fernández *et al*., 2012). This may, in turn, lead to antioxidant mechanisms becoming overwhelmed causing a suppression in antioxidant enzyme activities (Zhang *et al*., 2004). Similar observations were made by Zhu *et al*. (2011) who reported an initial induction of SOD activities at lower TiO_2 NP concentrations (0.1 and 1 mg/L) followed by inhibition at the highest NP concentration (10 mg/L).

GST is an important phase II enzyme, the specific activity of which was measured by monitoring the formation of a conjugate L-glutathione to CDNB through the thiol group of the glutathione (Pinho *et al*., 2005). In crustaceans, GST activity is generally found in the gills and hepatopancreas, which have high metabolic rates (Lavarias *et al*., 2011). In aquatic organisms, direct ingestion or passage across epithelial boundaries such as gills are the main routes of NP exposure (Moore, 2006). GST activities were significantly affected by AgNP exposure. GST activity in exposed crabs was suppressed in the gills at both AgNPs concentrations (7% and 1% lower in the 10 µg/mL and 100 µg/mL AgNP groups, respectively when compared to the control). However, GST activity in the hepatopancreas was induced in both AgNP concentrations (75% and 36% higher in the 10 µg/mL and 100 µg/mL AgNP groups, respectively when compared to the control). A higher GST activity implies a greater detoxification capacity (Pinho *et al*., 2005) or conjugation of oxidative

products. It can, therefore, be postulated from our present study that GST was involved in antioxidant defences as a biotransformation enzyme to detoxify impairment to the hepatopancreas. Previous studies have also reported similar results when comparing GST activity in hepatopancreas of estuarine crab *Chasmagnathus granulatus* (Pinho *et al*., 2003; Pinho *et al*., 2005).

As mentioned above, crustacean hepatopancreas is regarded as the main site for toxicant metabolism and biotransformation of ROS (Livingstone, 1998). At the same exposure concentration, enzymatic activities in the hepatopancreas were generally higher when compared to the gills suggesting that the hepatopancreas might be a more sensitive organ (Hao *et al*., 2009) to AgNP exposure and also implies a lower ability to scavenge O_2^- (Pan and Zhang, 2006).

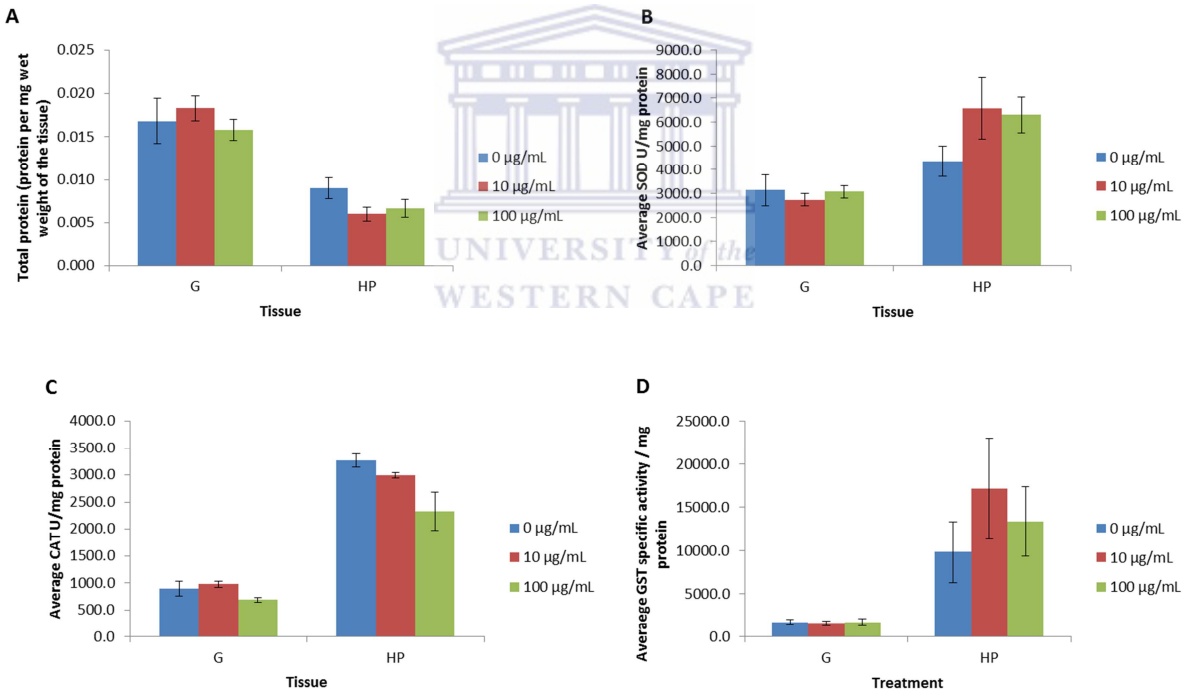


Figure 4.2: Effects of AgNPs (10 µg/mL and 100 µg/mL) at 21 °C on total protein (A), SOD (B), CAT (C) and GST (D) activity in the gills (G) and hepatopancreas (HP) of *P. perlatus* following a seven-day exposure period. Data are presented as means ± S.E.M. Statistical significance (indicated by *) was denoted by $p < 0.05$ versus the respective control crabs.

3.3.Modulating effects of temperature stress

Several factors influence the behaviour of AgNPs in the environment: the inherit NP characteristics as well the environmental (abiotic) properties. In freshwater ecosystems, seasonal temperature extremes are common, and the severity of temperature extremes is predicted to increase due to the global climate change (IPCC, 2007; IPCC, 2013). The observed results, for the first time, enhanced the importance of both AgNPs and temperature as influential variables in the oxidative stress responses in *P. perlatus*. The reduced protein content observed in the hepatopancreas at 28 °C (Figure 4.3) suggests possible mitochondrial damage (Qin *et al*., 2012), indicating the obstruction and/or modulation of protein participation in various biological processes as a result of AgNP/temperature stress (Vijayavel *et al*., 2006; Singaram *et al*., 2013), or due to proteolysis due to lack of food during AgNP/temperature stress (Elumalai and Balasubramanian, 1999). Collectively, the SOD–CAT system provides the first defence against oxidative toxicity at a cellular level. Enzymatic activities were significantly affected by temperature in all the tissues analysed (Figure 4.3). Since the dissolution of oxygen is generally higher at lower temperatures (and vice versa), it is expected that oxidative stress responses to be inversely correlated to temperature (Vinagre *et al*., 2012). This is because of the higher availability of oxygen in support of ROS generating processes at lower temperatures. However, the present study indicates that oxidative stress responses were generally lowest at conditions of lowest stress (*i.e*. at 21 °C and 18 °C) and highest at conditions of highest stress (*i.e*. 100 µg/mL AgNPs at 28 °C). Overall, SOD, CAT and GST activities were generally suppressed at 18 °C and induced at 28 °C in both tissues, the latter suggesting that AgNP and temperature co-exposure induced oxidative damage in the examined tissues. In a previous study, Gomes *et al*. (2015) reported increased oxidative damage with higher AgNP concentrations. Similarly, Vinagre *et al*. (2012) showed that *Dicentrarchus labrax* experienced thermal stress at 28 °C, as indicated by increased MDA and catalase activity. The response of the oxidative stress biomarkers analysed was generally higher in the hepatopancreas than in the gills (Figure 4.3), indicating that the hepatopancreas might be a more sensitive organ to the collective effects of AgNPs and elevated temperatures. This is in contrast to what has been reported by Vinagre *et al*. (2014), who generally found that the gills presented higher constitutive levels of oxidative stress biomarkers. Since gills are in direct contact with the external environment, the expected induction of antioxidant enzyme activity is indicative of continuous antioxidant mechanisms. In the hepatopancreas co-exposure to higher AgNP concentrations (*i.e*. 100 µg/mL) and higher temperature (*i.e*. 28 °C) induced activities of SOD (statistically significant

($p < 0.05$) at 100 µg/mL), CAT (statistically significant ($p < 0.05$) at 100 µg/mL) and GST ((statistically significant ($p < 0.05$) at 10 µg/mL and 100 µg/mL). This indicates activation of the antioxidant activity of *P. perlatus* by increasing AgNP concentrations and elevated temperature.

The simultaneous SOD, CAT and GST activity increases observed in crabs exposed to the same AgNP concentration and same temperature regime represents a reinforcement of the defence mechanisms, providing an additional evidence of a serious environmental health risk. Several studies have confirmed that the antioxidant defence system could be significantly induced under periods of stressful conditions resulting from NP or temperature stress. For example, Madeira *et al*., (2013) has given evidence of temperature effects on oxidative stress, reporting a relation between thermal stress response and oxidative stress response. Paital and Chainy (2013) observed seasonal variations in oxidative stress responses in mud crabs (*Scylla serrata*) and reported a marked increase in the antioxidative defences during the summer months, which might be associated with changes in environmental temperature. Previous studies have reported that augmentation of antioxidant capacity in the tissues of crabs at higher temperatures could be a reflection of an adaptive response to counteract xenobiotics (both chemical and environmental stress) induced ROS and oxidative stress levels (Paital and Chainy, 2010; Paital and Chainy, 2013). Extreme temperature increases or decreases influence metabolic rates and oxygen consumption which frequently cause oxidative stress. As such, the activation of antioxidant defences is an essential constituent of stress responses against oxidative stress.

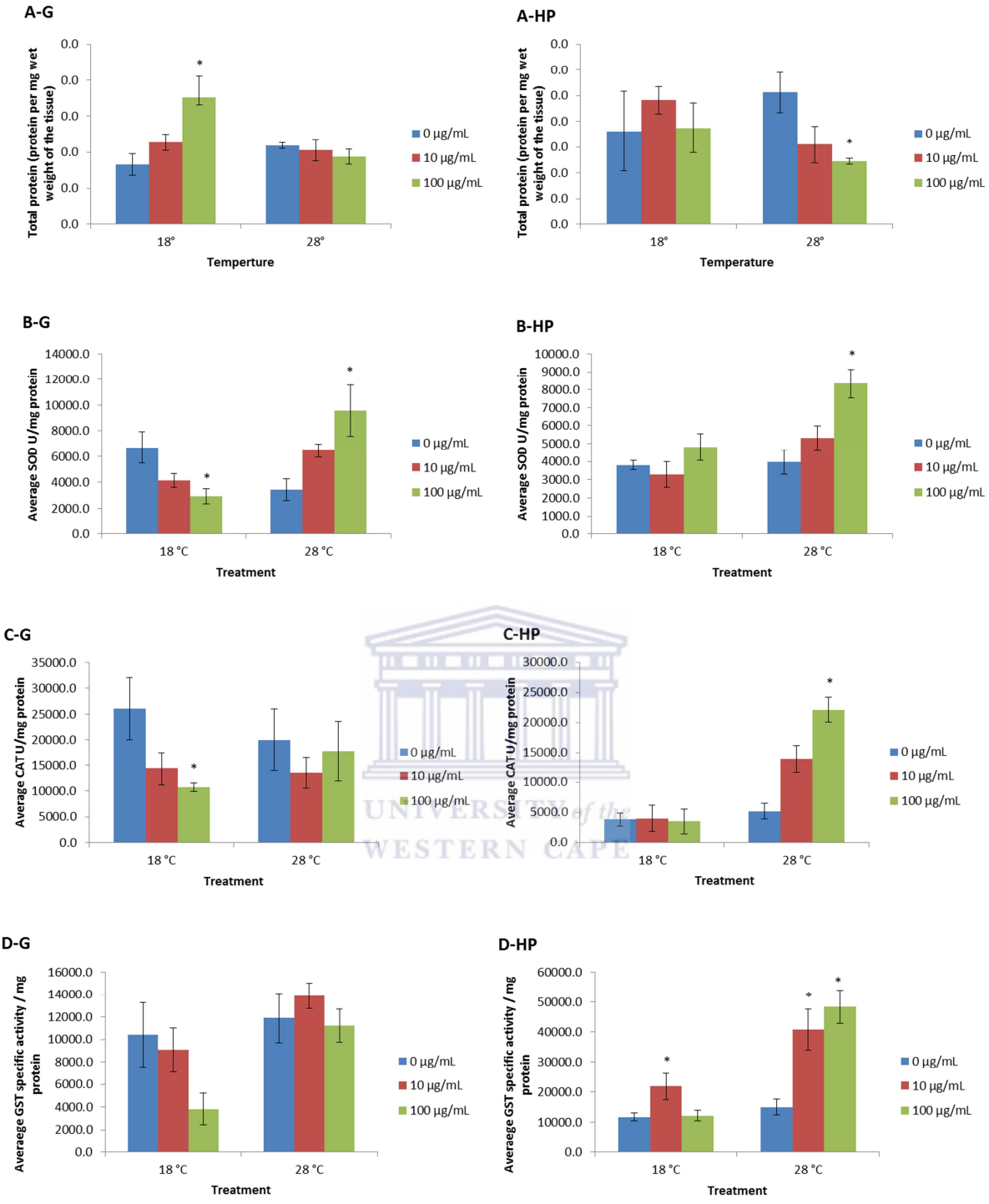


Figure 4.3: Effects of AgNPs (10 µg/mL and 100 µg/mL) and temperature (18 °C and 28 °C) on total protein (A), SOD (B), CAT (C) and GST (D) activity in the gills (G) and hepatopancreas (HP) of *P. perlatus* following a seven-day exposure period. Data are presented as means ± S.E.M. Statistical significance (indicated by *) was denoted by $p < 0.05$ versus the respective control crabs.

4. Principal component analysis (PCA)

Principal Component Analysis (PCA) was applied in order to statistically define the differences of antioxidant defence enzyme activities between AgNP concentrations and temperature stress in the gills and hepatopancreas. This multivariate approach to analysing the data indicated that biomarker responses varied with AgNP concentration and temperature. The overall PCA indicates different biomarker responses between control and AgNP exposed crabs, and between tissues. For the gills (Figure 4.4), the two principal components represent 95.08% of total variance, with PC1 representing 81.24% and PC2 13.84%. As for AgNP–exposed crabs, a clear separation of the 28 °C temperature regime occurred, suggesting a specific response of both tissues due to temperature exposure. Co-exposure regimes with AgNP at 28 °C showed strong correlations of SOD with at 100 µg/mL AgNP, whereas GST and CAT activities were associated with 10 µg/mL AgNP exposure (Figure 4.4). Exposures at 18 °C and 21 °C showed no association with antioxidant enzymes.

As for the hepatopancreas the two principal components represent 98.48% of total variance, with PC1 representing 81.94% and PC2 16.54% (Figure 4.5). Similar to the gills, the overall PCA indicates a clear separation between unexposed and Ag–exposed crabs. PC1 divides crabs exposed to different temperature regimes, showing a dissimilarity of biomarkers response during the course of the experiment, dependent on the exposure temperature. Exposures at 18 °C and 21 °C showed no association with antioxidant enzymes. In this second biplot, CAT and GST activities were closely associated with co-exposure to 28 °C and both AgNP concentrations (10 µg/mL and 100 µg/mL). These results show a significant correlation between antioxidant efficiency to counteract Ag NPs exposure.

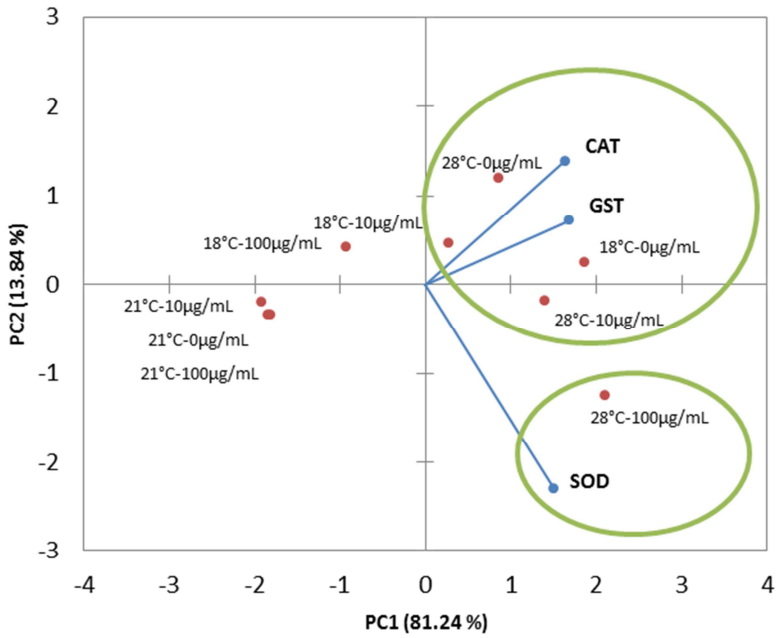


Figure 4.4: Biplot of the first two components of Principal Component Analysis (PCA) including all measured markers (SOD, CAT and GST) in the gills of *P. perlatus* exposed to AgNPs (0 µg/mL; 10 µg/mL and 100 µg/mL) at difference temperatures (18 °C, 21 °C and 28 °C).

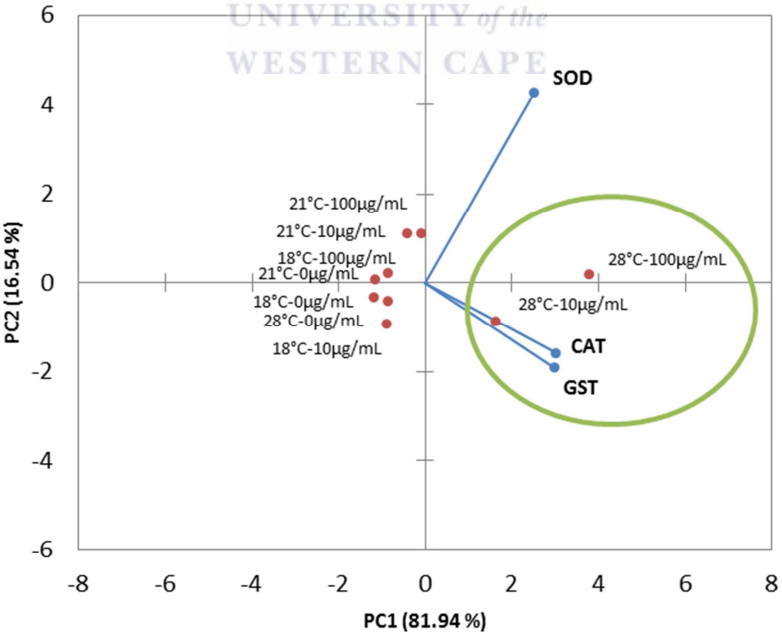


Figure 4.5: Biplot of the first two components of Principal Component Analysis (PCA) including all measured markers (SOD, CAT and GST) in the hepatopancreas of *P. perlatus* exposed to AgNPs (0 µg/mL; 10 µg/mL and 100 µg/mL) at different temperatures (18 °C, 21 °C and 28 °C).

5. Conclusions

To our knowledge, this study represented the first of its kind to examine the co-effects of AgNPs and temperature on the antioxidant competence of *P. perlatus*. Silver NP is present in the aquatic environments primarily from discharge of industrial and municipal effluents. The increasing use of nanotechnology highlights the need to understand and clarify the environmental impacts of nanomaterials. In this study, the oxidative stress of AgNPs in the freshwater crab *P. perlatus* and modulation by elevated temperatures were assessed. The overall results reported from this study showed that exposure of *P. perlatus* to concentrations of AgNPs influence the antioxidant enzyme capacity. In addition, the results of this study suggests that oxidative stress biomarkers were modulated by temperature stress, which is of great relevance to environmental monitoring the effects of nano-toxins. The distinct antioxidant efficiency in the gills and hepatopancreas of the exposed crabs are dependent on the concentration of AgNP used, as well as the exposed temperature, reflecting the individual physiological and metabolic functions of the two tissues. It can be concluded that any modulation of the antioxidant enzymes could have an effect on the survivability of aquatic organisms upon exposure to contaminants. The knowledge gained from the information generated in this study could contribute to our knowledge of the potential harmful effects of AgNPs on aquatic ecosystems and provide a more comprehensive understanding of their environmental behaviours. Given the widespread use of AgNPs, a systematic, coherent, and tested foundation for managing the uncertain health and environmental aspects of AgNPs is required. Another important finding of this study is that the hepatopancreas seem to be more susceptible to oxidative stress than the gills (contrary to other studies (Pan and Zang, 2006), as alterations of enzymatic activities were more pronounced. The distinct antioxidant efficiency in the gills and hepatopancreas of exposed crabs are dependent on both AgNP concentration and temperature. The results also reflect the dissimilar physiological and metabolic function of the these tissues. Furthermore, this study showed that temperature is an important variable in the oxidative stress response of crabs exposed to AgNPs.

6. Acknowledgments

This research was supported by the National Research Foundation (NRF) Thuthuka Fund (Grant no. TK20110905000026731) and the Council for Scientific and Industrial Research

(CSIR). The authors are grateful to the assistance of Dewald Schoeman (University of the Western Cape) and Christoff Truter (University of Stellenbosch).

7. References

Abele, D., Heise, K., Pörtner, H.O., Puntarulo, S., 2002. Temperature-dependence of mitochondrial function and production of reactive oxygen species in the intertidal mud clam *Mya arenaria*. J. Exp. Biol. 205, 1831–1841.

Ahmed, R.G., 2005. Is there a balance between oxidative stress and antioxidant defense system during development. Med. J. Islamic World Academy Sci. 15(2), 55–63.

Bates, B.C., Z.W. Kundzewicz, S. Wu and J.P. Palutikof, Eds., 2008: Climate Change and Water. Technical Paper of the Intergovernmental Panel on Climate Change, IPCC Secretariat, Geneva, 210 pp.

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248-254.

Blaser, S.A., Scheringer, M., MacLeod, M.; Hungerbühler, K., 2008. Estimation of cumulative aquatic exposure and risk due to silver: Contribution of nano–functionalized plastics and textiles. Sci. Tot. Environ. 390, 396–409.

Elumalai, M., Balasubramanian, M.P., 1999. Influence of naphthalene on esterase activity during vitellogenesis of marine edible crab, *Scylla serrata*. Bull. Environ. Contam. Toxicol. 62(6), 743–748.

Falfushynska, H., Gnatyshyna, L., Yurchak, I. and Sokolova, I., 2015. The effects of zinc nanooxide on cellular stress responses of the freshwater mussels *Unio tumidus* are modulated by elevated temperature and organic pollutants. Aquat. Toxicol. 162, 82-93.

Freire, C.A., Welker, A.F., Storey, J.M., Storey, K.B., Hermes-Lima, M., 2011. Oxidative stress in estuarine and intertidal environments (temperate and tropical). Oxidative Stress in Aquatic Ecosystems. John Wiley & Sons Ltd. 41–57.

Gaiser, B.K., Fernandes, T.F., Jepson, M.A., Lead, J.R., Tyler, C. R., Baalousha, M., Biswas, A., Britton, G.J., Cole, P.A., Johnston, B.D., Ju-Nam, Y., Rosenkranz, P., Scown, T.M. and Stone, B.D., 2012. Interspecies comparisons on the uptake and toxicity of silver and cerium dioxide nanoparticles. Environ. Toxicol. Chem. 31(1), 144–154.

Gomes, S.I.L., Hansen, D., Scott-Fordsmand, J.J. and Amorim, M.J.B., 2015. Effects of silver nanoparticles to soil invertebrates: Oxidative stress biomarkers in *Eisenia fetida*. Environ. Poll. 199, 49-55.

Griffit, R.J., Brown-Peterson, N.J., Savin, D.A., Manning, C.S., Boube, I., Ryan, R.A. and Brouwer, M., 2012. Effects of chronic nanoparticulate silver exposure to adult and juvenile sheepshead minnows (*Cyprinodon variegatus*). Environ. Toxicol. Chem. 31(1), 160–167.

Gubbins, E.J., Batty, L.C. and Lead, J.R., 2011. Phytotoxicity of silver nanoparticles to *Lemna minor L*. Environ. Poll. 159, 1551–1559.

Halliwell, B., 1994. Free radicals, antioxidants and human disease: curiosity, cause or consequence. Lancet. 344, 721–724.

Halliwell, B., Gutteridge, J.M., 1999. Free Radicals in Biology and Medicine. Oxford University Press, New York.

Halliwell, B. and Gutteridge, J.M.C., 2007. Free Radicals in Biology and Medicine. Oxford University Press, Oxford.

Hao, L., Wang,Z., Xing,B., 2009. Effect of sub-acute exposure to TiO_2 nanoparticles on oxidative stress and histopathological changes in Juvenile Carp (*Cyprinus carpio*). J.Environ.Sci. 21, 1459–1466.

Hayashi, Y., Engelmann, P., Foldbjerg, R., Szabo, M., Somogyi, I., Pollak, E., Molnar, L., Autrup, H., Sutherland, D.S., Scott-Fordsmand, J., Heckmann, L.H., 2012. Earthworms and humans in vitro: characterizing evolutionarily conserved stress and immune responses to silver nanoparticles. Environ. Sci. Technol. 46, 4166-4173.

IPCC (Intergovernmental Panel on Climate Change), 2007. Climate change 2007: the physical science basis. In: Solomon S, Qin D, Manning M, Chen Z and others (eds) Contribution of Working Group I to the 4th assessment report of the Intergovernmental Panel on Climate Change. Cambridge University Press, Cambridge.

Jayaseelan, C., Rahuman, A.A., Ramkumar, R., Perumal, P., Rajakumar, G., Kirthi, A.V., Santhoskumar, T. and Marimuthu, S., 2014. Effect of sub-acute exposure to nickel nanoparticles on oxidative stress and histopathological changes in Mozambique tilapia, *Oreochromis mossambicus*. Ecotox. Environ. Saf. 107, 220-228.

Lapresta–Fernández, A.; Fernández, A.; Blasco, J., 2012. Nanoecotoxicity effects of engineered silver and gold nanoparticles in aquatic organisms. Trends Anal. Chem. 32, 40–59.

Lavarias, Sl., Heras, H., Pedrini, N., Tournier, H. and Ansaldo, M., 2011. Antioxidant response and oxidative stress levels in *Macrobrachium borellii* (Crustacea: Palaemonidae) exposed to the water-soluble fraction of petroleum. Comp. Biochem. Physiol. C. 153, 415-421.

Levard, C., Hotze, E.M., Lowry, G.V., Brown, G.E., 2012. Environmental transformations of silver nanoparticles: impact on stability and toxicity. Environ. Sci. Technol. 46, 6900-6914.

Livingstone, D.R., 1998. The fate of organic xenobiotics in aquatic ecosystems: quantitative and qualitative differences in biotransformation by invertebrates and fish. Comp. Biochem. Physiol. A 120, 43–49.

Lushchak, V.I., 2011. Environmentally induced oxidative stress in aquatic animals. Aquat. Toxicol. 101, 13–30.

Madeira, D., Narciso, L., Cabral, H.N., Vinagre, C., Diniz, M.S., 2013. Influence of temperature in thermal and oxidative stress responses in estuarine fish. Comp. Biochem. Physiol. A. 166, 237-243.

Massarsky, A., 2014. Characterizing the biochemical and toxicological effects of nanosilver in vivo using zebrafish (*Danio rerio*) and in vitro using rainbow trout (*Oncorhynchus mykiss*) (PhD thesis). University of Ottawa, Canada.

Massarsky, A., Abraham, R., Nguyen, K.C., Rippstein, P. and Tayabali, A.F., 2014. Nanosilver cytotoxicity in rainbow trout (*Oncorhynchus mykiss*) eryhtocytes and hepatocytes, Comp. Biochem. And Physiol. C. 159, 10-21.

Moore, M.N., 2006. Do nanoparticles present ecotoxicological risks for the health of the aquatic environment? Environ. Int. 32: 967–976.

Navarro, E., Piccapietra, F., Wagner, B., Marconi, F., Kaegi, R., Odzak, N., Sigg, L., Behra, R., 2008. Toxicity of Silver Nanoparticles to *Chlamydomonas reinhardtii*. Environ. Sci. Technol. 42, 8959-8964.

Novo,M.S.,Miranda, R.B., Bianchini, A., 2005. Sexual and seasonal variations in osmoregulation and ion regulation in the estuarine crab *Chasmagnathus granulatus* (Crustacea, Decapoda). J. Exp. Mar. Biol. Ecol. 323, 118–137.

Paital, B., Chainy, G.B.N., 2010. Antioxidant defences and oxidative stress parameters in tissues of mud crab (*Scylla serrata*) with reference to changing salinity. Comp. Biochem. Physiol.C. 151, 142–151.

Paital, B., Chainy, G.B.N., 2013. Seasonal variability of antioxidant biomarkers in mud crabs (*Scylla serrata*). Ecotox. Environ. Saf. 87, 33-41.

Pan, L. and Zhang, H., 2006. Metallothionein, antioxidant enzymes and DNA strand breaks as biomarkers of Cd exposure in a marine crab, *Charybdis japonica*. Comp. Biochem. Physiol. C. 144, 67-75.

Pan, L.Q., Ren, J.Y., Wu, Z.W., 2004 Effects of heavy metal ions on SOD, CAT activities of hepatopancreas and gill of the crab *Eriocheir sinensis*. J. Ocean. 34, 189-194.

Park, S-Y. and Choi, J., 2010, Geno- and ecotoxicity evaluation of silver nanoparticles in freshwater crustacean *Daphnia magna*. Environ. Eng. Res. 15(1), 23–27.

Pereira, P., Pablo, H., Subida, M.D., Vale, C., Pacheco, M., 2009. Biochemical responses of the shore crab (*Carcinus maenas*) in a eutrophic and metal-contaminated coastal system (Óbidos lagoon, Portugal). Ecotoxicol. Environ. Saf. 72, 1471–1480.

Pham, C.H., Yi, J. and Gu, M.B., 2012. Biomarker gene response in male Medaka (*Oryzias latipes*) chronically exposed to silver nanoparticle. Ecotox. Environ. Saf. 78, 239-245.

Piao, M.J., Kang, K.A., Lee, I.K., Kim, H.S., Kim, S., Choi, J.Y., Choi, J., Hyun, J.W., 2011. Silver nanoparticles induce oxidative cell damage in human liver cells through inhibition of reduced glutathione and induction of mitochondria-involved apoptosis. Toxicol. Lett. 201, 92-100.

Pinho, G.L.L., de Rosa, C.M., Yunes, J.S., Luquet, C.M., Bianchini, A., Monserrat, J.M., 2003. Toxic effects of microcystins in the hepatopancreas of the estuarine crab *Chasmagnathus granulatus* (Decapoda, Grapsidae). Comp. Biochem. Physiol. 135, 459–468.

Pinho, G.L.L., Da Rousa, C.M., Maciel, F.E., Bianchini, A., Yunes, J.S., Proenca, L.A.O. and Moserrat, J.M., 2005. Antioxidant responses and oxidative stress after microcystin exposure in the hepatopancreas of an estuarine crab species. Ecotoxicol. Environ. Saf. 61, 353-360.

Piccinno, F., Gottschalk, F., Seeger, S., Nowack, B., 2012. Industrial production quantities and uses of ten engineered nanomaterials in Europe and the world. J. Nanoparticle Res. 14, 1109-1119.

Qin, Q., Qin, S., Wang, L. and Lei, W., 2012. Immune responses and ultrastructural changes of hemocytes in freshwater crab. Aquat. Toxicol. 106-107, 140-146.

Reinecke, A.J., Snyman, R.G. and Nel, J.A.J., 2003. Uptake and distribution of lead (Pb) and cadmium (Cd) in the freshwater crab *Potamautes perlatus* (Crustacea) in the Eerst River, South Africa. Water, Air, Soil Poll. 145, 395–408.

Scown, T.M., Santos, E., Johnston, B.D., Gaiser, B., Baalousha, M., Mitov, S., Lead, J.R., Stone, V., Fernandes, T.F., Jepson, M., van Aerle, R., Tyler, C.R., 2010. Effects of

aqueous exposure to silver nanoparticles of different sizes in rainbow trout. Toxicol. Sci. 115(2), 521–534.

Sies, H., 1993. Strategies of antioxidant defense. Eur. J. Biochem. 215, 213–219.

Snyman, R. G., Reinecke, A. J. and Nel, J. A. J.: 2002, 'Uptake and distribution of copper in the freshwater crab, *Potamonautes perlatus* (Crustacea) in the Eerste River, South Africa', African Zoology 81–89.

Vander, O. R., Beyer, J., Vermeulen, N. P. E., 2003. Fish bioaccumulation and biomarkers in environmental risk assessment: A review. Environ. Toxicol. Pharm. 13: 57–149.

Vijayavel, K. and Balasubramanian, M.P., 2006. Fluctuations of biochemical constituent and marker enzymes as a consequence of naphthalene toxicity in the edible estuarine crab, *Scylla serrata*. Ecotoxicol. Environ. Saf. 63, 141-147.

Vinagre, C., Madeira, D., Narciso, L., Cabral, H., Diniz, M., 2012. Effect of temperature on oxidative stress in fish: lipid peroxidation and catalase activity in the muscle of juvenile seabass, *Dicentrarchus labrax*. Ecol. Indic. 23, 274-279.

Vinagre, C., Madeira, D., Mendonca, V., Dias, M., Roma, J. and Diniz, M., 2014. Effect of increasing temperature in the differential activity of oxidative stress biomarkers in various tissues of the Rock goby, *Gobius paganellus*. Mar. Environ. Res. 97, 10-14.

Walters, C., Pool, E. and Somerset, V., 2013. Aggregation and dissolution of silver nanoparticles in a laboratory-based freshwater microcosm under simulated environmental conditions, Toxicol. Environ. Chem. 95:10.

Wang, W., Wang, A., Liu, Y., Xiu, J., Liu, Z., Sun, R., 2006. Effects of temperature on growth, adenosine phosphates, ATPase and cellular defense response of juvenile shrimp *Macrobrachium nipponense*. Aquaculture 256, 624–630.

Zhang, J.F., Sun, Y.Y., Shen, B., Liu, H., Wang, X.R., Wu, J.C., Xue, Y.Q., 2004. Antioxidant response of *Daphnia magna* exposed to no. 20 diesel oil. Chem. Spec. Bioavailab. 16, 139–144.

Zhao, C-M. and Wang, W-E., 2010. Biokinetic uptake and efflux of silver nanoparticles in *Daphnia magna*. Environ. Sci. and Technol. 44, 7699–7704.

Zhu, X., Zhou, J. and Cai, Z., 2011. The toxicity and oxidative stress of TiO_2 nanoparticles in marine abalone (*Haliotis diversicolor supertexta*). Mar. Poll. Bull. 63, 334–338.

CHAPTER 5: COMBINED SILVER NANOPARTICLES AND TEMPERATURE EFFECTS IN THE CAPE RIVER CRAB *POTAMONAUTES PERLATUS* - INTERACTIONS BETWEEN CHEMICAL AND CLIMATE STRESSORS

This chapter has been submitted as an article to Journal of Toxicology and Environmental Health, Part A: Current Issues

Abstract

Silver nanoparticles (AgNPs) are widely used in commercial and personal care products with its annual productions ranging from 5 – 1 000 tons. Due to its widespread application, AgNPs has the potential for entering surface waters from multiple sources thereby causing increasing environmental and health concerns. The influence of silver nanoparticles (AgNPs) and temperature variation on toxicity and oxidative stress responses were investigated in the tissues of the Cape River crab *Potamonautes perlatus* following a seven-day exposure period. Toxicity assessments of crabs exposed to different AgNP concentrations and temperature regimes showed that *Potamonautes perlatus* had a benchmark dose (BMD) of 782.77 µg/mL AgNPs and Critical thermal maximum (CTMax) of 25.37 °C. Biochemical analysis indicated that the superoxide dismutase (SOD), catalase (CAT) and glutathione S-transferase (GST) and cytochrome P450 (CYP450) activity was significantly affected by AgNPs. Contrary to other studies, our results show that the haemolymph are more susceptible to oxidative stress originated by AgNPs and temperature stress, whereas the gills constitutes the main storage organ for Ag. These findings suggest that seven day exposure to environmentally realistic concentrations of AgNPs and temperature stress caused induced antioxidant defences of *P. perlatus*.

Keywords: Crabs, Oxidative stress, *Potamonautes perlatus*, Silver nanoparticles, Temperature, Toxicity

1. Introduction

Aquatic ecosystems are susceptible to both anthropogenic (such as introduction of pollutants) and natural stressors (abiotic factors such as temperature variations). As such, organisms residing in such areas may experience alterations in biochemical and physiological processes related to the maintenance of homeostasis. Oxidative stress is almost an unavoidable characteristic of aerobic life, caused by an imbalance between production of reactive oxygen species (ROS) and antioxidant defence (Maria *et al*., 2009; Singaram *et al*., 2013; Jayaseelan *et al*., 2014). An over-production of ROS can damage DNA, protein and lipids (Halliwell and Gutteridge, 1999). To maintain homeostasis and prevent oxidative stress under stress conditions, aquatic organisms have an antioxidant defence system for the removal of excess ROS (Kong *et al*., 2012). The defence system is composed of antioxidative enzymes and non-enzymatic antioxidants. These include Phase I detoxification enzymes cytochrome P450 (CYP450), Phase II enzymes glutathione S-transferase (GST), and antioxidative enzymes superoxide dismutase (SOD) and catalase (CAT). When the ability to remove excess ROS is inhibited, organisms experience oxidative stress (Livingstone *et al*., 1992; Zhang *et al*., 2004; Pinho *et al*., 2005).

Currently, most of the research into the toxicity of NPs has focused on the effects of single stressors. For example, temperature has long been known to alter the chemistry of chemical pollutants resulting in significant alterations in their toxicities (Schiedek *et al*., 2007). Temperature affects both chemical and biological processes including aquatic organisms' sensitivity to toxic substances, alters physiological stress responses, and may lead to higher metabolism which increases production of ROS (Bagnyukova *et al*., 2007; Choi, 2010). Similarly, anthropogenic stressors have long been known to induce stress in aquatic organisms. In particular, emerging pollutants such as nanoparticles (NPs) have received particular attention. Of all NPs, silver NPs (AgNPs) have been one of the most studied NPs, since it is present in several commercially available products including footwear, paints, wound dressings, cosmetics and textiles due to their antibacterial properties. As such, they have the potential to enter drinking water systems, ground water systems, and other water systems. Due to their increased commercial applications, their presence in the aquatic environment has also increased (Zhu *et al*., 2011; Fabrega *et al*., 2012). Few studies have investigated the ecotoxicity of co-exposure of NPs with other common environmental stressors. In a recent study, Falfushynska *et al*., (2015) concluded that ZnO-NPs toxicity in the mussel *Unio tumidus* was modulated by organic pollutants and enhanced by elevated

temperatures. In another study, Martins *et al*. (2011) reported higher copper toxicity at salinity 2 ppt than at 30 ppt.

The Cape River crab *Potamonautes perlatus* is a predatory aquatic organism prevalent in rivers of the south-western region of the Western Cape of South Africa (Snyman *et al*., 2002), and is frequently exposed to multiple stressors. *P. perlatus* is a burrowing and opportunistic feeder, consuming a large variety of prey. Biomarkers measuring changes at the biochemical level have been used as effective early warning tools in ecological risk assessments. To evaluate the effect of both chemical and climatic stressors on aquatic organisms to conditions anticipated under predicted scenarios of climate change, the toxicity and biochemical responses in *P. perlatus* to a range of AgNP concentrations and temperatures were investigated. The experimental temperatures were chosen taking into account the predicted increases in mean atmospheric and aquatic water temperatures (Bates *et al*., 2008), since climate change projections indicate an increase in the frequency, intensity and duration of thermal extremes (IPCC, 2001). Detoxification (CYP450) and antioxidant (SOD, CAT, GST) enzyme activity were measured in the tissues (gills, hepatopancreas, haemolymph, haemocytes and muscles) of *P. perlatus*. These biomarkers were chosen as they are considered useful enzymes that play a significant role in reducing damage to cells caused by ROS. Although several studies have investigated the individual effects of temperature and AgNPs in the levels of oxidative stress biomarkers (Ronisz *et al*., 1999; Novo *et al*., 2005; Wang *et al*., 2006), to our knowledge, no studies have investigated the combined effects of AgNPs and temperature in freshwater crabs. This study aims to assess how environmental parameters (temperature) could affect the environmental distribution and biological effects of chemical toxicants (AgNPs).

2. Materials and methods

2.1.Characterization of the AgNPs samples

Commercially available AgNPs were purchased from a local supplier (Sigma Aldrich, MO, USA). It was supplied as a black powder with a purity of 99 % and a specific surface area of 5.0 m^2/g, as advertised by the manufacturer. The stock AgNP suspension was prepared by dispersing AgNPs in deionized water and sonicating for 5 min. From this stock suspension, AgNP suspensions were added to the experimental microcosms to obtain a final concentration of 0, 1, 10, 100, 1 000 and 10 000 µg/mL. The AgNP suspension was pipetted

on to the carbon surface of an SEM stub and characterized for particle size by scanning electron microscopy (SEM; EVO® MA15) (Walters *et al*., 2013). The size distribution of the dry AgNPs and AgNPs suspensions were determined by transmission electron microscopy (TEM). This was achieved by using a JEOL 1200-EX II electron microscope at an accelerating voltage of 120 kV (Walters *et al*., 2013). The specific surface area of the AgNP powder was analysed by BET using a using ASAP 2010 (Accelerated Surface Area and Porosimetry System; Micromeritics Instrument Corporation).

2.2.Animal collection and acclimation

The Cape River crab *P. perlatus* was used as test organism. Adult crabs, averaging 50 ± 5 mm in length and 75 ±10 g in body weight, were collected randomly in an unpolluted site on the Eerste River (Stellenbosch) using handmade traps comprising of a fishing rod fitted with a mesh net containing bait during Spring/Summer 2014. They were taken to the laboratory and kept in 2 L microcosms where they were allowed to acclimatize for three days at room temperature (21 ± 2 °C) prior to exposure. Test media were completely renewed daily. No food was provided during the acclimatization period.

2.3.Experimental protocol for acute toxicity test

There is little information relating to measurement of AgNP concentrations in surface waters. To determine the appropriate AgNP concentration and temperature of exposure, we performed acute toxicity and mortality tests based on modified US EPA protocols (Sheehan, 2001). The acute exposure study consisted of three experimental stages. Experiment 1 involved a temperature-dependant regime and consisted of five different temperature regimes (*i.e*. 16, 18, 22, 26 and 28 °C). During experiment 2, a total of 6 crabs per treatment were exposed for seven days to the benchmark dose (BMD) AgNP concentration (obtained in experiment 1) at the five pre-determined temperature regimes. Following experiment 2, the numbers of live and dead crabs were determined via visual inspection and the Critical thermal maximum (CTMax) were derived through LogProbit analysis (US EPA BMDS Program, version 2.5) and was used to estimate the temperature to be used in experiment 3. The CTMax is defined as “arithmetic mean of the collective thermal points at which the endpoint is reached” (Mora and Ospina, 2001), or that temperature for a given species above which most individuals respond with unorganized locomotion, subjecting the animal to likely death (McDiarmid and Altig, 1999).

Experimental 2 involved a concentration-dependant regime and comprised of crabs specimens exposed to five different AgNP concentrations including a control regime (*i.e.* 0, 1, 10, 100, 1 000 and 10 000 µg/mL AgNPs). In order to cover a wide range of contamination levels that may be reported for polluted environment, a total of 6 crabs per treatment were exposed for seven days to a wide range of concentrations. Temperature (21 ± 2 °C) and photoperiod (12 h alternating light/dark cycle) were fixed. Following experiment 1, the numbers of live and dead crabs were determined via visual inspection and the BMD were derived through LogProbit analysis (US EPA BMDS Program, version 2.5). This BMD was used to estimate the BMD AgNP concentration to be used in experiment 2.

Experiment 3 involved the assessment of the role of oxidative stress in AgNP induced toxicity, a total of 6 crabs per treatment were exposed to the corresponding BMD and CTMax values obtained during the preceding experimental stages. The experiment was conducted in 2 L plastic tanks (three crabs per tank with 200 mL of water, total of two tanks per treatment regime) with a 12 h alternating light/dark cycle following modified methods described by Cheng (2005). For all experimental stages, crabs were exposed for seven days and were unfed during the acclimatization and exposure periods. Every 24 h during the experiments 1 and 2, live crabs were counted and the dead crabs were removed. Death was assumed when no movement occurred when mechanically stimulated. No food was provided during the exposure period.

2.4.Preparation of tissue samples for biochemical assays

Tissue samples were collected at the end of Experiment 3. Approximately 1 - 2 mL of haemolymph were drawn from the first abdominal appendage using a 5 mL sterile syringe fitted with an 18 gauge hypodermic needle and transferred to 4.5 mL sodium citrate vials to prevent coagulation on ice. The haemolymph was centrifuged at 1 500 rpms at 4 °C for 5 minutes to pellet the haemocytes. The resultant supernatant (or cell free haemolymph – CFH) was carefully aliquoted for enzymatic assays and stored at -80 °C. The pelleted cells were washed once in 100 µL. After removal of the haemolymph, crabs were cryoanaethesized and the remaining tissues (gills, hepatopancreas and muscle) were removed. Approximately 80 mg of tissue, the cell free haemolymph and the pelleted haemocytes were homogenized (Omni-Ruptor 400 (Omni Internation Inc.) in 800 µL phosphate buffer containing 5% protease inhibitor cocktail (Sigma Aldrich, MO, USA), which contained 4-(2 aminoethyl)benzenesulfonyl fluoride (AEBSF), pepstatin A, E-64, bestatin, leupeptin, and

aprotinin. Homogenization was done using a 30% 12 second pulse cycle. The homogenate cycle was centrifuged for 2 minutes at 13 000 rpm at 4 °C (Universal 32R, Hettich Zentrigugen, Germany). All tissue samples were stored at -80 °C until enzymatic analysis.

2.5.Preparation of tissues for chemical analyses by ICP-OES and ICP-MS

Tissue samples for trace metal analysis were collected from each crab per treatment regime and pooled. Trace metal (Ag, Ca, Fe, Mg, Na and Zn) analyses were performed on the gills, hepatopancreas, haemolymph, haemocytes and muscles. Tissues were analysed for total metals using ICP-OES (Agilent Technologies, 7500 CX, Chemetrix, Midrand, RSA) for Ag, Ca, Fe, Mg, Na, and ICP-MS (Agilent Technologies, 7500 CX, Chemetrix, Midrand, RSA) for Zn. Prior to analysis, tissues were digested with a 5:1 mixture of 55 % nitric acid and 70 % perchloric acid (Sanders *et al*. 1998). It was then aspirated with a concentric nebuliser into a quartz spray chamber cooled by a Peltier cooler into the Inductively Coupled Plasma.

2.6.Enzyme activity assays

Assays were performed in triplicate on the gills, hepatopancreas, haemolymph, haemocytes and muscles for each crab specimen. The protein content of each sample extracts was determined according to Bradford (1976) using bovine serum albumin as standard. Cytochrome P450 (CYP450) enzyme activity was determined using a commercially available kit (Vivid® CYP2C8 Green, catalogue no. PV6141, Life Technologies, Carlsbad, CA, USA) and followed the manufacturer's protocols. In brief, the Vivid® Substrate and fluorescent standards were reconstituted. A known volume of test compound, positive inhibition control (Montelukast P450 Inhibitor, Cayman Chemical, MI, USA) and solvent controls were added to each well. The fluorescence is read using a microplate reader (FLUOstar Omega, BMG Labtech, Ortenberg, Germany) with an excitation wavelength of 490 nm and an emission wavelength of 520 nm. Results are expressed as % activity per mg protein. SOD activity was measured using a commercially available kit (Sigma-Aldrich, MO, USA) and followed the manufacturer's protocols. The absorbance was read at 450 nm using a microplate reader (FLUOstar Omega, BMG Labtech, Ortenberg, Germany). The SOD activity was expressed in units per mg protein. CAT activity was measured in the tissues *P. perlatus* in samples using a commercially available kit, following the manufacturer's protocols (Arbor Assays, MI, USA). The absorbance was read at 560 nm using a microplate reader (FLUOstar Omega, BMG Labtech, Ortenberg, Germany). Results are expressed as CAT units per mg protein.

GST activity was measured using a commercially available kit (Sigma Aldrich, MO, USA) and following the manufacturer's protocols. Activity was measured spectrophotometrically at 340 nm (FLUOstar Omega, BMG Labtech, Ortenberg, Germany). Activity was expressed as GST specific activity per mg protein.

2.7.Statistical Analysis

The BMD, CTMax and associated 95% confidence intervals were calculated using the US EPA BMDS Program (version 2.5). Statistical differences between the control and exposed crabs were determined by a univariate one-way ANOVA followed by Tukey (HSD) test. Differences were statistically significant when $p < 0.05$. To explore the patterns in correlations between data, Principal Component Analysis (PCA) was used to assess the interrelationships among the parameters used.

3. Results and Discussion

3.1.Characterization of AgNPs

To determine the physico-chemical properties of AgNPs, particle size distribution and shape were examined by SEM and TEM. The SEM and TEM micrographs revealed that the dry AgNPs formed small, loosely packed aggregates no more than 100 nm in size (Figure 5.1). The TEM micrograph also confirmed that the morphology of the AgNPs was observed to be spherical in shape. The TEM micrograph of the AgNP in suspension showed the formation of large aggregates. The PXRD pattern of the AgNPs showed diffraction peaks at 2Θ from 3° to 90°. EDX confirmed the presence of Ag. The specific surface area of the AgNPs was determined to be 7.5329 $m^2/g \pm 0.0028$ (Walters *et al*., 2013).

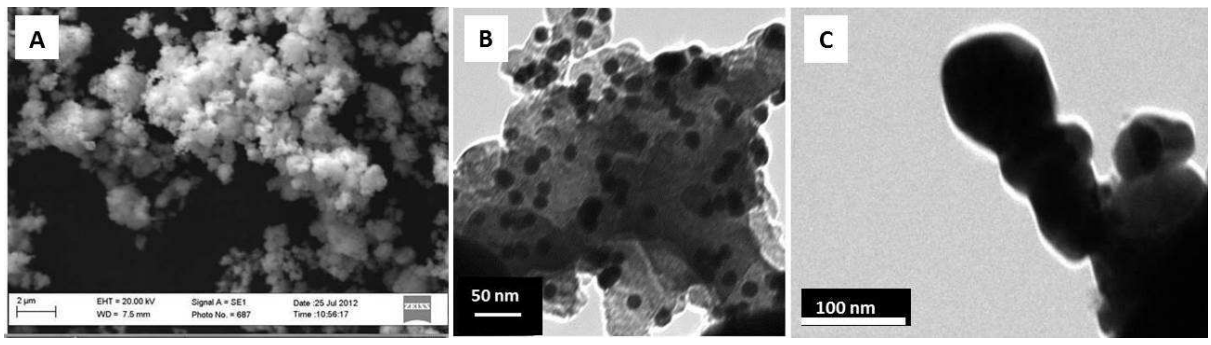


Figure 5.1: SEM micrograph of dry AgNP (A). TEM micrograph of dry AgNPs (B) and AgNP in suspension (C) (Walters *et al*., 2013).

3.2.Trace metal levels in the tissues of *P. perlatus*

Figure 5.2 shows the Ag concentrations in the tissues of *P. perlatus* exposed to AgNPs. To detect uptake of AgNPs, the content of Ag in the gills, hepatopancreas, haemolymph, haemocytes and muscle were measured. Ag concentrations were significantly higher in the exposed group when compared to the controls, with a 10-fold increase observed (average Ag content in the AgNP exposed group was 36 397.93 µg/kg). The elevated Ag content observed in exposed tissues implies that the Ag loading is largely attributed to the ionic form of Ag (*i.e.* Ag^{+}) released from the AgNPs. This was supported by Navarro *et al*. (2008), studying the toxicity of AgNPs to a freshwater algae *Chlamydomonas reinhardtii*, and reporting that AgNPs served as an addition source of Ag^{+}. The liver is generally regarded as the main target organ for AgNPs (Kim *et al*. 2008; Kim *et al*. 2010). However, in the AgNP exposed group, Ag was largely accumulated in the gills. This suggests that the major route of AgNP entry is via direct passage across surface epithelia and that the gills are the key target organ for Ag accumulation. These results are in contrast with previous studies described in the literature. As an example, Gomes *et al*. (2014) recently reported Ag content in the digestive gland two to five-fold higher than that of the gills in exposed tissues of the mussel *Mytilus galloprovincialis*. The elevated Ag levels reported in this study for the haemocytes and haemolymph suggests that some of these particles had passed through the gills into the haemolymph and were subsequently distributed to other tissues and organs (Martins *et al*., 2011; Canesi *et al*., 2012). A possible interpretation for our finding is that the mechanisms of transport at the gill membrane could perhaps limit the Ag flux from the gill to the haemolymph, thus leading to a build-up of Ag inside the gill ion-transporting cells (Martins *et al*., 2011). Surprisingly, the lowest Ag concentration was measured in the hepatopancreas which is in contrast to other studies (Kulthong *et al*., 2012). This observation further supports that the large AgNP agglomerates in suspension may have prevented their hepatic absorption, which was evidenced by Kulthong *et al*. (2012).

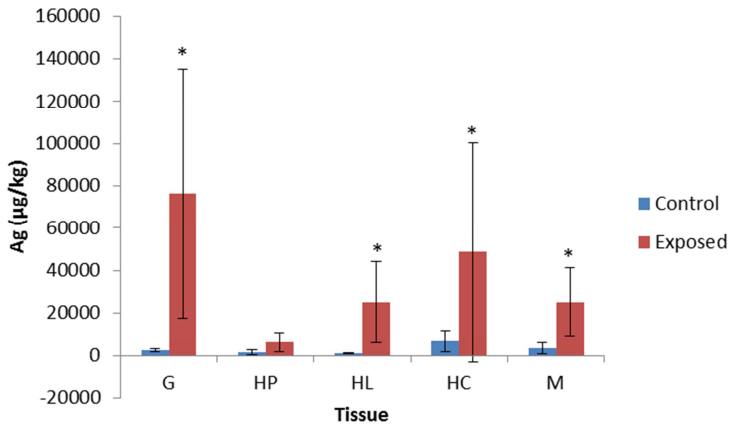


Figure 5.2: Silver concentrations in crab tissues (G = gills; HP = hepatopancreas, HL = haemolymph, HC = haemocytes, M = muscles). Data are presented as means ± S.E.M. Statistical significance (indicated by *) was denoted by $p < 0.05$ versus the respective control crabs.

3.3.Acute toxicity tests

Acute toxicity values of experiments 1 and 2 are compared in (Table 5.1). In the temperature-dependant experiment, 50% mortality was recorded after 2 days at 28 °C. No mortalities were observed during the 7 day experimental period in the 18 °C and 22 °C temperature groups. However, mortalities were observed for all other temperature-dependant regime (Figure 5.3) after 2 days. At the end of the exposure period, 75% had died in the temperature-dependant experiments.

Aqueous exposure to AgNPs caused crab mortality during the experimental periods indicating that the AgNPs and temperature combinations were toxic to the survival of the crabs. No mortalities were observed during the 7 day experimental period in the control, 10 µg/mL and 100 µg/mL AgNP groups. However, mortalities were observed for all other AgNP-dependant regime (Figure 3) after 2 days. In the AgNP-dependant experiment, crabs exposed to > 1 000 µg/mL showed signs of fatigue. Additional, approximately 25% of crabs in each of the 1 000 µg/mL and 10 000 µg/mL AgNP experimental groups had died after 2 days. At the end of the exposure period, 50% had died in the AgNP-dependant experiments. The validity of the tests was possible because the mortality in the control group (*i.e.* 0 µg/mL) was less than 10% in all of the cases (no mortalities were observed).

At the 7 day exposure period, the BMD value of AgNP was 782.77 µg/mL and the CTMax was 25.37 °C (Table 5.1), while the EC_{50} of AgNP was 4083.36 µg/mL and the CTMax was 23.56 °C. Based on these results, AgNPs appears to exert increased toxicity (as evidenced by the number of mortalities) with increasing AgNP concentration and temperature. Previous

studies have reported that AgNPs (10-200 nm) induced 2 h EC_{50} of 3300 nM ± 572 in *Chlamydomonas reinhardtii* (Navarro *et al*., 2008). Others have reported 48 h EC_{50} of 2.5 µg/mL and 4.9 µg/mL AgNPs in *Oncorhynchus mykiss* (Farkas *et al*., 2009). As such, these results suggest that AgNPs can generate different degrees of toxicity under different exposure conditions (such as NP size, coating and concentration, temperature, salinity, etc.) (Yang *et al*., 2012).

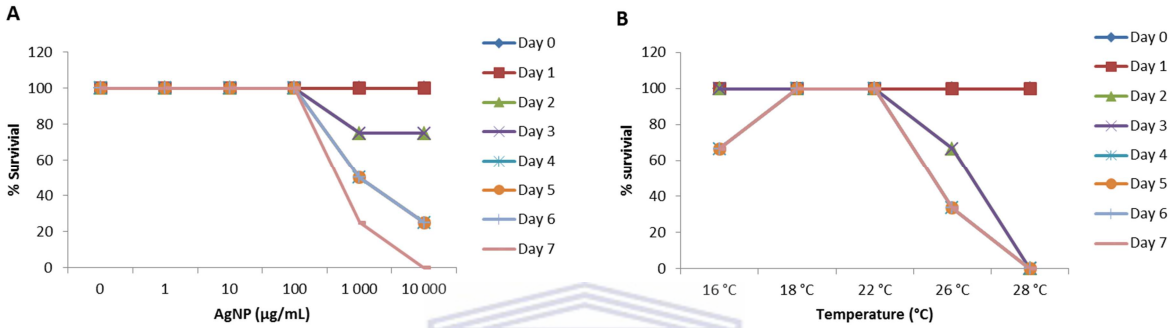


Figure 5.3: % Survival in the AgNP-dependant (A) and temperature-dependant (B) experiments.

Table 5.1: BMD, CTMax and, 95% confidence limits, and LogProbit line parameters for experiment 1 and experiment 2 experimental treatments for *P. perlatus* is shown.

Experiment	Exposure (h)	EC_{50} (µg/mL AgNP)	BMD (µg/mL AgNP)	95% confidence limit	Slope	Correlation coefficient
Experiment 1	24	-	-	-	-	-
	48	3689.16	18 827.00	$p < 0.05$	0.29	0.70
	72	3689.16	18 827.00	$p < 0.05$	0.29	0.70
	96	4209.83	1 947.73	$p < 0.05$	0.55	0.85
	120	4209.83	1 947.73	$p < 0.05$	0.55	0.85
	144	4209.83	1 947.73	$p < 0.05$	0.55	0.85
	168	4083.36	782.77	$p < 0.05$	2.75	0.82

Experiment	Exposure (h)	CTMax (°C)	95% confidence limit	Slope	Correlation coefficient
Experiment 2	24	-	-	-	-

48	26.14	$p < 0.05$	18.00	0.83
72	26.14	$p < 0.05$	18.00	0.83
96	25.37	$p < 0.05$	18.00	0.75
120	25.37	$p < 0.05$	18.00	0.75
144	25.37	$p < 0.05$	18.00	0.75
168	25.37	$p < 0.05$	18.00	0.75

3.4.Oxidative stress and antioxidant defence

Oxidative stress is a common pathway of toxicity in aquatic organisms. ROS have been reported to induce oxidative damage including enzyme inactivation, protein degradation, DNA damage and lipid peroxidation (Di Giulio *et al*., 1995). The generation of free radicals in response to AgNPs and temperature stress should be scavenged by the various antioxidant systems to serve as a protective response to detoxify the ROS generated (Singaram *et al*., 2013). In crabs, the defence system is equipped with enzymes to counteract free radicals produced during exposure to stressors (Kong *et al*., 2011; Singaram *et al*., 2013). To determine the enzyme activities occurring under a controlled AgNP and temperature regime, six crabs were exposed to 0 µg/mL (control group) and 782.77 µg/mL (exposed group) of AgNP at 25.3 °C for seven days (Table 2). The total protein content (protein concentration per gram of tissue) was found to be highest in the control tissues when compared to the exposed counterparts (Figure 5.4). The decrease in protein content observed in the exposed tissues could be attributed to mitochondrial damage (Qin *et al*., 2012). AgNPs are known to induce oxidative stress by triggering ROS through the mitochondrial electron transport chain (Hermes-Lima, 2005). Also, protein synthesis is generally down-regulated during oxidative stress (Vogel *et al*., 2011). This finding was supported by several authors. For example, Vogel *et al*. (2011) reported inhibition of protein synthesis in Saccharomyces cerevisiae following diamide-induced oxidative stress; while Lopez-Alonso *et al*. (2013) reported similar results for rat hepatocytes exposed to Cylindrospermopsin, a widely distributed freshwater cyanobacterial toxin. Another possibility for the observed protein reduction could be attributed to the proteolysis process for energy production and utilization (Balasubramanian, 1983; Vijayavel and Balasubramanian, 2006).

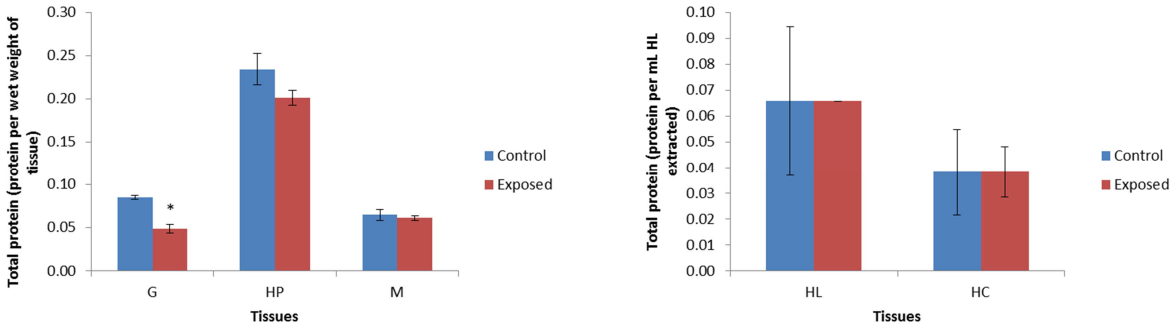


Figure 5.4: Effect of 7-day exposures to AgNPs (782.77 µg/mL) at 25.37 °C on total protein concentrations in tissues (G = gills; HP = hepatopancreas; M = muscles; HL = haemolymph; HC = haemocytes) in *P. perlatus*. Data are presented as means ± S.E.M. Statistical significance (indicated by *) was denoted by $p < 0.05$ versus the respective control crabs.

Cytochrome P450 (CYP450) belongs to the superfamily of hemeproteins and is one of the most important Phase I detoxification enzymes, which is largely responsible for the metabolism (degrading and elimination) of xenobiotics (Kulthong *et al*., 2012). Cytochrome P450 instigate the detoxification process, comprise largely of heme proteins which are predominantly located in the endoplasmic reticulum of the liver (Stegeman, 1992; Bucheli, 1995). CYP enzymes are expressed in several tissues including the liver, kidney, lung, adrenal, gonads and brain, and are regarded as the main enzymes involved in drug metabolism (Aguirre-Martínez *et al*., 2013). In crabs, the hepatopancreas is the major site of uptake and CYP enzyme-dependent biotransformation of lipophilic xenobiotics (Bucheli and Fent, 1995). Activity of CYP450 was significantly ($p < 0.05$) enhanced when compared to the control group (Figure 5.5). Conversely, previous studies have reported inhibition of hepatic CYP450 activity by AgNP in rats (Kulthong *et al*., 2012). Activity of CYP450 was tissue-specific. AgNPs strongly inhibited the CYP450 activity in the hepatopancreas and haemolymph, while activity was significantly induced in the gills ($p < 0.05$), haemocytes ($p < 0.05$) and muscles (Figure 5.5A). These findings suggest that CYP450 were up-regulated by AgNPs with the greater extent being seen with in gills and haemocytes possibly indicative of a protective mechanism to promote metabolism and excretion within these tissues (Lamb *et al*., 2010).

Superoxide dismutase (SOD) is the first Phase II enzyme to deal with oxyradicals (Linhua *et al*., 2009) and is responsible for catalyzing the dismutation of highly superoxide radical O2− to O2 and H2O2 (Linhua *et al*., 2009; Jayaseelan *et al*., 2014). It is very sensitive to toxins and can therefore be used as an oxidative stressed signal for the early warning of

environmental pollution (Linhua *et al*., 2009). Catalase (CAT) is also a key Phase II enzyme in the antioxidant defence system, converting the resulting free radicals H_2O_2 to water and oxygen (Linhua *et al*., 2009). Significant differences ($p < 0.05$) were observed between the control and exposed groups for most tissues (Figure 5). In the present study, SOD and CAT activities were induced in all tissues, with the highest activity observed in the haemolymph (Figure 5B). The induction of SOD and CAT activities observed are consistent with ROS being generated during the response to AgNPs and temperature stress. This is in accordance with Dissanayake *et al*. (2011) who reported lower oxidative stress (assessed through total haemolymph antioxidant status) of the shore crab *Carcinus maenas* during the warmer months. In another study, Ahamed *et al*. (2010) reported increased activities of SOD and CAT (in hepatopancreas) in Drosophila melanogaster exposed to AgNPs. Glutathione S-transferase (GST), Phase II biotransformation enzymes, are involved in the cellular detoxification of xenobiotic compounds (Pinho *et al*., 2005) that play a fundamental role in protection against endogenous and exogenous toxic chemicals (Sheehan *et al*., 2001), by conjugating the thiol group of the glutathione. GST activity is generally found in the gills and hepatopancreas, which are in direct contact with the external environment (Lavarias *et al*., 2011). In this study, induction of GST activity was observed in the haemolymph (4.05 times higher in the exposed group when compared to the control), haemocytes (4.84 times higher in the exposed group when compared to the control) and gills (1.19 times higher in the exposed group when compared to the control). The elevated GST levels in these tissues suggest activation of detoxification mechanisms (Singaram *et al*., 2013) owing to oxidative stress. Notably, inhibition (0.73 times lower when compared to the control) of GST activity was observed in the HP and also for the muscles (0.73 times lower when compared to the control) of the exposed group (Figure 5). The former result is in agreement with a previous work that shows induction of GST activity in gill tissues of the crustacean *Macrobrachium borellii* exposed to hydrocarbons (Lavarías *et al*., 2011), and inhibition in the hepatopancreas of C. maenas exposed to 5% of wastewater effluent (Ghedira *et al*., 2009). The observed results suggest differential and more pronounced responses by these cellular defence mechanisms (SOD, CAT and GST) in the haemolymph compared to the other tissues, especially for the gills and hepatopancreas, suggesting an enhanced effect of ROS in this tissue.

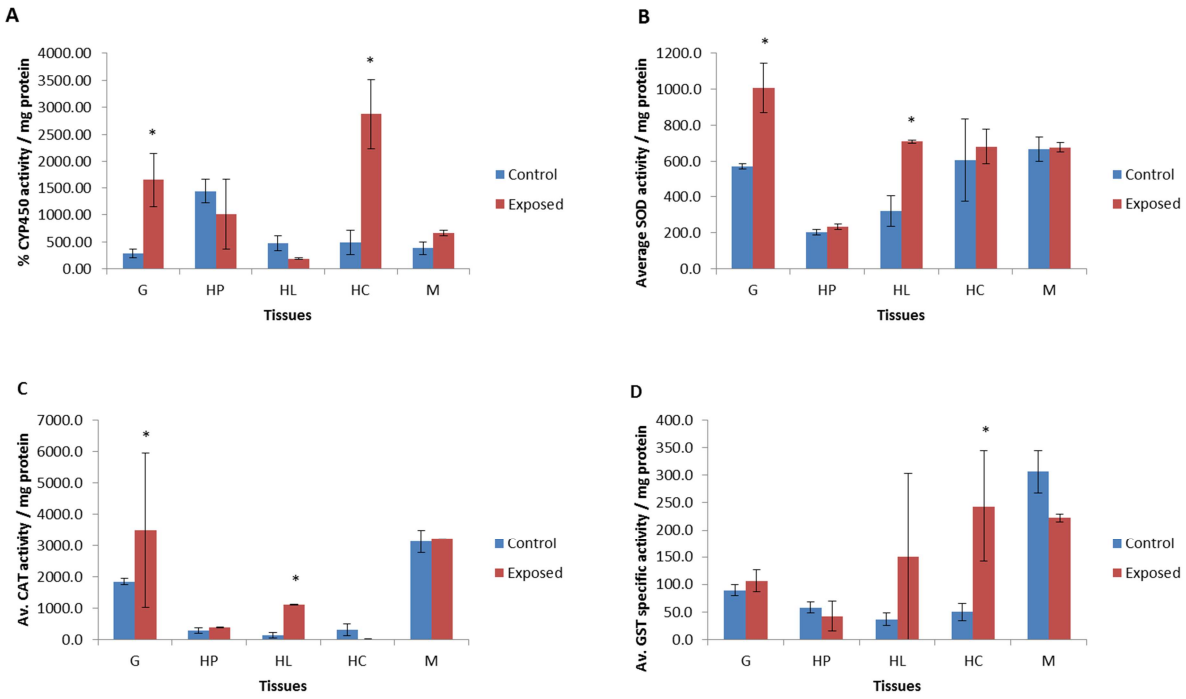


Figure 5.5: Effect of 7-day exposures to AgNPs (782.77 µg/mL) at 25.37 °C on enzymatic activity of CYP450 (A), SOD (B), CAT (C) and GST (D) in tissues (G = gills; HP = hepatopancreas, M = muscles; HL = haemolymph; HC = haemocytes) of *P. perlatus* following a seven-day exposure period. Data are presented as means ± S.E.M. Statistical significance (indicated by *) was denoted by $p < 0.05$ versus the respective control crabs.

From the above results, it can be concluded that AgNPs caused oxidative stress, affecting cellular enzymatic defences, which may be a ROS-induced toxicity mechanism. Other studies also found an important effect of environmental stressors in the oxidative stress response of various tissues of crabs. For example, Rodrigues *et al*. (2012) observed that temperature influenced oxidative stress biomarkers in the muscle and digestive gland of *Callinectes maenas* and Madeira *et al*. (2014) in the haemolymph of *Pachygrapsus marmoratus*. Similarly, Freire *et al*. (2011) observed that salinity influenced oxidative stress biomarkers in the gills, hepatopancreas, haemolymph and muscle of *C. danae* and *C. ornatus*. Co-exposure studies of NPs with other common environmental stressors have reported toxicity in the mussel *Unio tunidus* following exposures to ZnO-NPs, organic pollutants and temperature. These studies show the significant effects of co-exposure of NPs with environmental stressors, as well as the oxidative stress response of various aquatic organisms and the various tissues are affected.

4. Principal Component Analysis (PCA)

Principal component analysis (PCA) was used to assess the interrelationships amongst biochemical responses and metals content in tissues of the control and exposed crab groups. In the PCA, the first two PCA axes were selected because they explain the majority of variance. As shown on the PCA scatterplot (Figure 5.6), two principal components were defined for explaining the major amount of total variance (71.72%) when accumulated metals and biomarkers were considered. The first principal component (PC1) accounted for 41.48% of the variance while PC2 accounted for 30.24%. A close association between SOD, GST and Ag, with exposed gills was observed; while CAT, Mg and Ca where closely associated with exposed muscles. This PCA suggests a relationship between the antioxidant efficiency (particularly SOD and GST) of *P. perlatus* to counteract Ag. A close association between CYP450 and the haemocytes was observed, supporting the results observed for the antioxidant enzymes. Noticeably, hepatopancreas and haemolymph did not establish any association between biochemical responses or metal content.

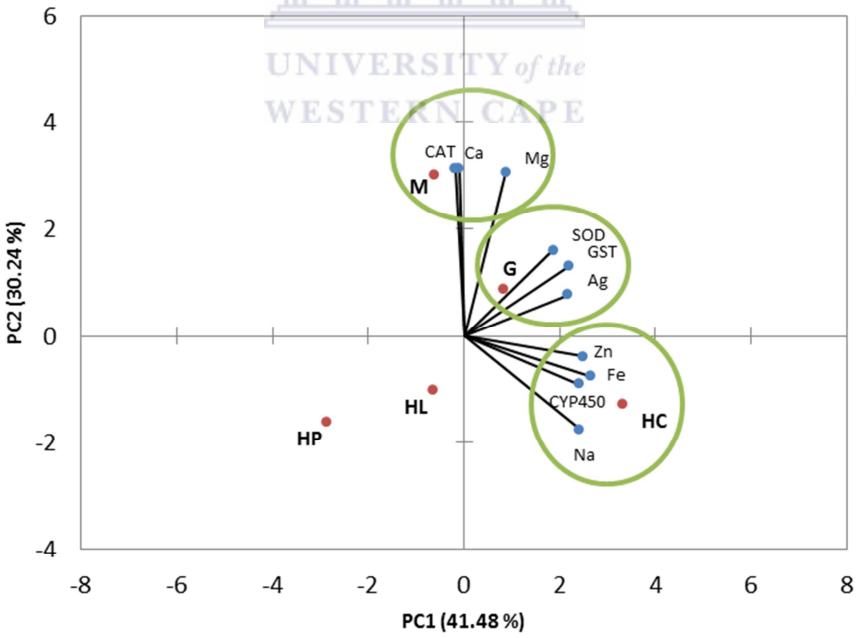


Figure 5.6: Principal component analysis (PCA) applied on data of control and exposed crab groups taking into account five variables (SOD, CAT, GST, CYP450, and trace metal concentrations).

A second PCA was constructed which incorporates tissues per treatment (control tissues (Figure 5.7A) and exposed tissues (Figure 5.7B) and a spectrum of metal concentrations (determined by ICP-OES and ICP-MS). In the control group, the two principal components represented 87.72% of total variance, with PC1 accounting for 63.23% of the variance and PC2 accounting for 24.49%. The scatter plot showed that muscles of the control group were largely associated with the metals Mn, K, Mg, Sr and Ca. The haemocytes of the control group were largely associated with the metals Ag, Fe, Pb, Cr, Al, Cd, V, Zn, Se, Na, Ni and Cu. Gills, hepatopancreas and haemolymph of the control group showed no association with metal content.

In the exposed group, the two principal components represented 82.91% of total variance, with PC1 accounting for 54.60% of the variance and PC2 accounting for 28.31%. As with the control group, exposed gills and hepatopancreas showed no association with metal content. Exposed muscles were closely associated with the metals Sr, Ca, K, Mg and Mn. Similar to the control haemocytes, exposed haemocytes were closely associated with the metals Ag, Fe, Pb, Cr, Al, Cd, V, Zn, Se, Na, Ni and Cu.

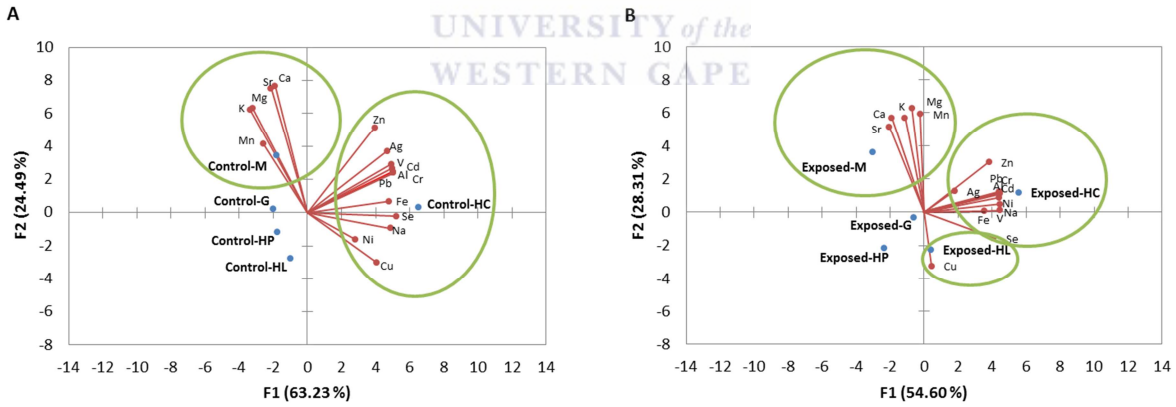


Figure 5.7: Principal component analysis (PCA) of tissues of control (A) and exposed group (B) with metal content.

5. Conclusions

This study shows the importance of temperature as an influential variable in the toxicity of *P. perlatus* exposed to AgNPs for all the tissues tested. In the acute toxicity assay, mortalities were observed at 1 000 µg/mL and 10 000 µg/mL AgNPs, and at temperatures 16 °C, 26 °C

and 28 °C, while no mortalities were observed at 18 °C and 22 °C (BMD 782.77 µg/mL; $P < 0.05$). In addition, the present study attempted to study co-effects of AgNP and temperature stress in biochemical activity in the tissues of the Cape River crab P. perlatus. Based on the results obtained, it can be concluded that AgNPs and temperature stress had an important effect of the levels of oxidative stress biomarkers in *P. perlatus*. It can further be concluded that the AgNPs/temperature combination induced an overall activation of Phase I and Phase II enzymes. For example, activity of CYP450 enzymes was significantly induced ($p < 0.05$) and showed up-regulation in gills and haemocytes. The exposed group displayed the following activity pathway for CYP450: HC > G > HP > M > HL; SOD: G > HL > HC > M> HP; CAT: G > M> HL > HP > HC; and for GST: HC > M > HL > G > HP. The distinct antioxidant efficiency in the haemolymph reflects the dissimilar physiological and metabolic function between the tissues. The haemolymph seemed to be more susceptible to oxidative stress (considering the significantly induced CYP450, SOD, CAT and GST levels), while the gill is the main tissue for Ag accumulation. The gills are in direct contact with the external environment, and thus, although not having high levels of oxidative stress biomarkers, have the highest Ag levels. These results support the hypothesis that AgNPs and elevated temperature in the environmentally relevant range increased the toxicity and cellular responses. Furthermore, the results of this study supplement the existing information on the toxicity of AgNPs in a valuable freshwater crab species *P. perlatus* and furthermore highlight this metal-NPs capacity to elicit oxidative stress in tissues. Significant aspects of climate change and pollutant interactions merit further studies to assess the effects on vulnerable species and exposing the nature of thresholds that might potentially trigger adverse events.

6. Acknowledgments

This research was supported by the CSIR. The authors wish to thank Christoff Truter (University of Stellenbosch), Dewald Schoeman (University of the Western Cape), Charlton van der Horst (CSIR) and Sebastian Brown (CSIR, Chemical and Analytical Services, Stellenbosch) for assisting in various aspects of the research.

7. References

Aguirre-Martínez, G.V., De Valls, T.A. and Martin-Diaz, M. L., 2013. Early responses measured in the brachyuran crab *Carcinus maenas* exposed to carbamazepine and novobiocin: Application of a 2-tier approach. Ectoxicol. Environ. Saf. 97, 47-58.

Ahamed, M., Posgai, R., Gorey, T.J., Nielsen, M., Hussain, S.M., Rowe, J.J., 2010. Silver nanoparticles induced heat shock protein 70, oxidative stress and apoptosis in *Drosophila melanogaster*. Toxicol. Appl. Pharmacol. 242, 263-269.

Bagnyukova, T.V., Lushchak, O.V., Story, K.B. and Lushchak, V.I., 2007. Oxidative stress and antioxidant defense responses by goldfish tissue to acute change of temperature from 3 to 23 °C. J. Therm. Biol. 32, 227-234.

Balasubramanian, M.P, Dhandayuthapani, S., Neelaiappan, K. and Ramalingam, R., 1983. Comparative studies on phosphomonoesterase in helminths. . Helminthologia 20, 111–120.

Bates, B.C., Z.W. Kundzewicz, S. Wu and J.P. Palutikof, Eds., 2008. Climate Change and Water. Technical Paper of the Intergovernmental Panel on Climate Change, IPCC Secretariat, Geneva, 210 pp.

Brouwer, M., Brouwer, T.H., 1998. Biochemical defense mechanisms against copper induced oxidative damage in the blue crab, *Callinectes sapidus*. Arch. Biochem. Biophys. 351, 257–264.

Bucheli, T.D.A.F.K., 1995. Induction of cytochrome P450 as a biomarker for environmental contamination in aquatic ecosystems. Crit. Rev. Environ. Sci. Technol. 25, 201-268.

Canesi, L., Ciacci, C., Fabbri, R., Marcomini, A., Pojana, G. and Gallo, G., 2012. Bivalve molluscs as an unique target group for nanoparticle toxicity Mar. Environ. Res. 76, 16–21

Cheng, P.W., Liu, S.H., Hsu, C.J., Lin-Shiau, S.Y., 2005. Correlation of increased activities of Na^+, K^+-ATPase and Ca^{2+}-ATPase with the reversal of cisplatin ototoxicity induced by D-methionine in guinea pigs. Hear. Res. 205, 102-109.

Choi, J.E., Kim, S., Ahn, J.H., Youn, P., Kang, J.S., Park, K., Yi, J., Ryu, D.Y., 2010. Induction of oxidative stress and apoptosis by silver nanoparticles in the liver of adult zebrafish. Aquat. Toxicol. 100, 151-159.

Di Giulio, R.T., Benson,W.H., Sanders, B.M., Van Veld, P.A., 1995. Biochemical mechanisms: metabolism, adaptation, and toxicity. In: Rand, G.M. (Ed.), Fundamentals

of Aquatic Toxicology: Effects, Environmental Fate, and Risk Assessment. Taylor & Francis, Bristol, USA, pp. 523–561.

Dissanayake, A., Galloway, T.S., Jones, M.B., 2011. Seasonal differences in the physiology of *Carcinus maenas* (Crustacea: Decapoda) from estuaries with varying levels of anthropogenic contamination. Estuar. Coast. Shelf Sci. 93, 320-327.

Fabrega, J., Tantra, R., Amer, A., Stolpe, B., Tomkins, J., Fry, T., Lead, J.R., Tyler, C.R.,Galloway, T.S., 2012. Sequestration of zinc from zinc oxide nanoparticles and life cycle effects in the sediment dweller amphipod *Corophium volutator*. Environ. Sci. Technol. 46, 1128–1135.

Falfushynska, H., Gnatyshyna, L., Yurchak, I. and Sokolova, I., 2015. The effects of zinc nanooxide on cellular stress responses of the freshwater mussels *Unio tumidus* are modulated by elevated temperature and organic pollutants. Aquat. Toxicol. 162, 82-93.

Farkas, J., Christian, P., Urrea, J.A., Roos, N., Hassello,¨v M, Tollefsen, K.E., Thomas, K.V., 2010. Effects of silver and gold nanoparticles on rainbow trout (*Oncorhynchus mykiss)* hepatocytes. Aquat. Toxicol. 96, 44–52.

Freire, C.A., Togni, V.G., Hermes-Lima, M., 2011. Responses of free radical metabolism to air exposure or salinity stress, in crabs (*Callinectes danae* and *C. ornatus*) with different estuarine distributions. Comp. Biochem. Physiol. Mol. Integr. Physiol. 160, 291-300.

Ghedira, J., Jebali, J., Bouraoui, Z., Banni, M., Chouba, L. and Boussetta, H., 2009. Acute effects of chlorpyryphos-ethyl and secondary treated effluents on acetylcholinesterase and butyrylcholinesterase activities in *Carcinus maenas.* J. Environ. Sci. 21, 1467–1472.

Gomes, T. Pereiraa, C.G., Cardosoa, C., Sousab, V.S., Teixeirab, M.R., Pinheiroc, J.P. Bebiannoa, M.J., 2014. Effects of silver nanoparticles exposure in the mussel *Mytilus galloprovincialis.* Mar. Environ. Res. 101, 208–214

Halliwell, B., Gutteridge, J.M., 1999. Free Radicalsin Biology and Medicine. Oxford University Press, New York. Hermes

Hermes-Lima, M., 2005. Oxygen in biology and biochemistry: role of free radicals. Pp. 319-368. Ed. Storey, K.B. Functional Metabolism: Regulation Adaptation. John Wiley & Sons, Inc., Hoboken, NJ, USA. doi: 10.1002/047167558X.ch12.

IPCC (Intergovernmental Panel on Climate Change), 2007. Climate change 2007: the physical science basis. In: Solomon S, Qin D, Manning M, Chen Z and others (eds)

Contribution of Working Group I to the 4th assessment report of the Intergovernmental Panel on Climate Change. Cambridge University Press, Cambridge.

Jayaseelan, C., Rahuman, A.A., Ramkumar, R., Perumal, P., Rajakumar, G., Kirthi, A.V., Santhoshkumar, T. and Marimuthu, S., 2014. Effect of sub-acute exposure to nickel nanoparticles on oxidative stress and histopathological changes in Mozambique tilapia, *Oreochromis mossambicus*. Ecotoxicol. Environ. Saf. 107, 220–228.

Kim YS, Kim JS, Cho HS, Rha DS, Kim JM, Park JD, Choi BS, Lim R, Chang HK, Chung YH, Kwon IH, Jeong J, Han BS, Yu IJ., 2008. Twenty-eight-day oral toxicity, genotoxicity, and gender-related tissue distribution of silver nanoparticles in Sprague-Dawley rats. Inhal. Toxicol. 20, 575–583.

Kim YS, Song MY, Park JD, Song KS, Ryu HR, Chung YH, Chang HK, Lee JH, Oh KH, Kelman BJ, Hwang IK, Yu IJ., 2010. Subchronic oral toxicity of silver nanoparticles. Part. Fibre Toxicol. 7:20.

Kong, X., Wang, G. and Li., 2012. Effects of low temperature acclimation on antioxidant defenses and ATPase activities in the muscle of mud crab (*Scylla paramamosain*). Aquaculture. 370-371, 144-149.

Kvitek L., Vanickova M., Panacek A., Soukupova J., Dittrich M., Valentova E., Prucek R., Bancirova M., Milde D. & Zboril R., 2009. Initial Study on the Toxicity of Silver Nanoparticles (NPs) against *Paramecium caudatum*. J. Phys. Chem. C. 113, 4296-4300.

Kulthong, K., Maniratanachote, R., Kobayashi, Y., Fukami, T., and Yokoi, T., 2012. Effects of silver nanoparticles on rat hepatic cytochrome P450 enzyme activity. Xenobiotica. 42(9), 854-862.

Lavarias, Sl., Heras, H., Pedrini, N., Tournier, H. and Ansaldo, M., 2011. Antioxidant response and oxidative stress levels in *Macrobrachium borellii* (Crustacea: Palaemonidae) exposed to the water-soluble fraction of petroleum. Comp. Biochem. Physiol. C.153, 415-421.

Linhua, H., Zhenyu, W. and Baoshan, X., 2009. Effect of sub-acute exposure to TiO_2 nanoparticles on oxidative stress and histopathological changes in Juveline Carp (*Cyprinus carpio*). J. Environ. Sci. 21, 1459-1466.

Livingstone, D.R., Lips, F., Martinez, P.G., Pipe, R.K., 1992. Antioxidant enzymes in the digestive gland of the common mussel *Mytilus edulis*. Mar. Biol. 112, 265–276.

Lopez-Alonso, H., Rubiolo, J.A., Vega, F., Vieytes, M.R. and Botana, L.M., 201). Protein synthesis inhibition and oxidative stress induced by *Cylindrospermopsin elicit* apoptosis in primary rat hepatocytes. Chem. Res. Toxicol. 26(2), 203-212.

Martins, C.M.G., Fernanda Barcarolli, I., Jaime de Menezesb, E., Mussoi Giacominb, M., Wood, C.M. and Bianchinia, A., 2011 Acute toxicity, accumulation and tissue distribution of copper in the blue crab *Callinectes sapidus* acclimated to different salinities: In vivo and in vitro studies. Aquat. Toxicol. 101, 88-99.

Madeira, D., Narciso, L., Cabral, H., Diniz, M., Vinagre, C., 2014. Role of thermal niche in the cellular response to thermal stress: lipid peroxidation and HSP70 in coastal crabs. Ecol. Indic. 36, 601-606.

Maria, V.L., Santos, M.A. and Bebianno, M.J., 2009. Contaminant effects in shore crabs (*Carcinus maenas*) from Ria Formosa Lagoon. Comp. Biochem. Physiol. C. 150, 196-208.

McDiarmid, R.W. and Altig, R., 1999. Tadpoles: The Biology of Anuran Larvae, University of Chicago Press ISBN 0-226-55762-6, p 202.

Mora, C., Ospina, A., 2001. Tolerance to high temperatures and potential impact of sea warming on reef fishes of Gorgona Island (tropical eastern Pacific). Mar. Biol.139, 765-769.

Navarro, E.; Piccapietra, F.; Wagner, B.; Marconi, F.; Kaegi, R.; Odzak, N.; Sigg, L.; Behra, R., 2008. Toxicity of silver nanoparticles to *Chlamydomonas reinhardtii* Environ. Sci. Technol. 42(23), 8959– 8964.

Novo, M.S., Miranda, R.B., Bianchini, A., 2005. Sexual and seasonal variations in osmoregulation and ion regulation in the estuarine crab *Chasmagnathus granulatus* (Crustacea, Decapoda). J. Exp. Mar. Biol. Ecol. 323, 118–137.

Pinho, G.L.L., da Rosa, C.M., Maciel, F.E., Bianchini, A., Yunes, J.S., Proenca, L.A.O. and Monserrat, J.M., 2005. Antioxidant responses and oxidative stress after microcystin exposure in the hepatopancreas of an estuarine crab species. Ecotoxicol. Environ. Saf. 61, 353–360.

Qin, Q., Qin, S., Wang, L. and Lei, W., 2012. Immune responses and ultrastructural changes of hemocytes in freshwater crab. Aquat. Toxicol. 106-107, 140-146.

Rodrigues, A.P., Oliveira, P.C., Guilhermino, L., Guimarães, L., 2012. Effects of salinity stress on neurotransmission, energy metabolism, and anti-oxidant biomarkers of *Carcinus maenas* from two estuaries of the NW Iberian Peninsula. Mar. Biol. 159, 2061-2074.

Ronisz, D., Larsson, D.G.J., Forlin, L., 1999. Seasonal variations in the activities of selected hepatic biotransformation and antioxidant enzymes in eelpout (*Zoarces viviparus*). Comp. Biochem. Physiol. C, 124, 271–279.

Schiekek, D., Sundelin, B., Readman, J.W. and Macdonald, R.W., 2007. Interactions between climate change and contaminants. Mar. Poll. Bull. 54, 1845-1856.

Sheehan, D., Meade, G., Foley, V.M. and Dowd, C.A., 2001. Structure, function and evolution of glulathionein transferases: implications for classification of non-mammalian members of an ancient enzyme superfamily. Biochem. J. 360, 1-16.

Singaram, G., Harikrishnan, T., Chen, F-Y., Bo, J. and Giesy, J.P., 2013. Modulation of immune-associated parameters and antioxidant responses in the crab (*Scylla serrata*) exposed to mercury. Chemoshere. 90, 917-928.

Snyman, R. G., Reinecke, A. J. and Nel, J. A. J., 2002. Uptake and distribution of copper in the freshwater crab, *Potamonautes perlatus* (Crustacea) in the Eerste River, South Africa, Afr. Zoology. 81–89.

Stegeman, J.J., Brouwer, M., Richard, T.D.G., Forlin, L., Fowler, B.A., Sanders, B.M., van Veld, P.A.., 1992. Molecular responses to environmental contamination: enzyme and protein systems as indicators of chemical exposure and effect. In: Huggett, R. J., Kimerly, R.A., Mehrle, P.M., JR, Bergman, H.L. (ed.) Biomarkers: Biochemical, Physiological and Histological markers of Anthropogenic Stress. Chelsea, MI, USA: Lewis Publishers.

Vijayavel, K. and Balasubramanian, M., 2006. Fluctuations of biochemical constituents and marker enzymes as a consequence of naphthalene toxicity in the edible estuarine crab *Scylla serrata*. Ecotox. Environ. Saf. 63, 141-147.

Vogel, C., Silva, G.M. and Marcotte, E.M., 2011. Protein expression regulation under oxidative stress. Mol. Cell. Proteomics, 10(12), M111.009217.

Walters, C., Pool, E. and Somerset, V., 2013. Aggregation and dissolution of silver nanoparticles in a laboratory-based freshwater microcosm under simulated environmental conditions, Toxicol. Environ. Chem. 95:10, 1690-1701.

Wang, W., Wang, A., Liu, Y., Xiu, J., Liu, Z., Sun, R., 2006. Effects of temperature on growth, adenosine phosphates, ATPase and cellular defense response of juvenile shrimp *Macrobrachium nipponense*. Aquaculture 256, 624–630.

Yang, X., Gondikas, A.P., Marinakos, S.M., Auffan, M., Liu, J., Hsu-Kim, H. and Meyer, J.N., 2012. Mechanism of silver nanoparticle toxicity is dependent on dissolved silver and surface coating in *Caenorhabditis elegans*. Environ. Sci. Technol. 46, 1119−1127.

Zhang, J.F., Shen, H., Wang, X.R., Wu, J.C., Xue, Y.Q., 2004. Effects of chronic exposure of 2,4-dichlorophenol on the antioxidant system in liver of freshwater fish *Carassius auratus*. Chemosphere. 55, 167–174.

Zhu, X., Zhou, J. and Cai, Z., 2011. The toxicity and oxidative stress of TiO_2 nanoparticles in marine aboalone (*Haliotis diversicolor supertexta*). Mar. Poll. Bull. 63, 334-338.

CHAPTER 6: CONCLUSIONS AND RECOMMENDATIONS

1. General conclusion

The present study was designed to investigate the behaviour of AgNPs in aqueous suspension under different environmental parameters with particular focus on environmental conditions such as temperature, and the concomitant effects on AgNP uptake, toxicity and antioxidant defence mechanisms in a freshwater crab species *Potamonautes perlatus*, common in the waters south-western region of the Western Cape (Snyman et al., 2002). Nano-products are increasingly being used in various products and, consequentially, the potential adverse effects associated with exposure to nanomaterials (NMs) are of concern. The risks associated with NMs (i.e. its fate and behaviour in the environment) are largely unknown and difficult to predict. As the ultimate sink for conventional contaminants, the aquatic ecosystem is therefore predisposed to the potential effects of NPs. Although our knowledge on the toxicity of various NMs in the aquatic environment has increased over the past few years, there is still a lack of knowledge regarding exposure concentrations, bioaccumulation in tissues, as well as environmental factors which could potentially affect its toxicity or bioaccumulation. The present thesis emerges in this context, centring on the effects of the most commonly used and commercial available AgNP using a freshwater crab species *P. perlatus* as a sentinel species.

Chapter 1 discussed nanomaterials, freshwater crabs, oxidative stress, biomarkers and the antioxidant defence system. This chapter also introduced the research hypothesis of this study and described the structure of the thesis and what each chapter entails. The aims and objectives of this study are also highlighted.

Chapter 2 reviewed the available research pertaining to nanomaterials and specifically, silver nanoparticles in the aquatic environment (in plants, aquatic invertebrates and fish). It further focussed on the use of crabs in biomarkers studies, and concluded with providing recommendations on future nano-toxicological studies using invertebrates. This chapter was published in Journal of Environmental Science and Health, Part A: Toxic/Hazardous Substances and Environmental Engineering (2014) volume 49: 13 by C. R. Walters, with co-authors E. J. Pool and V. S. Somerset.

The behaviour of NPs is influenced by several factors, such as the inherent properties of the NP (size, shape, surface area, surface charge, crystal structure, coating, and solubility/dissolution) and environmental properties of the dispersed media (pH, ionic strength, salinity, organic matter). These properties of NPs are particularly important for assessing their fate and ecotoxicity in the environment. Chapter 3 presented a study on the effects of AgNPs in its natural state and in solution under different environmental conditions. This chapter concluded that higher temperatures resulted in higher toxicity due to the formation of smaller aggregates at elevated temperatures, and that AgNP dissolution and sedimentation contributed to a higher availability and toxicity of AgNP (and Ag+) to *P. perlatus*. This chapter was published in Toxicological & Environmental Chemistry volume 95: 10 by C. R. Walters, with co-authors E. J. Pool and V. S. Somerset.

Several studies have previously investigated the effects of NPs in mussels, benthic organisms, and fish. These studies have all generally reported induction of oxidative stress. However, in the natural environment, AgNPs are not present in isolation. As such, it is important to consider the presence of other environmental stressors. For this purpose, Chapter 4 aimed to investigate the modulating effects of temperature as an environmental stressor. In this chapter it was observed that temperature is an important variable in the oxidative stress response of crabs exposed to AgNPs. Overall, this in vivo study suggests that: (1) the toxicity of AgNPs to *P. perlatus* is both concentration and temperature dependant; (3) co-exposure to AgNPs and temperature results in oxidative stress, (4) temperature modulated the oxidative stress effects in *P. perlatus* exposed to AgNPs; (5) (6) Ag from AgNP is largely sequestered in the gills.

Chapter 5 investigated the mechanisms of uptake, target tissues and toxicity of AgNPs in *P. perlatus*, assays using environmental realistic concentrations of AgNPs. The results show that the haemolymph are more susceptible to oxidative stress originated by AgNPs and temperature stress, whereas the gills constitutes the main storage organ for Ag. This chapter concluded overall that AgNPs and elevated temperature in the environmentally relevant range increased the toxicity and cellular responses.

2. Concluding remarks

In this thesis, the use of traditional biomarkers of oxidative stress, metal exposure and tissue distribution were combined to evaluate the effects of AgNPs exposure in a freshwater crab species *P. perlatus*. NP characterization in experimental media was also undertaken to link NPs properties and behaviour to toxic responses. Given the capacity of AgNPs to generate oxidative stress and induce oxidative damage, and the ability of temperature to modulate these effects, it becomes apparent that although oxidative stress is a predictable outcome for NP toxicity, several factors are responsible for determining their modes of action. Overall, these findings have shed light on the poorly understood field of nanotoxicology, and furthermore, improve our understanding of risk assessment of metal NPs using the data collected in this project.

The following are specific conclusions for AgNPs resulting from this study:

- The formation of smaller aggregates at higher temperatures suggests higher toxicity.
- The release of free metal ions from NPs and NPs aggregates contribute to a higher toxicity towards aquatic organisms.
- The freshwater crab *P. perlatus* has proved to be a significant target for AgNP exposure and, furthermore, has proved to be a suitable species to assess the ecotoxicity of AgNP in the aquatic environment.
- Overall, these results suggest that oxidative stress is a significant mechanism of AgNP toxicity, suggested by the alteration on the antioxidant capacity of cells against ROS formation and consequently enzymatic activation / inhibition with increasing AgNP concentration and temperatures.
- Oxidative stress responses to AgNPs particles were significantly modulated by temperature stress in *P. perlatus* as antioxidant levels were generally induced at higher AgNP and higher temperatures.
- In the acute toxicity study, mortality was observed from day 2 with maximum mortality achieved at day 7.
- In Chapter 4, the hepatopancreas was observed to be a more sensitive organ to the collective effects of AgNPs and elevated temperatures due to the higher antioxidant levels measured in this tissue.

- The haemolymph is more susceptible to oxidative stress from AgNPs and temperature co-exposure than its internalization, as evidenced by a stronger oxidative response (Chapter 5), whereas the gills are the main tissue for their storage (Chapter 5).
- Results indicated that enzymes involved in detoxification, *i.e.* CYP450, has functional significance in the haemocytes.
- Antioxidant enzymes activities (SOD and CAT), and GST are valuable tools to assess the oxidative status of crab tissues co-exposed to AgNPs and temperature.

3. Future Perspectives and Recommendations

- The work conducted in this thesis has focussed largely on the collective effects of AgNPs and temperature on the oxidative stress defences of the Cape River crab *P. perlatus*. Whether the reported results could be applicable in a more environmentally realistic setting has to be investigated, however these findings do offer several directions for future research:
- In Chapter 2 sedimentation was observed at elevated temperatures. This suggests that AgNPs could potentially pose a threat to benthic organisms. Future studies should thus consider the use of benthic organisms in nanotoxicological assessments.
- As evidenced in this thesis, AgNPs are not in present in isolation. It is therefore important that future studies consider the effects of multiple toxins such as emerging pollutants (pharmaceuticals, etc.). Other abiotic factors are also worthwhile considering in future co-exposure studies.
- South Africa's National Nanotechnology Strategy (DST, 2007) envisages the exploitation of nanotechnology in South Africa. Future studies should consider the transformation of AgNPs (both coated and uncoated) as it passes through wastewater treatment plants. Since the levels of AgNPs are expected to increase in the environment, one should question whether our wastewater treatment plants are capable of managing the elevated levels, in terms of their treatment capacity and efficiency. As such, the evaluation of the removal of selected NPs in wastewater by different water treatment processes should be undertaken in order to estimate the concentrations of NPs in reclaimed wastewater for potable reuse.
- Chronic in vivo exposures should be considered. In Chapter 5 it showed that haemolymph was largely affected by AgNP and temperature. Perhaps then more

effects would be observed in other tissues. Since aquatic organisms are exposed to contaminants for longer periods (even for an entire lifetime), future studies should consider a more realistic scenario in terms of exposure timeframes.

- Previous studies have reported notable differences between responses of male and female individuals, suggesting that there may be some gender-specific effects to NP exposure. Different responses amongst gender should thus be considered.
- Future investigations should assess the combined oxidative stress responses of AgNPs and lower temperature limits, *i.e*. critical thermal minima (CTMin).