

Combined Silver Nanoparticles and Temperature Effects in the Cape River Crab *Potamonautes Perlatus* - Interactions between Chemical and Climatic Stressors

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Abstract

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 *P. 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), 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 concentrations of AgNPs and temperature stress caused induced antioxidant defences of *P. perlatus*.

Keywords

Silver nanoparticles; Homeostasis; Biomarkers

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 [1-3]. An over-production of ROS can damage DNA, protein and lipids [4]. To maintain homeostasis and prevent oxidative stress under stress conditions, aquatic organisms have an antioxidant defence system for the removal of excess ROS [5]. 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 [6-8].

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 [9]. 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 [10,11]. 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 [12,13]. Few studies have investigated the ecotoxicity of co-exposure of NPs with other common environmental stressors. In a recent study, Falfushynska et al. [14] 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. [15] 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 [16] 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 [17], since climate change projections indicate an increase in the frequency, intensity and duration of thermal extremes [18]. 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 [19-21] 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).

Materials and Methods

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 m²/g,

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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 $\mu\text{g}/\text{mL}$. 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) [22]. 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 [22]. The specific surface area of the AgNP powder was analysed by BET using a ASAP 2010 (Accelerated Surface Area and Porosimetry System; Micromeritics Instrument Corporation).

Animal collection and acclimation

The Cape River crab *P. perlatius* 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 ($21 \pm 2^\circ\text{C}$) prior to exposure. Test media were completely renewed daily. No food was provided during the acclimatization period.

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 [23]. 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" [24], or that temperature for a given species above which most individuals respond with unorganized locomotion, subjecting the animal to likely death [25].

Experiment 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 $\mu\text{g}/\text{mL}$ 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 ($21 \pm 2^\circ\text{C}$) and photoperiod (12 h alternating light/dark cycle) were fixed. Following experiment 2, 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 [26]. 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.

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 cryoanaesthetized and the remaining tissues (gills, hepatopancreas and muscle) were removed. Approximately 80 mg of tissue, the CFH and the pelleted haemocytes were homogenized (Omni-Ruptor 400 (Omni International 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 Zentrifugen, Germany). All tissue samples were stored at -80°C until enzymatic analysis.

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 [27]. It was then aspirated with a concentric nebuliser into a quartz spray chamber cooled by a Peltier cooler into the Inductively Coupled Plasma.

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 [28], 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.

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.

Results and Discussion

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 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 calculated size distribution histogram of the dry AgNP revealed that the size of AgNPs ranged from 10 to 55 nm ($n=276$) (Figure 1D), while the corresponding histogram for suspended AgNPs revealed AgNP size from 20 to 1000 nm (Figure 1E). The PXRD pattern of the AgNPs showed diffraction peaks at 2θ from 3° to 90° (Figure 1D). EDX confirmed the presence of Ag. The specific surface area of the AgNPs was determined to be $7.5329 \text{ m}^2/\text{g} \pm 0.0028$ (Figure 1E) [22].

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

Figure 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 $36397.93 \mu\text{g}/\text{kg}$). 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., [29] studying the toxicity of AgNPs to a freshwater algae *Chlamydomonas*

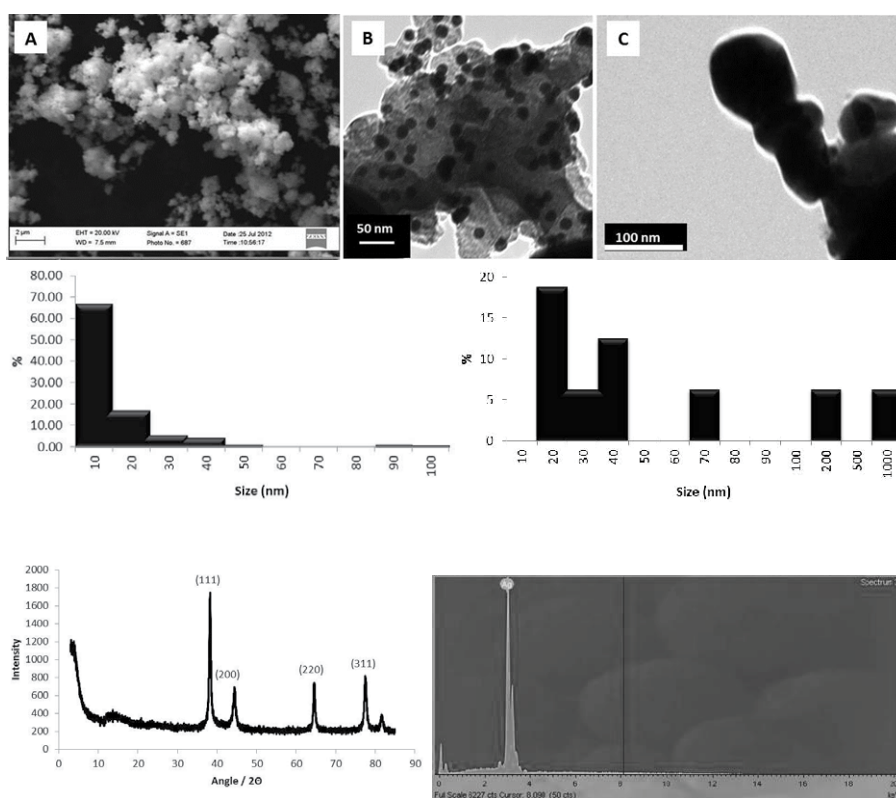
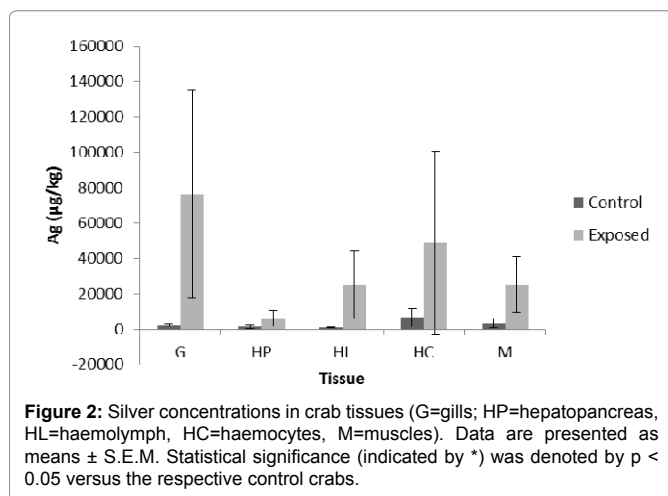


Figure 1: SEM micrograph of dry AgNP (A). TEM micrograph of dry AgNPs (B) and AgNP in suspension (C). Histogram of the size distribution for dry (D) and suspended (E) AgNPs. The XRD (F) and EDX spectrum showing elemental Ag composition (G) [22].



reinhardtii, and reporting that AgNPs served as an additional source of Ag^+ . The liver is generally regarded as the main target organ for AgNPs [30,31]. 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. [32] 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 [15,33]. 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 [15]. Surprisingly, the lowest Ag concentration was measured in the hepatopancreas which is in contrast to other studies [34]. This observation further supports that the large AgNP agglomerates in suspension may have prevented their hepatic absorption, which was evidenced by Kulthong et al. [34].

Acute toxicity tests

Acute toxicity values of experiments 1 and 2 are compared in Table 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 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).

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 [35]. 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. In crabs, the defence system is equipped with enzymes to counteract free radicals produced during exposure to stressors [2,5]. To determine the enzyme activities occurring under a controlled AgNP and temperature regime, six crabs were exposed to 0 µg/mL (control group) and 787.77 µg/mL (exposed group) of AgNP at 25.3 °C for seven days. 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 4). The decrease in protein content observed in the exposed tissues could be attributed to mitochondrial damage [36]. AgNPs are known to induce oxidative stress by triggering ROS through the mitochondrial electron transport chain [37]. Also, protein synthesis is generally down-regulated during oxidative stress [38]. This finding was supported by several authors. For example, Vogel et al. [38] reported inhibition of protein synthesis in *Saccharomyces cerevisiae* following diamide-induced oxidative stress; while Lopez-Alonso et al. [39] 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 [40,41].

Cytochrome P450 (CYP450) belongs to the superfamily of hemoproteins and is one of the most important Phase I detoxification enzymes, which is largely responsible for the metabolism (degrading and elimination) of xenobiotics [34]. Cytochrome P450 instigate the detoxification process, comprise largely of heme proteins which are predominantly located in the endoplasmic reticulum of the liver [42,43]. 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 [44]. In crabs, the hepatopancreas is the major site of uptake and CYP enzyme-dependent biotransformation of lipophilic xenobiotics [43]. Activity of CYP450 was significantly ($p < 0.05$) enhanced when compared to the control group (Figure 5). Conversely, previous studies have reported inhibition of hepatic CYP450 activity by AgNP in rats [34]. 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 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 [45].

Superoxide dismutase (SOD) is the first Phase II enzyme to deal with oxyradicals [46], and is responsible for catalyzing the dismutation of highly superoxide radical O_2^- to O_2 and H_2O_2 [3,46]. It is very sensitive to toxins and can therefore be used as an oxidative stressed signal for the early warning of environmental pollution [46]. 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 [46]. 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

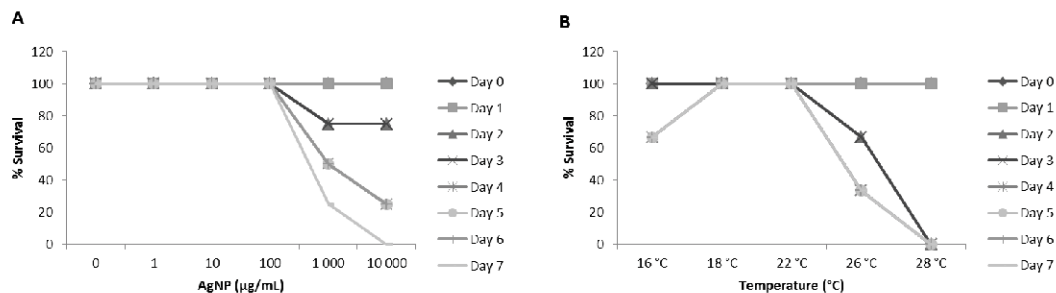


Figure 3: % Survival in the AgNP-dependant (A) and temperature-dependant (B) experiments.

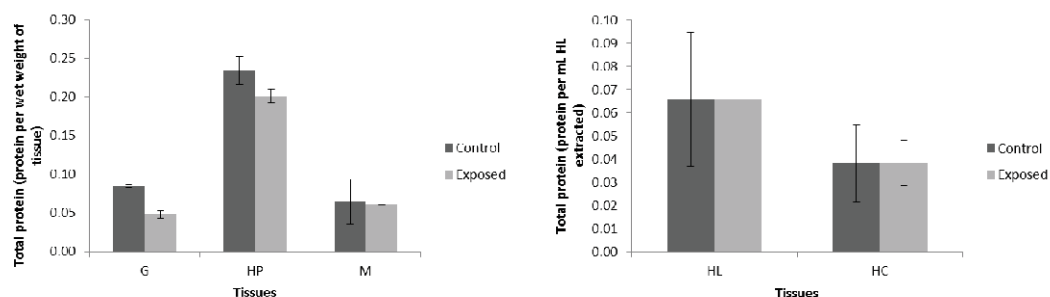


Figure 4: Effect of 7-day exposures to AgNPs (782.77 µg/mL) at 25.37°C on total protein concentrations in tissues (G=gills; HP=hepatopancreas; M=muscles; HL=haemolymph; HC=haemocytes) in *P. perlatus*. Data are presented as means ± S.E.M. Statistical significance (indicated by *) was denoted by $p < 0.05$ versus the respective control crabs.

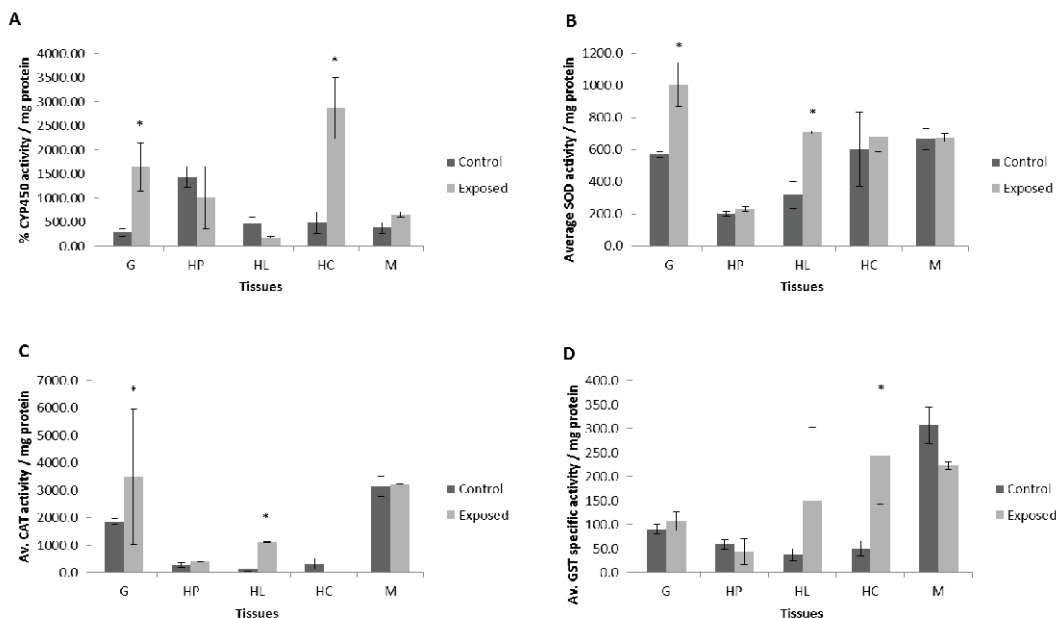


Figure 5: Effect of 7-day exposures to AgNPs (782.77 µg/mL) at 25.37 °C on enzymatic activity of CYP450 (A), SOD (B), CAT (C) and GST (D) in tissues (G=gills; HP=hepatopancreas, M=muscles; HL=haemolymph; HC=haemocytes) of *P. perlatus* following a seven-day exposure period. Data are presented as means ± S.E.M. Statistical significance (indicated by *) was denoted by $p < 0.05$ versus the respective control crabs.

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. [47] 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. [48] 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 that play a fundamental role in protection against endogenous and exogenous toxic chemicals [23] 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 [49]. 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).

Data are presented as means ± S.E.M. Statistical significance (indicated by *) was denoted by $p < 0.05$ versus the respective control crabs. 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 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 [49], and inhibition in the hepatopancreas of *C. maenas* exposed to 5% of wastewater effluent [50]. 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.

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. [51] observed that temperature influenced oxidative stress biomarkers in the muscle and digestive gland of *Callinectes maenas* and Madeira et al. [52] in the haemolymph of *Pachygrapsus marmoratus*. Similarly, Freire et al. [53] 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.

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 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 were closely associated with exposed muscles. This PCA suggests a relationship between the antioxidant efficiency (particularly SOD and GST) of *P. perlatius* 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.

A second PCA was constructed which incorporates tissues per treatment (control tissues (Figure 7A) and exposed tissues (Figure 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.

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

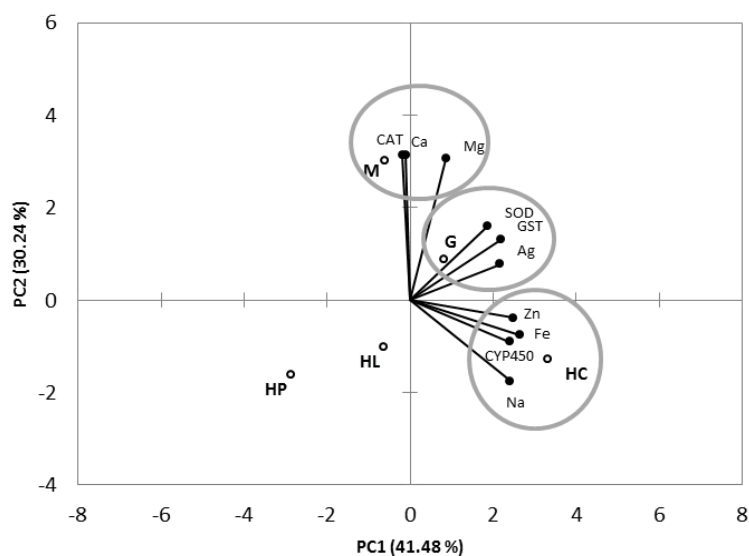


Figure 6: Principal component analysis (PCA) applied on data of control and exposed crab groups taking into account five variables (SOD, CAT, GST, CYP450, and trace metal concentrations).

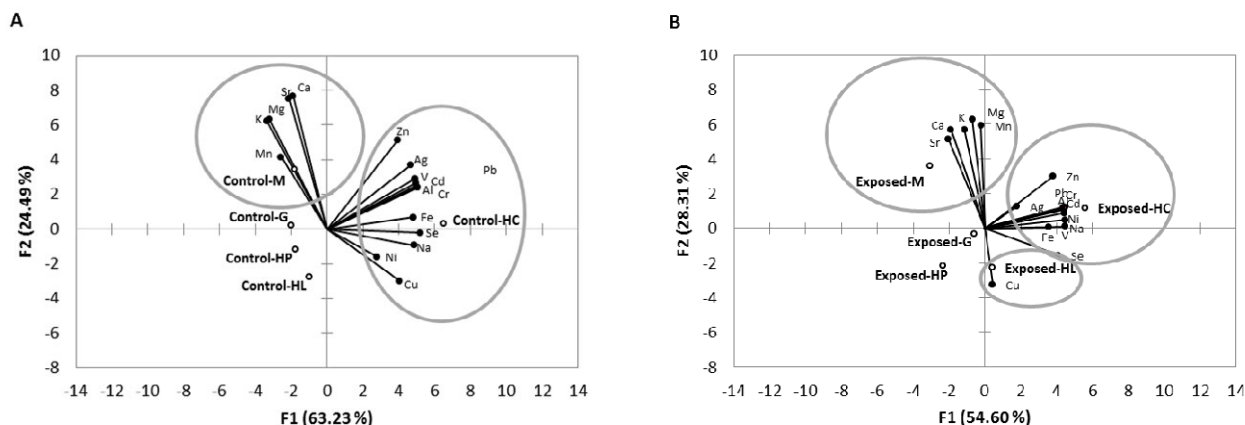


Figure 7: Principal component analysis (PCA) of tissues of control (A) and exposed group (B) with metal content.

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.

Conclusions

This study shows the importance of temperature as an influential variable in the toxicity of *P. perlatius* 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. perlatius*. 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. perlatius*. 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.

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