Chapter 2

Nanotoxicity in Aquatic Invertebrates

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Additional information is available at the end of the chapter

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Abstract

Due to their unique properties, nanomaterials (NMs) are being incorporated in several applications including consumer products, electronics, pesticides and the pharmaceutical industry. As such, the rapid development and large-scale production of NMs has inspired concerns regarding their environmental health risks. In order to address these concerns, there has been a rapid development in the methods of toxicity testing of NMs, specifically in aquatic organisms. Understanding the unique properties of nanoscale materials has proven to be a particular important aspect of their toxicity. Properties such as surface area, surface coating, surface charge, particle reactivity, aggregation and dissolution may affect cellular uptake, *in vivo* reactivity and distribution across tissues. The behaviour of NPs is influenced by both the inherent properties of the NP as well as environmental properties (such as temperature, pH, ionic strength, salinity, organic matter). As such, this chapter describes methodologies of NM characterization in exposure media and NM *in vivo* toxicity experimental procedures under variable environmental conditions (with special emphasis on temperature).

Keywords: Toxicity, Nanotoxicity, Nanomaterials, Nanoparticles, Silver nanoparticles, Temperature, Aquatic invertebrates

1. Introduction

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 [1] through intentional and unintentional releases. Like many other toxins, the aquatic environment is particularly vulnerable as it acts as a sink for nanoparticles (NPs) [2]. 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.

Silver nanoparticles (AgNPs) are the most widely used metal NPs, present in several consumer products largely due to their antibacterial properties. It is estimated that the annual production exceeds 1000 tons/year [3]. The increased use 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 increased release into the environment, thereby posing an environmental risk and human health concern.

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 [4]. In addition, it is predicted that concentrations of AgNPs in natural waters range from 0.03 to 500 ng/L [5]. A fundamental question is whether AgNPs remains in the particle phase in the environment following dissolution or whether it poses an additional risk.

Silver NPs are known to induce the production of reactive oxygen species (ROS) [6–8]. 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 [9, 10]. 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 that reduce the damaging effects of hydrogen peroxide (H_2O_2) to prevent the production of hydroxyl radicals ($HO\cdot$), the most damaging oxygen species [11]. These oxidative stress biomarkers have been widely used as "early warning" signs of environmental stress.

Assessing the ecotoxicity of NMs is a challenging task. Inexpensive, rapid and reproducible methods are preferred, and a coordinated standardization could help in avoiding the waste of resources. Standardized tests established by the Organization for Economic Co-operation and Development (OECD) and the U.S. Environmental Protection Agency (U.S. EPA) have protocols for testing aquatic, terrestrial and microbial organisms. However, these tests were established considering conventional chemicals and not NMs. The general consensus in the scientific community is that the basis of these standardized tests (i.e. test organism, endpoints) may generally work for NMs but would require some modifications. Nevertheless, NMs remain very poorly tested in contrast with their larger counterparts; the main difficulties in assessing toxicity are due to their colloidal nature and unique properties. The behaviour of NPs is collectively influenced by inherent (NP size, shape, surface area, surface chard, crystal structure, coating, solubility/dissolution) and environmental factors (temperature, pH, ionic strength, salinity, organic matter).

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 (such as

extreme temperatures), thus mimicking the conditions experienced naturally in the environment during potentially extreme conditions. The research areas which this research aims to address include NP fate and transport, bioavailability and ecotoxicology (or nanoecotoxicology).

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 a result of 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 ($\cdot O_2^-$), hydroxyl radicals ($\cdot OH$) and the nonradical 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 $\cdot O_2^-$, $\cdot OH$ and H_2O_2 that form during the metabolism of oxygen [12].

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) [13]. Figure 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.

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 [14] (Equation 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 2), which can in turn be reduced to water (H_2O) and O_2 by CAT (Equation 3) or glutathione peroxidase (GPx) (Equation 4) [15].

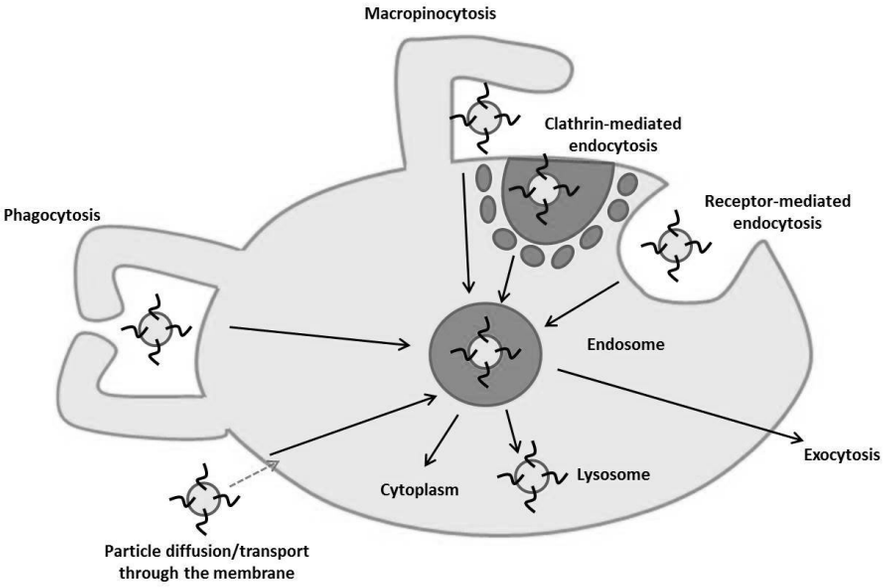

Figure 1. Schematic diagram of oxidative stress (adapted from www.sigmaaldrich.com).

$$H + O_2 + 2e^- + 2H^+ \rightarrow OH + H_2O \tag{1}$$

$$2O_2^- + 2H^+ \rightarrow 2H_2O + O_3 \tag{2}$$

$$2H_2O_2 \rightarrow 2H_2O + O_2 \tag{3}$$

$$2GSH + H_2O_2 \rightarrow GSSG + 2H_2O \tag{4}$$

Nonenzymatic 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 a substrate and is determined by the ratio of GSSG:GSH. This ratio is an indication of the redox state of cells [16] and is important to ROS detoxification.

1.3. Uptake and accumulation of silver nanoparticles

Once introduced into aquatic ecosystems, the fate and transport of AgNPs and its uptake by aquatic biota depends 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 or adsorb to suspended particles [17].

In aquatic organisms, the major routes of entry are via ingestion or direct passage across the gills and other external surface epithelia. Recent studies with *Daphnia magna* have indicated that AgNPs may be internalised by these routes [18]. At the cellular level, internalisation of NP occurs via endocytosis. Mechanisms of cellular uptake of NPs are described in Figure 2. Three main mechanisms are responsible for NP uptake: phagocytosis, macropinocytosis and receptor-mediated endocytosis [19]. During phagocytosis (a specific form of endocytosis), particles are taken up the invagination of the plasma membrane. Jayaseelan et al. [20] 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 [21].

The accumulation of NPs by aquatic organisms is dependent on both the uptake and the elimination (detoxification) of the NP out of the organism [22]. Processes which regulate the bioaccumulation (and bioavailability) of AgNPs include: the concentration of the AgNP, the physicochemical 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 [23].

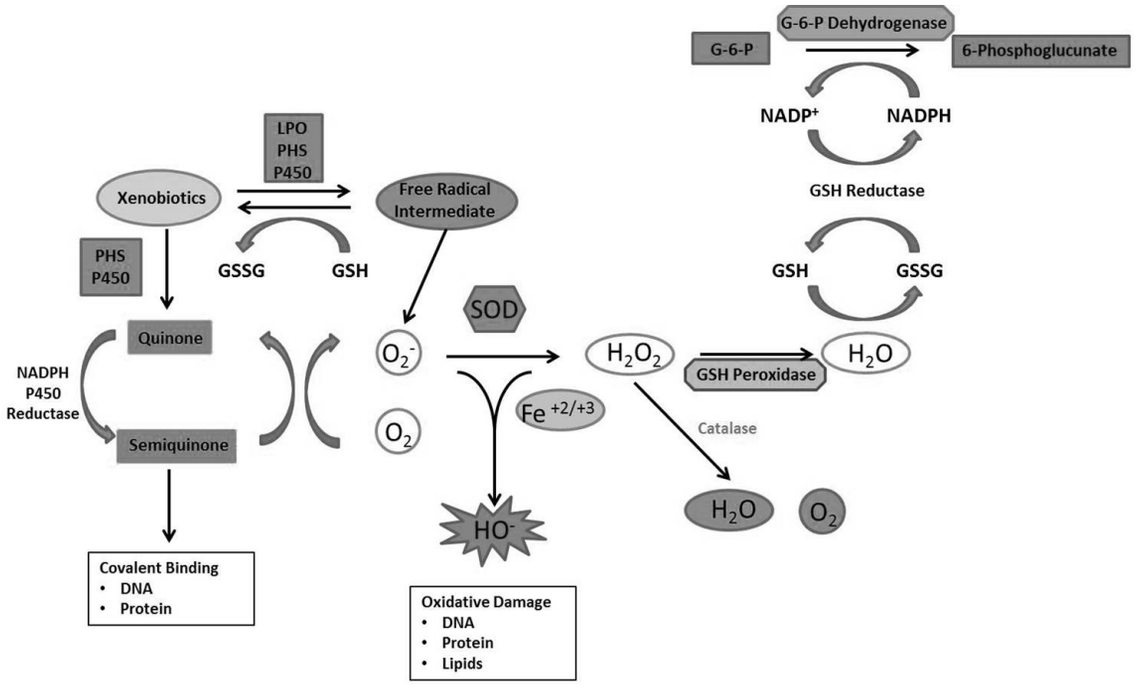

Figure 2. Mechanisms of cellular uptake of NPs.

2. Crustaceans and exposure routes in ecotoxicology

Potamonautes is a genus of freshwater crabs in the family Potamonautidae. They represent a fairly common species, having 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 *Potamonautes 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 [24]. 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 [24].

Crustaceans show a high sensitivity to environmental stressors [25] and are therefore found to be useful bioindicators for monitoring the pollution state in aquatic environments [26]. 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. [27] 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 [28]. Nanoparticles may also diffuse into the circulating haemolymph and settle in a target organ.

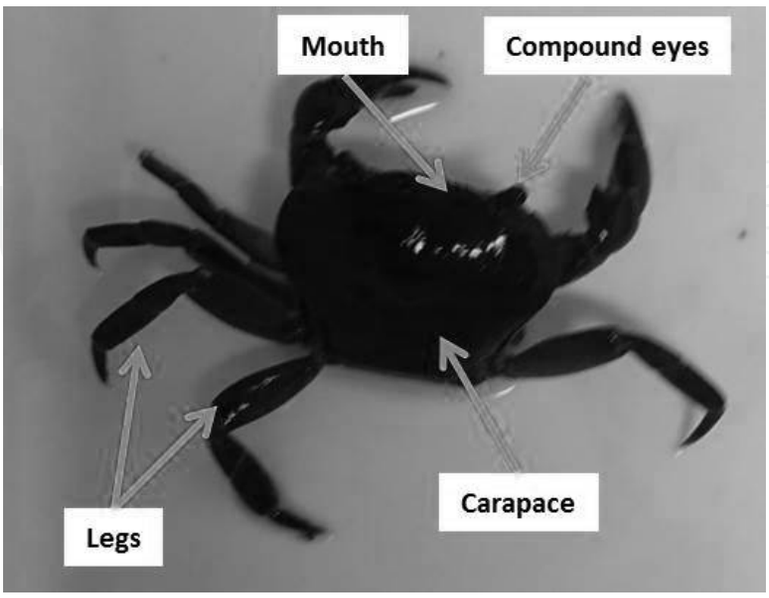

Figure 3. Dorsal view of *P. perlatus*.

3. Methodology

3.1. Characterization of nanomaterials in solution prior to *in vitro* exposure

3.1.1. Stock suspension and media preparation

AgNPs (Cat. No. 7440-22-4) were purchased from Sigma-Aldrich (Sigma Aldrich, South Africa). The product information indicated a particle size of <100 nm diameter, purity of 99.5%, a specific surface area of 5 m^2/g and density of 10.5 g/cm^3. The stock AgNP suspension (1 mg/mL) was prepared by dispersing AgNPs (1 mg dry AgNPs powder) in deionized water (1 mL) and sonicating for 5 min in an ultrasonic bath in order to disrupt any possible aggregates. The 1 mg/mL AgNP stock suspension was added to each of the plastic aquaria to obtain the appropriate final concentration.

3.1.2. SEM of nanoparticles

Scanning electron microscopy (SEM) with energy-dispersive X-ray (EDX) spectrometry capabilities was used to characterize particle size and confirm the presence of Ag. Sample preparation for SEM analysis included sonicating a dilute suspension of the AgNP in ethanol for 1 hour and dropping a portion onto a carbon surface of a SEM stub with a pipette. The droplet was allowed to dry and the SEM analysis was performed on an EVO® MA15. 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.

3.1.3. TEM of nanoparticles

Transmission electron microscopy (TEM) characterization was performed to obtain the NP size and morphology on 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 × 1032, and two seconds exposure time. A suspension of AgNP was dissolved in ethanol, and subsequently deposited onto copper grids and air-dried. ImageJ software was used to generate a particle size distribution based on the TEM images.

3.1.4. Powder X-Ray Diffraction (PXRD)

A Panalytical X'pert Pro diffractometer was used to collect a PXRD pattern for the AgNP. The PXRD pattern was collected between the angles of 2θ from 3° to 90°.

3.1.5. BET-specific surface area measurements

Brunauer–Emmet–Teller (BET) surface areas were determined using ASAP 2010 (Accelerated Surface Area and Porosimetry System; Micromeritics Instrument Corporation, GA, USA). Prior to surface area analysis, samples were heated to 100°C and degassed overnight.

3.2. Specimen collection and acclimatization

Adult *P. perlatus* samples were collected from an uncontaminated area of the Eerste River (Stellenbosch, South Africa) during spring 2014. Individuals were collected using handmade traps comprising of a fishing rod fitted with mesh net containing bait. Capturing the individuals during spring ensured that they had not been exposed to heat wave conditions prior to their collection, thereby assuring that their recent thermal history did not include exposures to high temperatures. After capture, *P. perlatus* (with a mean length of 50 ± 5 mm and weight of 75 ± 10 g) were transported to the laboratory where they were kept unfed in aquaria (21 ± 2°C), and at a natural photoperiod for three days to acclimatise before exposure experiments began.

3.3. Experimental setup

In this study, laboratory experiments were conducted to investigate the physiochemical properties and temperature-dependent solubility of AgNPs that potentially influence their toxicity in aqueous environments and to aquatic organisms.

Details of the experimental setup are summarised in Table 1. The experimental setup comprised three experiments: (A) AgNP characterization in the dry state and in solution, (B) acute toxicity study, and (C) *in vivo* study. Details of each experiment are given in the sections below. Upon arrival in the laboratory, the crabs were kept in plastic aquaria composed of 2-L tanks and allowed to acclimatize for three days. There were six individuals per tank (10 systems with 6 individuals per tank = total of 60 crabs). 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.

	Nanoparticle characterization			**In vivo study - Experiment A**			**Acute toxicity test - Experiment B**		
	A.1	**A.2**	**A.3**	**B.1**	**B.2**	**B.3**	**C.1: AgNP - dependant**	**C.2: Temperature - dependant**	**C.3**
AgNP conc. (μg/gL)	1	1	1	0, 10, 100	0, 10, 100	0, 10, 100	0, 10, 100, 1 000, 10 000	782.77	782.77
Temp. (°C)	21	18	28	18, 18, 18	21, 21, 21	28, 28, 28	21 °C (RT)	16, 18, 22, 26, 28	25.37
Number of individuals (n)	6	6	6	6, 6, 6	6, 6, 6	6, 6, 6	6, 6, 6, 6, 6	6, 6, 6, 6, 6	6

Table 1. Experimental conditions for evaluating the effect of AgNPs (RT = room temperature)

3.3.1. Silver nanoparticle characterization (Experiment A)

Experiment A investigated the behaviour of AgNP (1 μg/mL) in three laboratory microcosms: (1) a control regime kept at room temperature (i.e. 21°C; A,1), (2) a low-temperature regime (i.e. 18°C; A.2) and (3) a high-temperature regime (i.e. 28°C; A.3).

3.3.2. Acute toxicity study (Experiment B)

The acute exposure study consisted of three experimental stages. Stage 1 involved a concentration-dependent regime comprised of crab specimens exposed to five different AgNP concentrations, including a control regime (i.e. 0, 1, 10, 100, 1000 and 10,000 μg/mL AgNPs), in order to cover a wide range of contamination levels that may be reported for polluted environments. A total of six crabs per treatment were exposed for seven days to room temperature. At the start of each day, the numbers of live and dead crabs were determined via visual inspection and the benchmark dose (BMD) AgNP concentration was derived through LogProbit analysis (U.S. EPA BMDS Program, version 2.5).

During stage 2, a total of six crabs per treatment were exposed for seven days to the final BMD AgNP (7 days) concentration (obtained in stage 1) at five pre-determined temperature regimes (i.e. 16°C, 18°C, 22°C, 26°C and 28°C). The experimental temperatures were chosen, taking into account the predicted increases in mean atmospheric and aquatic water temperatures (Bates et al.), since climate change projections indicate an increase in the frequency, intensity and duration of thermal extremes [29]. At the start of each day, the numbers of live and dead crabs were determined via visual inspection and the Critical thermal maximum (CTMax) were derived through LogProbit analysis (U.S. EPA BMDS Program, version 2.5) and was used to estimate the temperature to be used in stage 3. The CTMax is defined as "arithmetic mean of the collective thermal points at which the endpoint is reached" [30], or that temperature for a given species above which most individuals respond with unorganized locomotion, subjecting the animal to likely death [31].

Stage 3 involved the assessment of the role of oxidative stress in AgNP-induced toxicity, a total of six crabs per treatment were exposed to the corresponding BMD (782.77 μg/mL) and CTMax (25.37°C) values obtained during the preceding experimental stages. The experiment was conducted in 2-L plastic tanks (Group B1: six crabs at 0, 1, 10, 100, 1000 and 10,000 μg/mL at 21°C (room temperature) and Group B.2: six crabs at 782.77 μg/mL at 25.37°C) with a 12-h alternating light/dark cycle following modified methods described by Cheng [32]. Tissue samples for biochemical analysis were collected from each crab specimen at the end of Stage 3.

For all experimental stages, crabs were exposed for seven days and were unfed during the acclimatization and exposure periods. Every 24 h during stages 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.

3.3.3. In vivo study (Experiment C)

The experimental temperatures for experiment A were 18°C, 21°C (room temperature) and 28°C. These temperatures were chosen to reflect the predicted increases in mean atmospheric and aquatic water temperatures [33]. Crabs were divided into three temperature-dependent regimes (i.e. 18°C, 21°C (control temperature) and 28°C) each containing three AgNP-dependent regimes (i.e. Group C.1 at 18°C: 0 μg/mL, 10 μg/mL and 100 μg/mL AgNPs; Group C.2 at 21°C: 0 μg/mL, 10 μg/mL and 100 μg/mL AgNPs and Group C.3 at 28°C: 0 μg/mL, 10 μg/mL

and 100 μg/mL AgNPs). There were six individuals per regime (nine regimes with six crabs each = 54 crabs in total).

3.4. Tissue preparation

At the end of the exposure period, crabs were cryoanaethesized and tissues (gills and hepatopancreas) were excised from each crab sample. Tissues (gills and hepatopancreas) were homogenized (1:10 w/v) using an Omni-Ruptor 400 (Omni International Inc., GA, USA) homogenizer in a 1:20 protease inhibitor cocktail (Sigma Aldrich, MO, USA) prepared with a phosphate buffer (PBS). Homogenates were centrifuged (Universal 32R, Hettich Zentrigugen, Germany) at 4°C for 2 min at 13,000 rpm. Supernatants were removed and used for determination of enzyme activity.

3.5. Biochemical analysis

Biochemical analyses were done using commercially available kits purchased from a local supplier and were performed in triplicate following the manufacturer's protocols.

The enzymatic assay of SOD activity (Sigma-Aldrich, MO, USA), using nitroblue tetrazolium (NBT) and xanthine oxidase (XO), was carried out by reading the absorbance at 450 nm.

The enzymatic assay of CAT (Arbor Assays, MI, USA) was measured by following the increase in catalase in the sample with decreasing H_2O_2 concentration and by measuring the absorbance of 200 mM H_2O_2 at 520 nm. The reaction mixture consists of colorimetric detection reagent, horseradish peroxidase concentrate and H_2O_2.

The GST assay (Sigma-Aldrich, MO, USA) was measured at 340 nm following the conjugation of GSH with CDNB (1-chloro-2,4-dinitrobenzene). The reaction mixture consisted of Dulbecco's phosphate-buffered saline (DPBS), 200 mM L-glutathione reduced and 100 mM CDNB. The GST activity was measured by measuring the change in absorbance every minute for six minutes.

For normalization purposes, the results were divided by the total amount of protein (expressed in mg/g wet mass of tissue), calculated through the Bradford method (Bradford, 1976), to obtain enzymes activity in SOD units per mg protein (for SOD), CAT units per mg protein (for CAT) and GST-specific activity per mg protein (for GST).

3.6. Data integration

All data values are given as the mean ± SEM (standard error of means). Univariate one-way ANOVA was used to compare means between treatments followed by Dunnett's test to discriminate differences from the control group using XLSTat (Microsoft Excel and XLStat2015®). A minimum significance level of $p < 0.05$ was accepted. Benchmark dose (BMD) and the critical thermal maximum (CTMax) were calculated with LogProbit analysis (U.S. EPA BMDS Program, version 2.5).

4. Results and discussion

Although beneficial, advances in nanotechnology are also associated with expectations of growing potential toxicity and ecotoxicity largely due to their unusual properties. These properties that render NPs suitable in numerous applications are also ultimately the same properties responsible for unpredictable effects in the environment and produce adverse cellular effects and damage to living organisms. For example, small particle size and high specific surface area allows for higher reactivity and use in several applications, but also allows for their passage across biological barriers thereby entering cells [2,34]. It is therefore vital that NPs are correctly and accurately characterized in environmental media in order to ensure the reliability and reproducibility of toxicity tests.

Silver NPs are currently very widely used in industry largely due to their antibacterial properties, with applications in several consumer products. Once released into the environment, the state and behaviour of NP in the environment is dependent on environmental conditions (including temperature, pH, and ionic strength) in which the NP occurs. 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's behaviour and potential effects on the environment.

Commercial AgNPs were analysed in the dry state (TEM, SEM, BET, PXRD) to characterize surface composition and coatings, surface area, agglomeration state and size of the nanoparticles, in solution (TEM) to characterize particle size and aggregation potential in solution. The primary and aggregate size of the AgNPs in the dry state was characterized using TEM analysis (Figure 4a). A particle size distribution was generated from the TEM images by measuring the diameter of more than 500 nanoparticles (Figure 4b). The TEM image in Figure 4a verified the spherical nature of the AgNPs, while the size distribution histogram (Figure 4b) showed a majority of smaller particles measuring 10 nm and a small quantity of larger particles 30–50 nm. The image also shows that the smaller particles are primarily isolated but form agglomerates with diameters of 20 nm.

In the aquatic environment, AgNPs interact with natural water components, which can lead to chemical or biological alterations (such as size distribution, aggregation or disaggregation. This, in turn, will influence the potential transport of NPs in the water column and, consequently, their fate and toxicity. TEM images for each experimental regime (control, low and high temperature) are shown in Figure 5. It is known that larger aggregates imply reduced bioavailability and toxicity [35]. With this said, the enlarged aggregates evidenced in the TEM images at 18°C suggest that NP toxicity could be somewhat reduced. However, the TEM image at 28°C suggests a greater potential for toxicity since aggregation of the AgNPs are minimal. The propensity of NPs to aggregate in the aquatic environment can lead to sedimentation, thereby making NPs more available to interact with sediment-dwelling and benthic organisms than with pelagic (water dwelling) species.

The bioavailability of AgNPs in water and sediment in the different treatment regimes was assessed at the end of the exposure period (i.e. seven days). The available concentration of Ag

was found to decrease with increasing temperature (4.7% at 18°C versus 1.1% at 28°C). The loss of Ag suggests aggregation, sedimentation and dissolution of the particles in the exposure media that resulted in lower AgNP concentrations in the water column and reduced bioavailability of AgNP to *P. perlatus*. The bioavailability of Ag is a critical element influencing its toxicity to aquatic organisms and is dependent on several factors such as aggregation, sedimentation and internalization within the test organism. With this said, it can be assumed that, from the TEM image at 18°C, AgNPs formed large aggregates, whereas at 28°C, aggregation was minimal. The lack of obvious aggregation on the water surface in the high-temperature regime likely reflects the internalization of AgNP at elevated temperatures and possible sedimentation.

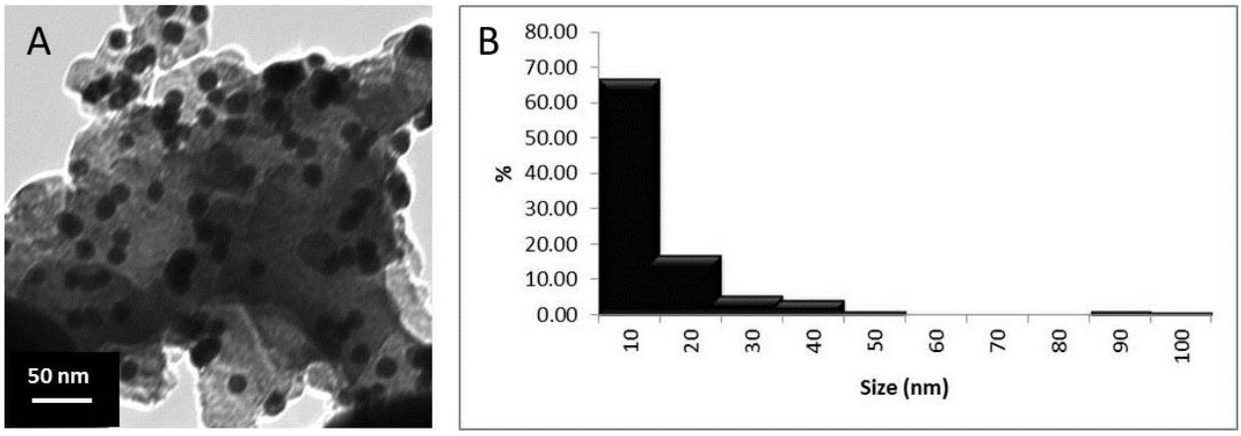

Figure 4. TEM image of dry AgNP (left) with the corresponding particle size distribution (right).

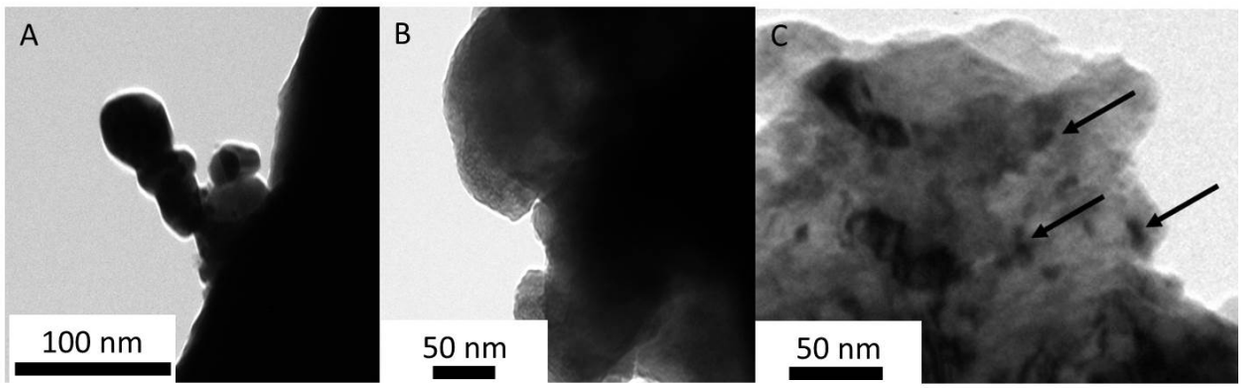

Figure 5. TEM AgNPs suspensions at (a) room temperature, (b) low temperature and (c) high temperature.

Silver NPs are capable of causing acute toxicity in *P. perlatus*; however, the toxicity differed significantly according to AgNP concentration and exposure temperature. The results of the seven-day acute toxicity tests performed with AgNPs and temperature, expressed as BMD values, are summarized in Table 2. In the acute toxicity test, survival in the control group was well above 90%, and thus met biological validity criterion. In Experiment B.1 (AgNP-depend-

ent regime), no mortalities were observed between 0 and 100 μg/mL AgNP. Mortalities were only observed in the 1000 μg/mL and 10,000 μg/mL treatment regimes, where mortality was observed from day 2 and increased until 75% and 100% mortality was confirmed at the end of the exposure period for the 1000 μg/mL and 10,000 μg/mL groups, respectively. In Experiment B.2 (temperature-dependent regime), 50% mortality was observed after 2 days at 28°C (Figure 4). No mortalities were observed in the 18°C and 22°C temperature groups. No significant differences in mortality between the two experimental stages were found. At the end of the exposure period, 75% had died in the temperature-dependent experiments. The mortality data during the experimental periods indicate that the AgNPs and temperature combinations were toxic to the survival of *P. perlatus*.

		SOD		CAT		GST	
Temperature (°C)	**AgNP (μg/mL)**	**G**	**HP**	**G**	**HP**	**G**	**HP**
Acute toxicity study (Experiment B)							
25.37	0	570.6 ± 18.0	203.5± 14.1	1842.8 ± 41.4	278.3 ± 41.4	89.6 ± 10.3	58.3 ± 10.3
25.37	787.77	1005.5 ± 136.3	233.6± 15.1	3490.9 ± 2468.5	388.7 ± 0.4	106.3 ± 19.9	42.8 ± 27.0
In vivo study (Experiment C)							
18	0	6698.4 ± 1221.7	3797.3 ± 249.5	896.5 ± 136.7	3269.8 ± 118.4	10426.2 ± 2858.0	11679.1 ± 1288.0
	10	4119.1 ± 535.0	3286.9 ± 693.9	977.7 ± 56.9	2989.2 ± 49.7	9066.5 ± 1975.7	21783.6 ± 4279.9
	100	2924.6 ± 591.7	4788.3 ± 740.2	684.3 ± 40.2	2327.9 ± 351.3	3826.1 ± 1368.2	12187.4 ± 1807.2
21	0	3135.4 ± 655.8	4357.4 ± 646.1	26061.6 ± 5963.0	3825.6 ± 1064.7	1683.9 ± 278.0	9802.4 ± 3540.7
	10	2728.4 ± 261.3	6584.0 ± 1284.9	14350.5 ± 3145.1	4016.8 ± 2150.3	1569.6 ± 267.8	17188.5 ± 5827.2
	100	3071.7 ± 250.4	6304.3 ± 756.0	10732.3 ± 835.3	3496.9 ± 2022.3	1669.7 ± 386.1	13342.8 ± 4038.7
28	0	3445.0 ± 836.3	3968.8 ± 673.2	20028.0 ± 6017.6	5184.7 ± 1301.1	11890.4 ± 2221.6	15018.7 ± 2708.8
	10	6458.3 ± 519.0	5297.3 ± 685.6	13526.1 ± 3048.6	13884.9 ± 2291.4	13865.6 ± 1127.6	40822.1 ± 6828.5
	100	9570.0 ± 1989.6	8339.1 ± 796.1	17765.2 ± 5873.4	22142.6 ± 2078.8	11197.1 ± 1483.0	48342.8 ± 5463.5

Table 2. Activities of antioxidant enzymes SOD (SOD units per mg protein), CAT (CAT units per mg protein)and GST (GST specific activity per mg protein)in tissues (G = gills; HP = hepatopancreas) of *P. perlatus* exposed to AgNP (10 μg/mL and 10 μg/mL) Data are presented as mean ± SEM (n = 6). Statistical significance (indicated by *) was denoted by $p < 0.05$ versus the respective control crabs.

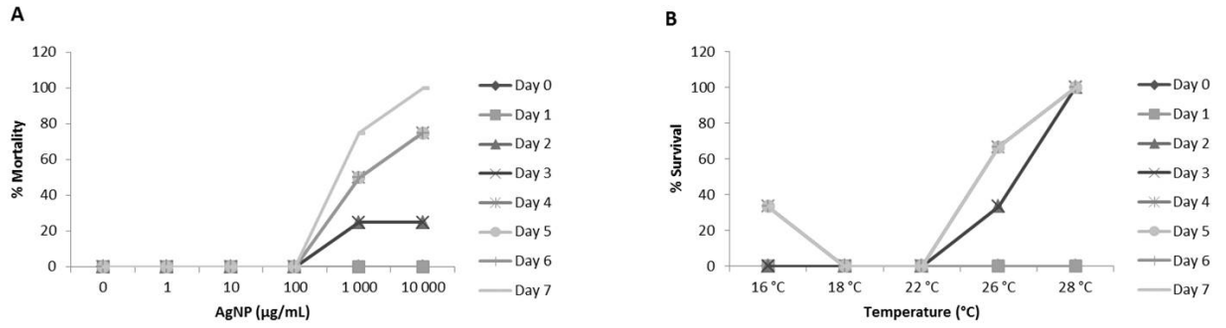

Figure 6. Mortality of *P. perlatus* exposed to various concentrations of AgNP (A) and various temperatures (B).

Based on these results, AgNPs appear to exert increased toxicity with increasing AgNP concentration and temperature. Previous studies have reported the toxic impacts of different NPs on crustaceans. In a recent study on *Chlamydomonas reinhardtii*, Navarro et al. reported that AgNPs (10–200 nm) induced 2 h EC_{50} of 3300 ± 572 nM [35]. Others have reported 48 h EC_{50} of 2.5 µg/mL and 4.9 µg/mL AgNPs in *Oncorhynchus mykiss* [36]. 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 and salinity) [37]. As such, these results suggest that AgNPs can generate different degrees of toxicity under different exposure conditions (such as NP size, concentration and temperature).

Oxidative stress is an important component of the stress responses in aquatic organisms, which are often exposed to a wide variety of environmental stressors (such as temperature variations and anthropogenic contamination). Biomarkers of oxidative stress are among the most commonly used biomarkers of cellular stress. These include superoxide dismutase (SOD), catalase (CAT) and glutathione-S-transferase (GST), which are used mainly as indicators of cellular stress resultant from both environmental contamination and environmental variables.

The crustacean antioxidant system is based on the collective actions of SOD, CAT and GST to counteract the overproduction of ROS. An important objective of this research was to assess the differential responses among different tissues. Because different tissues are located in different locations and perform different functions, each tissue may be more or less exposed to contaminants and will therefore have different cellular responses. To date, the gills and hepatopancreas have been the most used tissues for the determination of oxidative stress in crustaceans. The hepatopancreas is the major site for toxicant uptake and oxyradical-generating biotransformation enzymes [38]. The gills have a large exchange area and are in direct contact with the external environment [38]. For this reason, these tissues were chosen to assess the antioxidant defence mechanisms following AgNP and temperature exposures. In this study, in response to AgNP concentrations (i.e. 10 µg/mL and 100 µg/mL), antioxidant enzymes (SOD, CAT and GST) activity were significantly higher in the hepatopancreas when compared to the gills, suggesting that the hepatopancreas might be a more sensitive organ to the effects of AgNPs. Similarly, Zhu et al. [39] reported significant stimulation of SOD activity in liver (when compared to the gill and brain) following exposure of juvenile carp to C60. This indicates that AgNPs have tissue-specific effects on redox metabolism in *P. perlatus*.

Enzyme activity in the higher AgNP treatment (i.e. 100 μg/mL) was generally lower than the lower AgNP treatment (i.e. 10 μg/mL). SOD is the first defence against oxidative toxicity at a cellular level and is responsible for catalysing the dismutation of the superoxide radical O_2^- to O_2 and H_2O_2. The inductions of SOD and CAT in the higher-temperature and AgNP regimes suggest the production of superoxide anions by AgNP. The depletion of the antioxidant enzyme capacity (SOD, CAT and GST) suggests that the antioxidant defence system is overwhelmed by ROS [40] and further suggests that the antioxidant defence systems of these tissues were being stressed.

Experiment	Exposure (d)	BMD (μg/L) AgNP	95% confidence limit	Slope	Correlation coefficient
Experiment B.1					
	24	-	-	-	-
	48	3689.16	18 827.00	$p < 0.05$	0.29
	72	3689.16	18 827.00	$p < 0.05$	0.29
	96	4209.83	1 947.73	$p < 0.05$	0.55
	120	4209.83	1 947.73	$p < 0.05$	0.55
	144	4209.83	1 947.73	$p < 0.05$	0.55
	168	4083.36	782.77	$p < 0.05$	2.75
Experiment	**Exposure (h)**	**CTMax (°C)**	**95% confidence limit**	**Slope**	**Correlation coefficient**
Experiment B.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

Table 3. BMD values of the performed acute toxicity tests with AgNPs and temperature on *P. perlatus*

Elevated temperature has been shown to result in oxidative stress and affect enzyme activity. As a consequence, the induction of antioxidant defences is an important component of the stress response against oxidative stress [41]. The results reported here generally support the fact that oxidative stress biomarkers are highly sensitive to temperature, likely due to temperature-induced ROS production. It is hypothesized that exposure to AgNPs and elevated temperature will generate ROS and elicit oxidative stress in *P. perlatus*. Oxidative stress

responses are generally lowest at conditions of lowest stress (i.e. at 21°C—similar to room temperature) and highest at conditions of highest stress (i.e. 100 μg/mL AgNPs at 28°C and 18°C). In our study, antioxidant enzymes (SOD, CAT and GST) activities were generally lower at lower AgNP concentrations and at lower temperatures (e.g. at 0 μg/mL and 100 μg/mL, and at 18°C and 21°C), and was highest at higher AgNP concentrations and at higher temperature (e.g. at 100 μg/mL and at 28°C). Since the dissolution of oxygen is generally higher at lower temperatures (and vice versa), it is expected that oxidative stress responses are inversely correlated to temperature [42]. 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). Other studies have also provided similar evidence of temperature effects on oxidative stress parameters. For example, Vinagre et al. [43] reported the effect in tissues of *Gobius paganellus* in an experiment at increasing temperatures. In an earlier study, Vinagre et al. [42] also reported variation in oxidative stress responses (i.e. increased catalase activity) due to temperature (28°C) in *Dicentrarchus labrax*. A similar conclusion was reported by Rodrigues et al. [44] in in the muscle and digestive gland of *Callinectes maenas*, while Paital et al. [45] reported the seasonal effects on oxidative stress biomarkers in the gills and hepatopancreas of *Scylla serrata*. The capacity for aggregation, sedimentation and solubility of AgNPs in aqueous environments can limit their transport within the water column, and thereby reduce their bioavailability to aquatic organisms [46]. As seen, smaller aggregates were formed at the higher temperature regime, thereby enhancing the potential for toxicity.

5. Conclusions and significance

Nanoparticle toxicity is a growing concern in freshwater habitats. However, understanding NP effects on aquatic organisms is largely impeded by the lack of the studies addressing these effects combined with other environmentally relevant stressors. 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 *P. perlatus*, common in the waters of southwestern region of the Western Cape [47]. Nanoproducts are increasingly being used in various products and, consequentially, the potential adverse effects associated with exposure to 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. This book chapter emerges in this context, centring on the effects of the most commonly used and commercially available AgNP using *P. perlatus* as a sentinel species.

Coexposure to AgNPs and elevated temperature resulted in a significant increase in ROS production rates and increase in antioxidant enzyme activity. Elevated temperatures often increase the negative impacts of pollutants in aquatic organisms, especially as the temperatures approach the upper tolerance limits. As such, it is imperative to assess the effects of pollutants (such as NPs) on sentinel species in the context of the environmentally relevant thermal variability.

6. Future perspectives

The work conducted here has focussed largely on the collective effects of AgNPs and temperature on the oxidative stress defences of *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:

- 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.
- As evidenced, AgNPs are not, at 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 worthy of consideration in future coexposure studies.
- Previous studies have reported notable differences between the responses of male and female individuals, suggesting that there may be some gender-specific effects to NP exposure. Different responses between genders 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).

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