Ecotoxicology

Ecotoxicity of Silver Nanomaterials in the Aquatic Environment: A Review of Literature and Gaps in Nano-toxicological Research

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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.
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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; Potamanautes warreni; silver nanoparticles

1. Introduction

The advancements of nanotechnology in the last few decades have seen nanomaterials (NMs) become a constituent in a wide range of manufactured commercial and domestic products. Nanoparticles (NPs) 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 1; Project on Emerging Nanotechnologies). 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). Silver NPs are widely used in several consumer products including personal care products, laundry addictives, 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.

Fig1. Nanomaterial growth trend 2005 – 2010 (Project on Emerging Nanotechnologies)

Fig2. Percentage of products associated with a specific material (Project on Emerging Nanotechnologies) accessed 4 July 2012

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).

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-Videaa 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 (D. 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 to 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 and Choi 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 *Potamanautes warreni*. 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'Melia 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 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 et al. 2011).

Fig.3 Typical examples of nanoparticles aggregation (A: Choi et al. 2010; B: Glaspell et al. 2005; C: Pham et al. 2012; D: Saini et al. 2011).

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).

Once released into the environment these NPs are available for uptake by aquatic organisms. The plasma membrane of living organisms limits the entry of foreign materials into cells (Fabrega et al. 2011). It is generally believed that a possible route of entry into the bodies of aquatic organisms is via endocytosis (a process by which protein carriers located in the plasma membrane engulf other molecules) (Moore 2006), which can result in the cellular uptake of molecules between 1 – 100 nm in size. Nanomaterials are also known to penetrate the semi-permeable membranes of some aquatic organisms (Baun 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.

2.1. Effects of AgNPs on aquatic organisms

2.1.1. 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. 2011). 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 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 (C. vulgaris)* and marine microalgae *Dunaliella tertiolecta (D. 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 1: A non-exhaustive summary of the toxic effects of AgNPs to aquatic plants

2.1.2. Aquatic invertebrates

Extensive research exists which investigates the toxicity of AgNPs in aquatic invertebrates (Table 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 and Muller (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 (Asghari et al. 2012; Figure 4). 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.

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.

Table 2: A non-exhaustive summary of the toxic effects of AgNPs to aquatic invertebrates

In toxicity tests with other aquatic invertebrates, Croteau et al. (2011) investigated the bioaccumulation dynamics in the snail *Lymnaea stagnalis (L. 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.

Fig.4 Light microscope images of daphnia exposed to AgNPs. A: control, B: live daphnia with pigmentation, C and D: bubbles visible under the carapace; nanoparticles visible on the antennae and body surface (adapted from Asghari et al. 2012)

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 (C. riparius)* and *Chironomus tentans (C. 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.

Toxic effects of AgNPs on reproduction and development have been reported (Ringwood et al. 2007). 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^{+}.

2.1.3. 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; Bilberg et al. 2010; Scown et al. 2010; Griffit et al. 2012 Pham et al. 2012). The vast majority of fish nano-toxicological

studies published are acute studies, while fewer papers report on chronic studies (Aschberger et al. 2011). 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 Park (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* (*D. 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 5), as well as mortalities with increasing NP concentration. Nanoparticle size is known to affect toxicity. Bilberg et al. (2010) and Scown et al. (2010) reported size dependant uptake of AgNPs (10 – 35 nm) and associated oxidative stress in the gills of *D. rerio*.

Table 3: A non-exhaustive summary of the toxic effects of AgNPs to fish

Fig.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)

3. 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-toxicolgoical 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.

4. 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 (WHO 2001; Figures 6 and 7). 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. warreni*.

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 (Shvedova et al. 2003). 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).

Fig.6 The three categories of biomarkers (biomarkers of exposure, biomarkers of effect and biomarkers of susceptibility) (Adapted from DeCaprio et al. 1997)

Fig.7 Schematic representation of the sequential order of response to pollutant stress within biological system (Adapted from Bayne et al. 1985)

4.1. Biomarkers used in conventional ecotoxicological studies involving crabs

Ecotoxicity studies based on biomarkers have already been developed using crabs (table 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 and Geraldine 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 (Viarengo et al. 1990). 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. 2011), 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 (Elumalai et al. 2007). 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 4: A non-exhaustive summary of biomarker studies involving crabs

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 (Liqing 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. (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 (Zhang et al. 2001). Acid phosphatase (ACP) is a hydrolytic lysosomal biomarker whose functions are distorted during stress (Rajalakshmi et al. 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 & 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 (Sanders 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 2000), 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. 2002; 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. 2011). The results showed dose-time relationships suggesting a significant increase in DNA single strand breaks when compared to the control set.

5. 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. warreni* be applied to elucidate the nano-toxicological effects of AgNPs.

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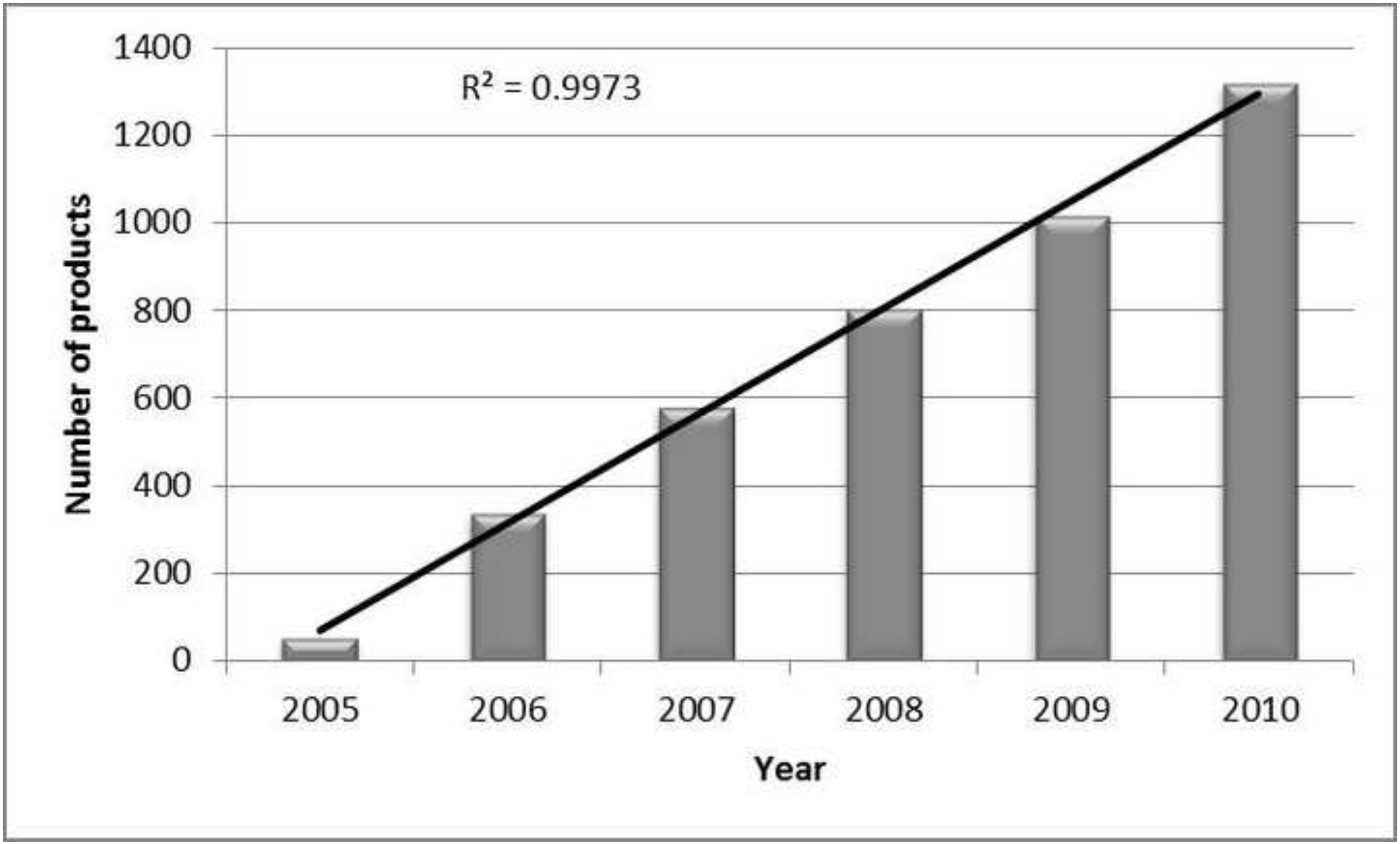

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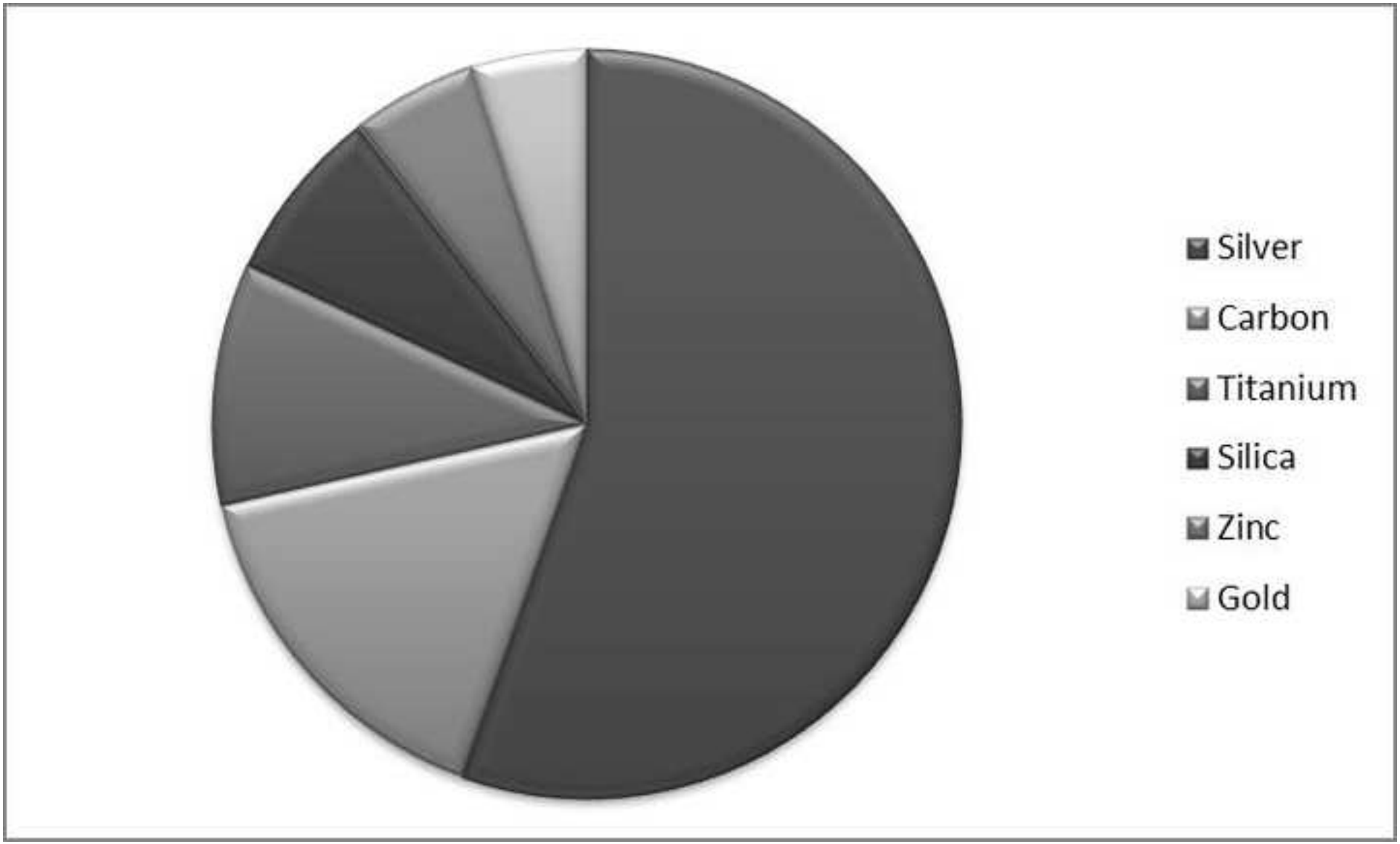

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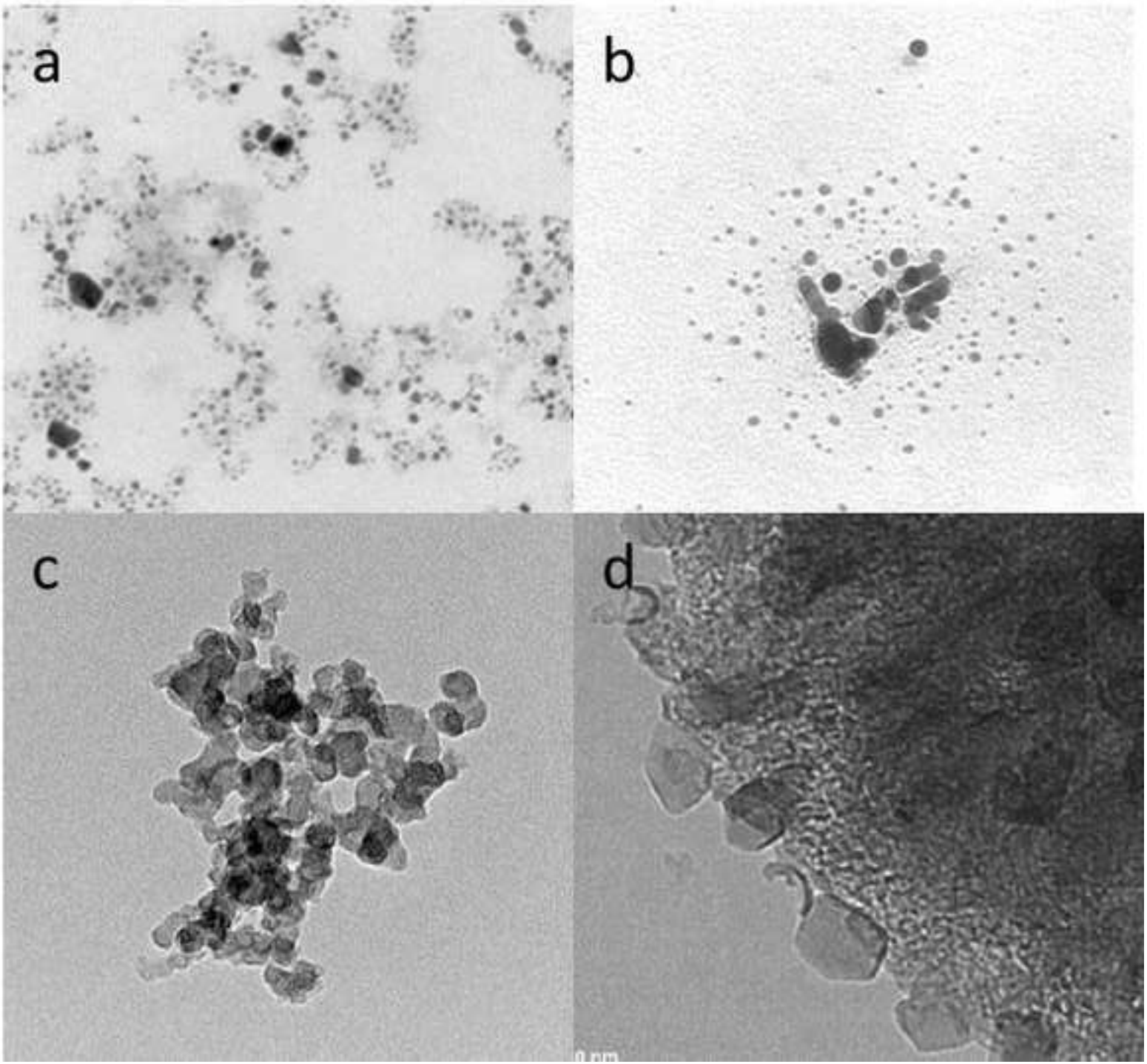

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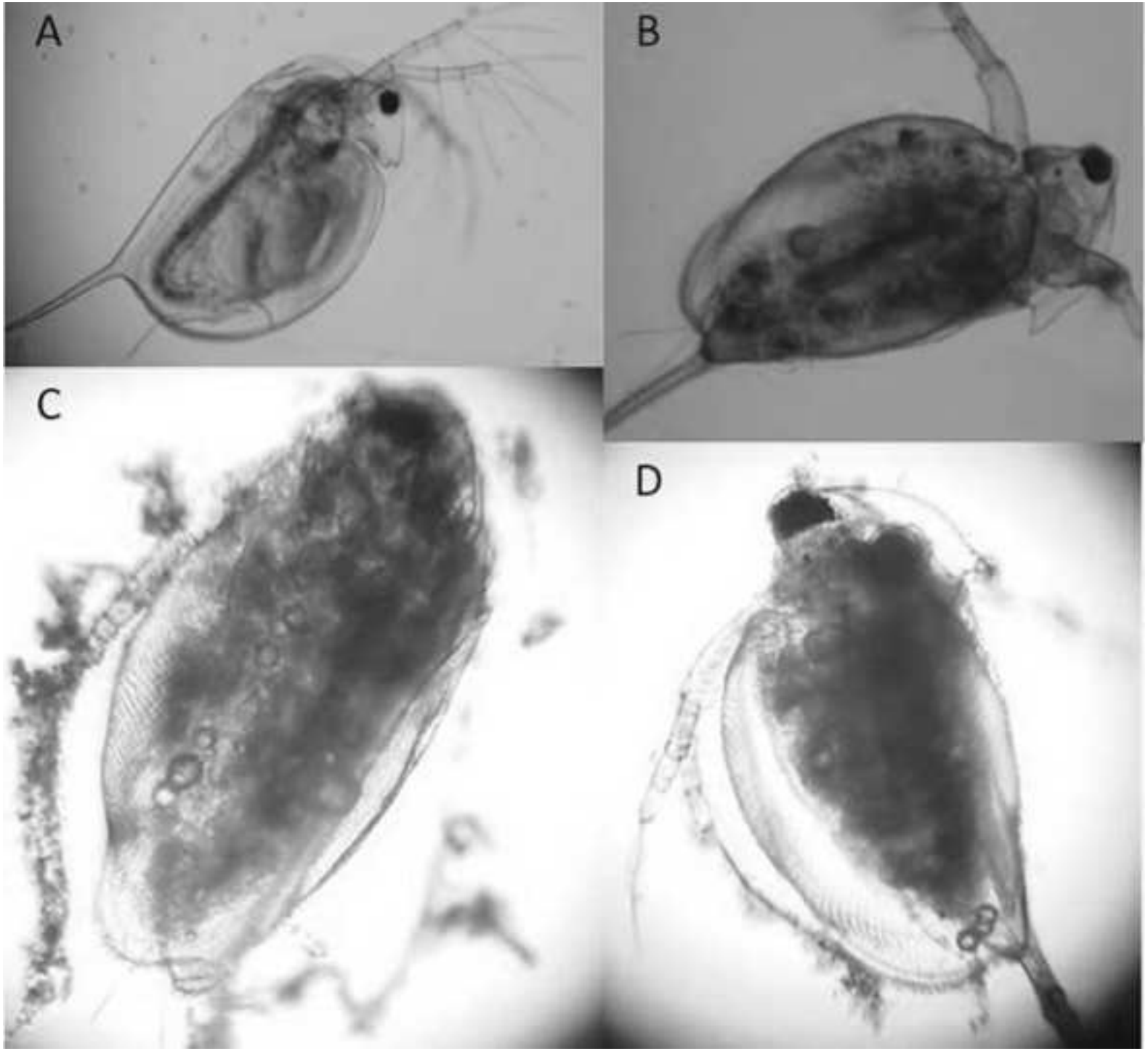

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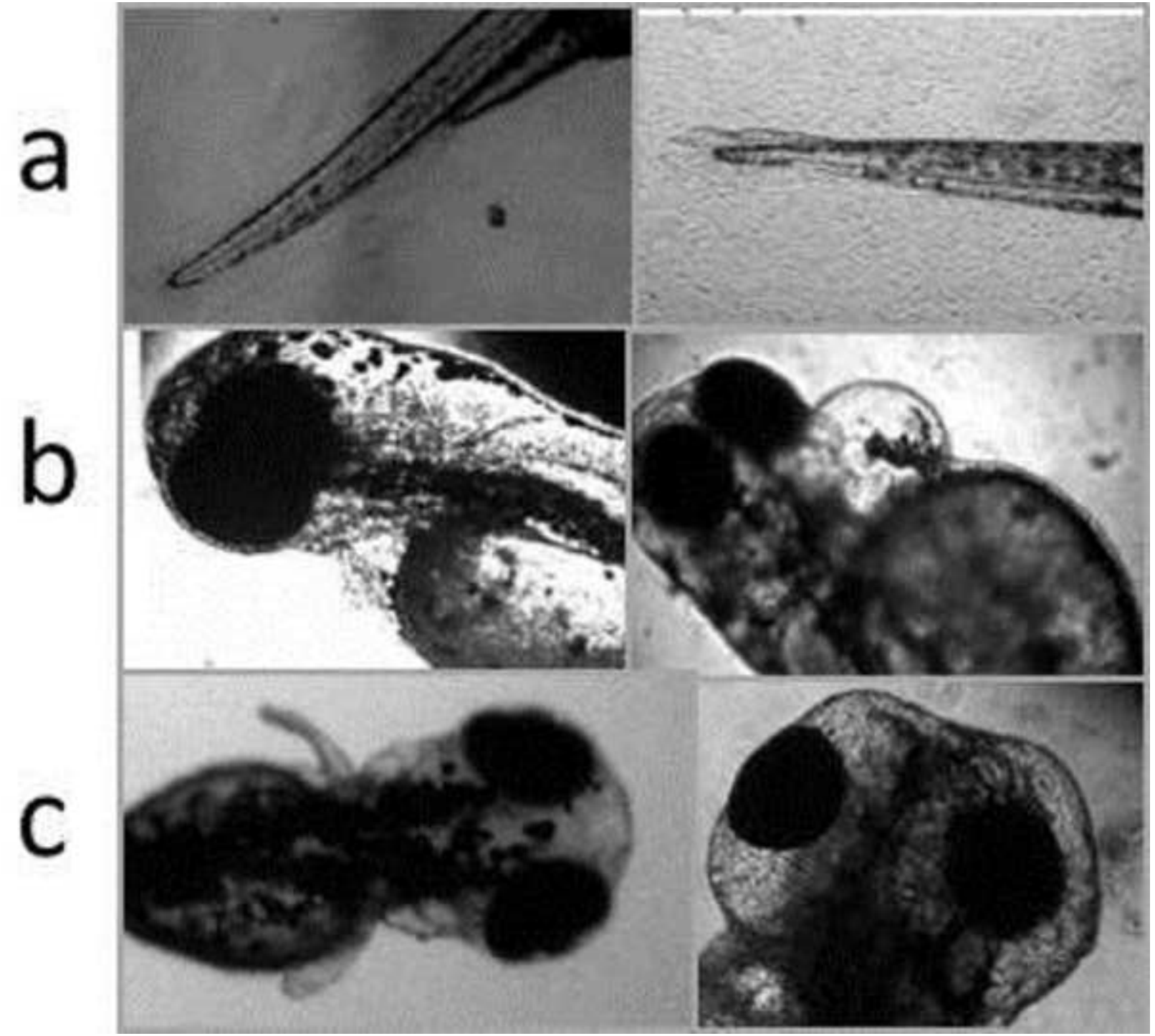

Figure 6
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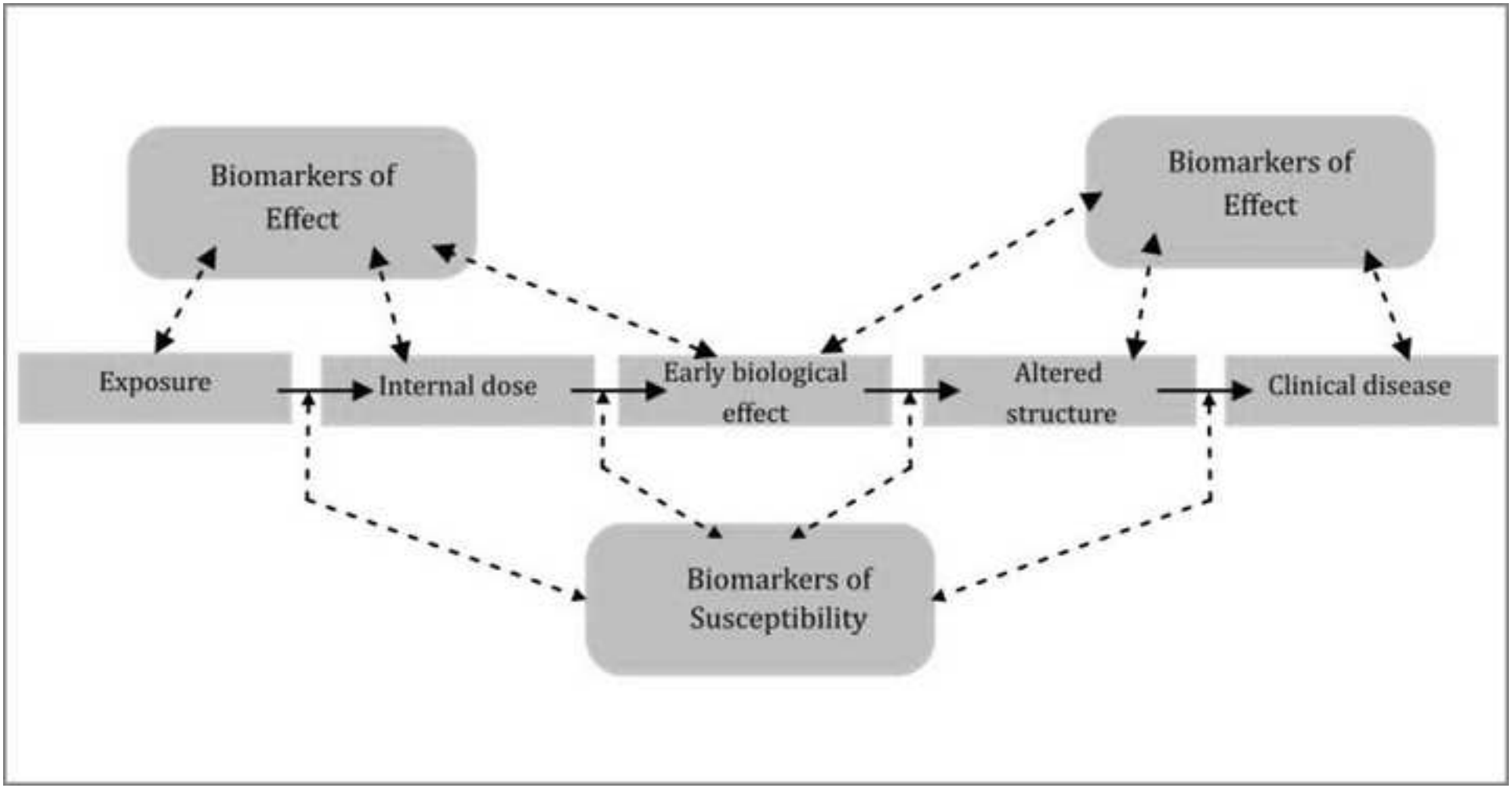

Figure 7
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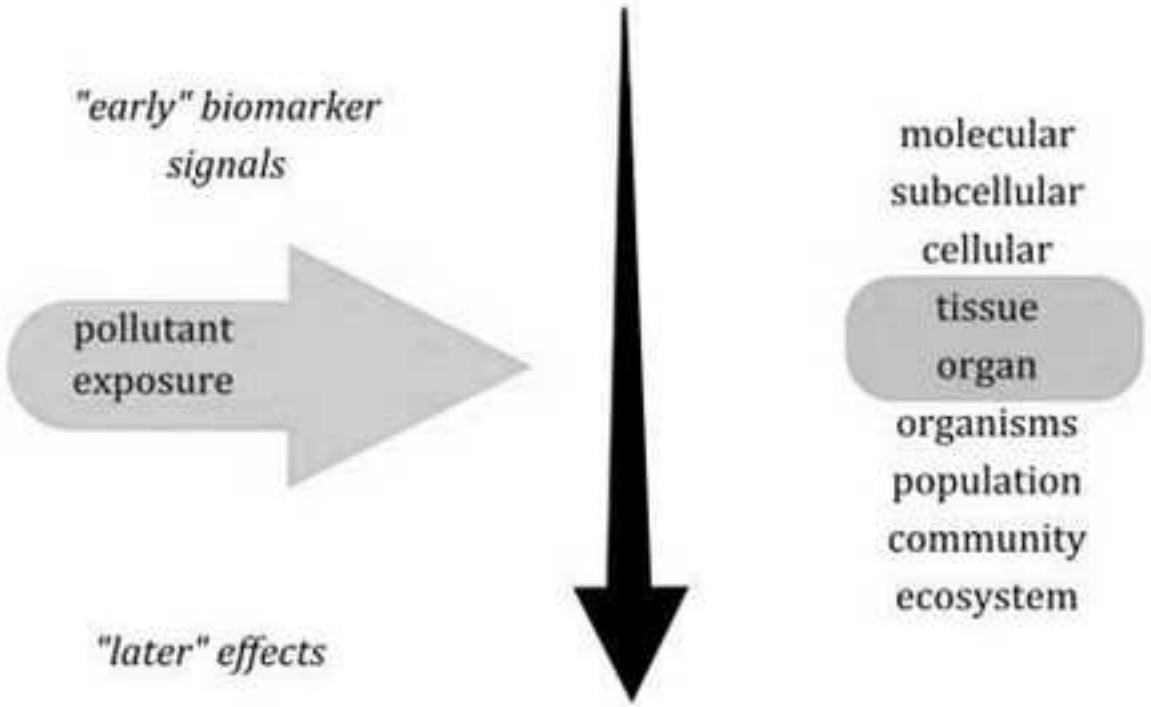

Table 1: A non-exhaustive summary of the toxic effects of AgNPs to aquatic plants

Test species	NP size (nm)	NP concentration	Major findings	Reference
Lemnar minor	20; 100	0, 5, 10, 20, 40, 80; 160 µg/ L	Plant growth inhibition at 5 µg/L AgNPs concentration	Gubbins et al. (2011)
Chlamydomonas reinhardtii	10 - 20	1 g/L	Similar EC50 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	0–10mg/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	10-200	10-100 000	Toxicity was	Navarro et

reinhardtii		nM	higher for $AgNO_3$ than for AgNP (in terms of EC50), when compared as a function of the Ag^+ toxicity of AgNP was much higher than that of $AgNO_3$.	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)

Table 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	20	2 – 500 µg/L	>70% of	Zhao and

magna			AgNPs were accumulated through ingestion	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)
Ceriodaphnia dubia	20-30	1 mg/mL	Increase in organic matter decreases AgNP toxicity	Gao et al. (2009)
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	7; 10; 20	2.2 mg/L	Loss of	Romer et al.

magna			mobility and fecundity	(2011)
Chironomus tentans	50-400	5-5000 ug/Kg	Percentage survival and growth length inhibition; catalase and peroxidase enzyme activity showed that toxicant stress of the NMs	Oberholster et al. (2011)

Table 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)
Danio rerio	11.6	0.08 nM	Increase in *D. rerio* mortalities; abnormalities in early life stages	Lee et al. (2007)
Perca fluviatilis	30–40	63, 129, 300 µg /L	Impairment of the tolerance to hypoxia; internal hypoxia during low	Bilberg et al. (2010)

			water oxygen tensions	
Oryzias latipes	49.6	1; 25 µg/L	Cellular and DNA damage, carcinogenic and oxidative stresses	Chae et al. (2009)
Danio rerio	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)

Table 4: A non-exhaustive summary of biomarker studies involving crabs

Test species	Toxin	Parameter / Biomark	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	Pan and Zhang (2006)

			tissue	
Carcinus maenas	Metals, PAHs		High gills LPO; hepatopancreas DNA integrity decreased in male crabs, 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	Liqing et al. (2011)
Chasmagnathus granulata	Cu, Zn, Cd, Pb	Survival curves	First zoeae were more sensitive than young crabs to acute exposure to metals.	Ferrer et al. (2006)
Scylla serrata	As	ACP, ALP	Inhibition of activity of ACP and ALP; dose dependent decrease in the activities of ACP and ALP	Saha et al. (2009)
Callinectes sapidus	Cu	Acute toxicity and	Acute dissolved Cu toxicity was	Martins et al. (2011)

		in vivo accumulation tests	higher at 2 ppt than at 30 ppt; Cu flux into the gills was higher than into other tissues analysed.	
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 and total haemocyte count (THC)	Li and Cheng (2008)
Callinectes sapidus	TBT	vivo effects of long-term	Respiration rates significantly	Oberdorster et al.

		exposure	decreased; hydroxylation of [14C]testosterone by hepatopancreas microsomes increased significantly	(1998)
Carcinus maenas	MT	Defence and damage biomarkers signals	Gills and hepatopancreas GST were reduced; MT induction occurred; High gills LPO; hepatopancreas DNA integrity decreased	Maria et al. (2002)