Toxicity of Engineered Nanoparticles to Marine Bivalve Molluscs

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Abstract

Engineered nanoparticles (ENPs) are increasingly used in manufacturing and consumer products. Their environmental release is a concern given their toxicological effects, which may include interference with cellular electron transport, reactive oxygen species generation, nucleic acid damage, protein oxidation, and cell membrane disruption. This thesis evaluated ENP effects on two tropical filter feeding bivalves (*Amiantis umbonella* and *Asaphis violascens*). Nano-silver and nano-titanium dioxide were evaluated over acute (48 hour; concentration 0.05, 0.2, 1, 2 mg/l) and chronic (4-weeks; concentration 0.05 mg/l) exposures. Chronic exposures were also conducted with nano- and bulk-copper and zinc oxides. Bivalve functional activity was measured as particle (microalgae) clearance rate. Simultaneously, a multi-biomarker approach (lipid peroxidation, catalase, glutathione peroxidase, glutathione reductase, glutathione S-transferase, and metallothionein) was used, supported by histopathology, to determine potential metabolic and cellular effects in gill and digestive gland tissues. Responses varied with nanoparticle type, concentration, exposure period, and species. Acute exposure triggered concentration dependent changes as clearance rate reduced with increasing concentration and exposure duration. Gills and digestive glands revealed oxidative injury; however, the respondent antioxidant defence biomarkers varied. Antioxidant enzyme levels were generally lower in the digestive gland with effects more evident at 0.05 mg/l. Bivalves, particularly *A. umbonella*, ceased filtering at higher concentrations but still registered oxidative injury. Acute exposure at lower concentrations enhanced antioxidant defence; however, cellular damage occurred. Chronically exposed animals were capable of ameliorating some of the damage. Membrane lipid peroxidation by nano-copper and zinc oxide was higher than their bulk states. Histopathology revealed morphological alterations in gills and digestive glands, particularly over longer exposures. Protein expression was examined (SDS-PAGE) in chronically exposed tissues with some changes common to all nanoparticles whereas others were nanoparticle specific. The present study indicates oxidative injury in marine bivalves exposed to ENPs with the gills and digestive gland as target organs.

Dedications

*I dedicate my work to my father (**Mohammad Akram Butt**), mother (**Tazeen Saeed Butt**), mentor and idol (**Mirza Umair Beg**), and grandmother (**Ameer Begum**) for their inspiration, guidance and support in my endeavours*

Motivational words

“Read! In the name of your Lord” (Al-Quran)

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1.1 Background to the Study

In this chapter, the science of nanoparticles and nanotechnology, their types, physico-chemical properties, exposure, and risks associated with their release into the environment are explained. The general biology of marine bivalve molluscs (the bioassay organisms of choice for this thesis) is discussed including their importance as filter feeders and biomonitoring organisms. This chapter will set out a sequential exploration of the subject of this thesis including the toxic effects of metal nanoparticles to marine bivalves, involving mechanisms of oxidative stress, lipid peroxidation, antioxidant enzymes and metallothioneins.

Production of engineered nanoparticles (ENPs) has grown continuously and is expected to reach 58,000 tons per year by 2020 according to an estimate by the United Nations Environment Programme. Among numerous ENPs, metallic nanoparticles (MNPs) are produced in large quantities and have been used widely in industry, agriculture, consumer products, and household goods (Joo and Zhao, 2017). Extensive applications of ENPs are generating new concern for policy makers and environmental managers regarding possible toxicological effects and pathological risks to human health and the environment (Canesi et al., 2015; Ju-Nam and Lead, 2008; Montes et al., 2012). As most spent ENPs will end up being released into environmental media, there have been significant concerns about their release into the aquatic environments via wastewater discharge and runoff. For instance, zinc oxide (ZnO) and titanium dioxide (TiO_2) nanoparticles have been extensively used in skincare products, with more than 33,000 tons of sunscreens produced containing up to 25% of ZnO nanoparticles (Joo and Zhao, 2017). At least 25% of the sunscreen used (~4000–6000 tons/year) was released in coral reef areas (Danovaro et al., 2008). The discharge volume of ZnO nanoparticles was estimated to be the second largest, second only to that of TiO_2 nanoparticles (Gottschalk et al., 2009).

The surface properties and the very small size of nanoparticles facilitate their biological uptake by direct ingestion or by entry across epithelial boundaries

such as in the gills or body wall (Bhatt and Tripathi, 2011; Canesi et al., 2015; Canesi et al., 2012). The toxic effects of nanoparticles are attributed to the generation of reactive radicals causing oxidative stress at the cellular level (Al-Subiai et al., 2012; Canesi et al., 2015; Rocha et al., 2015), and various factors have been reported to affect their potential toxicity (Joo and Zhao, 2017). According to the Organization for Economic Cooperation and Development (OECD), MNPs are of particular concern due to their unique physical and chemical properties, e.g., their ultra-small size, large surface area to volume ratio, high reactivity, and wide, versatile uses; yet information regarding the toxicity mechanisms of various MNPs has been lacking (Joo and Zhao, 2017). Therefore, it is important to generate information about the biological effects of MNPs on organisms within diverse marine ecosystems.

The first paper published on the toxicity of nanoparticles to aquatic organisms was by Moore in 2006. At the time that when this Ph.D. study was initiated, few research papers were available on the toxicity of ENPs to marine invertebrates (Figure 1.1). However, the frequency of relevant publications increased rapidly after 2010. The potential ecotoxicological risks of ENPs to aquatic organisms have been the subject of several recent reviews (Baker et al., 2014; Corsi et al., 2014; Grillo et al., 2015; Lapresta-Fernández et al., 2012; Ma and Lin, 2013; Matranga and Corsi, 2012; Maurer-Jones et al., 2013; Minetto et al., 2014; Misra et al., 2012) but despite this, their modes of action and biological risks are not yet fully understood (Joo and Zhao, 2017; Rocha et al., 2015).

Recently a comprehensive review has been prepared by a consortium of scientist discussing the issues related to the relevant test conditions for improved evaluation of ecological hazards of ENPs (Holden et al., 2016). Such debate was extremely important due to the specific behaviour of ENPs in aquatic environments, linked to their surface structure and reactivity, agglomeration, aggregation and dissolution which require test methodologies different from conventional toxicity testing or ecotoxicological methods. There are so many factors involved that it makes it challenging to study ENP toxicology. ENPs are a growing threat to marine ecosystems and it is essential to determine which aquatic ecosystems and compartments will be most at risk. Currently, knowledge of the

biological effects in aquatic environments is skewed toward studies on 'as-manufactured' nanoparticles in aqueous acute tests using pelagic organisms. The number of studies on environmentally modified (aged) ENPs, based on long-term chronic effects, bioaccumulation, and exposure of benthic (sediment) organisms is substantially fewer. Therefore, for the present study benthic bivalve molluscs were selected as there is not much toxicity data available on these organisms.

As filter feeders, bivalves are suitable test species for particulate pollutants. Two species (*Amiantis umbonella* and *Asaphis violascens*) prevalent in the local coastal areas were chosen to study the effects of varying concentrations of MNPs. In this experimental exposure study, nominal concentrations of MNPs were used and the internalization of the particles in the organism and confirmation of exposure was done through biological response assessment in the exposed organism (Selck et al., 2016). Determination of total metal content in the target tissues may indicate internalization of test particles but not the physical state of the ENP which is considered the main factor in exerting ENP toxicity. There are discrepancies in the literature about the ENP toxicity and their ionic form. In the authors' opinion the behaviour of ENP in the aqueous medium, its dissolution kinetics, ageing phenomenon and uptake kinetics has to be extensively determined in order to fully understand the mechanism of toxicity. The need for improved analytical approaches has been reviewed by Montaño et al. (2014) who discussed current analytical procedures for the measurement of particle size, surface group, particle number and elemental composition, as well as the problems involved in detection and characterization and potential need for further research in this area. It is therefore felt that monitoring of biological/biochemical responses to the given concentration of ENP in the surrounding water is the best way to understand their harmful effects.

The functional activity of exposed bivalves compared with unexposed animals was measured by determining filtration rate and the effect on key target organs (gills and digestive gland) was determined by measuring activities of oxidative defence enzymes and lipid peroxidation in the tissue. The oxidative defence system was examined in this study because of increasing evidence that ENPs exert oxidative stress in the exposed organism and therefore, specific

biomarkers were chosen to establish the effect of exposure to ENPs for this study. Histopathological studies of target organs also supported that the nature of tissue damage in the present exposure conditions was similar to that reported by several other authors and the effect obtained was concentration dependent. As regards to exposure concentrations, some researchers hold differing views as to whether ecotoxicological testing of ENPs should be conducted using environmentally relevant concentrations, where observing outcomes is difficult versus higher ENP doses, where responses are observable (Holden et al., 2016). Regarding environmentally relevant exposure concentrations it is to be mentioned that so far in the literature only predicted concentrations are reported that have been calculated based on the production and the use of modelling, and no report to the best of the authors' knowledge is available for onsite actual measurement of ENPs except for waste dumping sites. More so in Kuwait where no authentic data on the actual use of ENPs is available and no regulations have been promulgated, despite several cosmetic products containing ENPs being available on supermarket shelves.

1.2 Nanoparticle and Nanotechnology

A nanoparticle may be defined as a particle with at least one dimension between 1 and 100 nanometres (nm) in size (Rocha et al., 2015; Royal Society and Royal Academy of, 2004; Savolainen et al., 2010), where one nm is one billionth or 10^{-9} of a meter (Owen and Depledge, 2005). ENPs have unique physicochemical properties differing from their bulk forms due to their greater surface area to volume ratio (Vale et al., 2016). Several natural biological molecules, like proteins and DNA, fit well in the above definition of nanoparticles. Among them, viruses are the smallest naturally occurring functional nano-objects. To explain the orders of magnitude involved, the diameter of a human cell DNA molecule is 2-12 nm, a human red blood cell is 5,000 nm and a human hair is 10,000-50,000 nm (https://www.nano.gov/nanotech-101/what/nano-size).

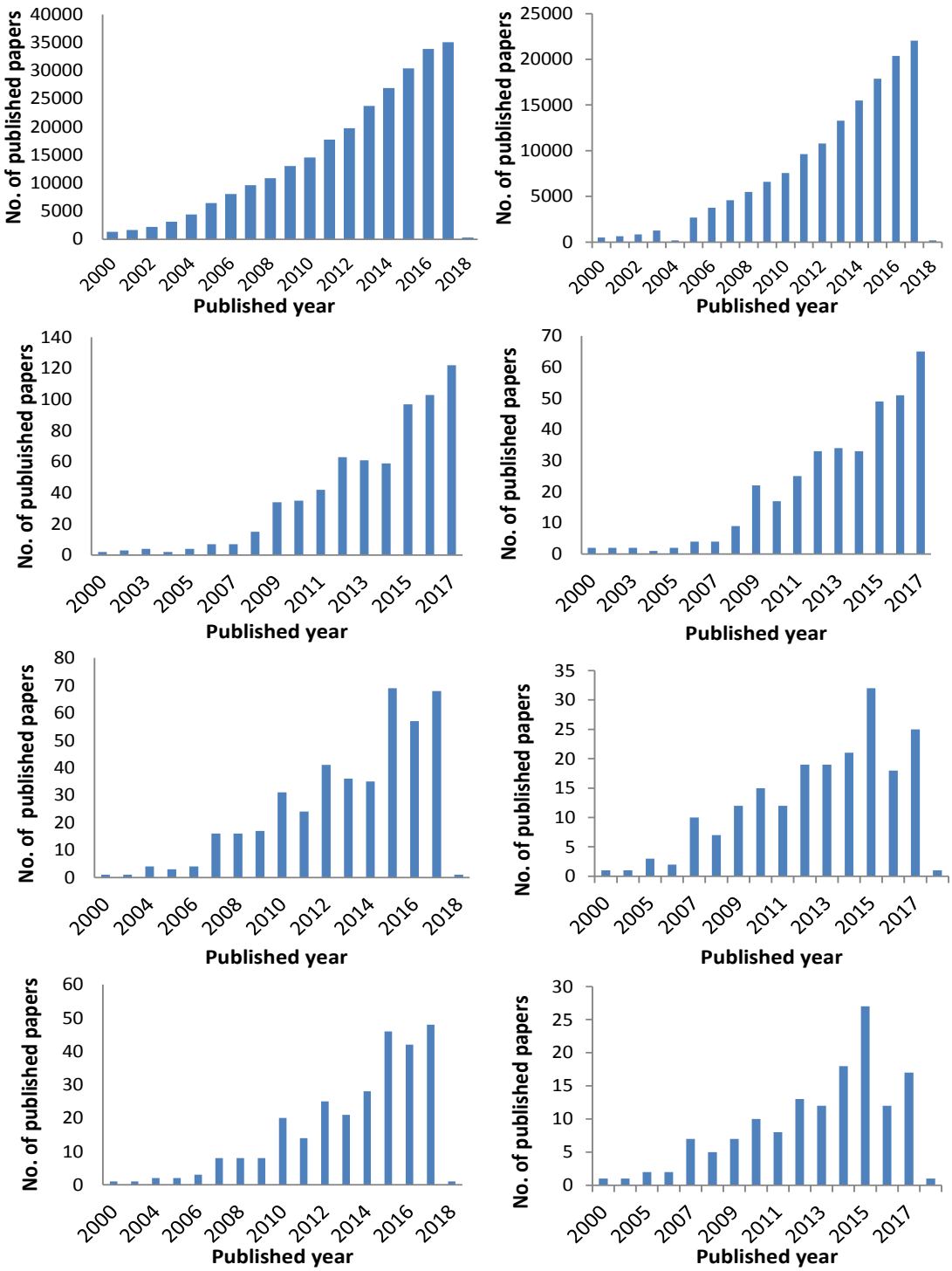

Figure 1.1. The number of papers identified by www.sciencedirect.com containing the keywords: published research papers on A) nanoparticles, B) engineered nanoparticles, C) nanoparticles and marine invertebrates, D) engineered nanoparticles and marine invertebrates, E) nanoparticles and bivalves, F) engineered nanoparticles and bivalves, G) toxicity of nanoparticles to bivalves, and H) toxicity of engineered nanoparticles to bivalves. Date of search was 21st Aug, 2017.

1.2.1 Natural versus engineered nanoparticles

Naturally occurring nanoparticles have always been present in the environment such as colloidal particles (I nm to 1 μm) present in freshwater (Lead and Wilkinson, 2006), volcanic dusts in the atmosphere (Ammann et al., 1990), and nm scale particles from soil erosion (Hasegawa et al., 2007). These naturally occurring nanoparticles are mainly created in the environment through geological processes like physicochemical weathering, authigenesis/ neoformation (e.g., in soils), and volcanic activity. Through geological processes inorganic nanoparticles are produced.

The other process to produce naturally occurring nanoparticles is through a biological mechanism which typically produces organic nanomolecules, although some organisms can produce mineral granules in the cells. The biological processes normally operate at the nanoscale and many biological molecules like proteins, peptides, DNA, RNA, ATP and many viruses are of nanosize. The release of organic nanomolecules into the environment is a common occurrence (e.g., mucoprotein exudates from algae and animals, dispersion of virus particles). The degradation of biological matter produces chemically active nanoparticles of 50–200 nm in size (e.g., humic and fulvic acids) occurring freely in natural waters and soils (Lead and Wilkinson, 2006). Thus the presence of such nanoparticles in the environment and also inside the cell suggests that living organisms evolved to adapt to exist with such materials.

There are many other factors that can differentiate naturally occurring nanoparticles and ENPs. The naturally occurring nanoparticles exist in the environment for a short period and, often disappear through dissolution, or become larger through particle growth or aggregation, whereas, some ENPs may persist because they are prepared with stabilizer, capping or fixing agents (Handy et al., 2008a). The naturally occurring nanoparticles may be toxic under certain circumstances, such as fine dust produced in volcanic eruptions (Lee and Richards, 2004), and so this raises concerns that ENPs could also be toxic. In fact, ENPs might represent a special case since they are designed to have particular surface properties and (surface) chemistries that are less likely to be found in natural particles. Thus, ENPs present enhanced or novel physico-chemical or

toxicological properties in comparison to natural nanoparticles and pose a challenge to environmental scientists to regulate their usage on a sound scientific basis. The natural and engineered nanoparticle present in the environment are given in Figure 1.2.

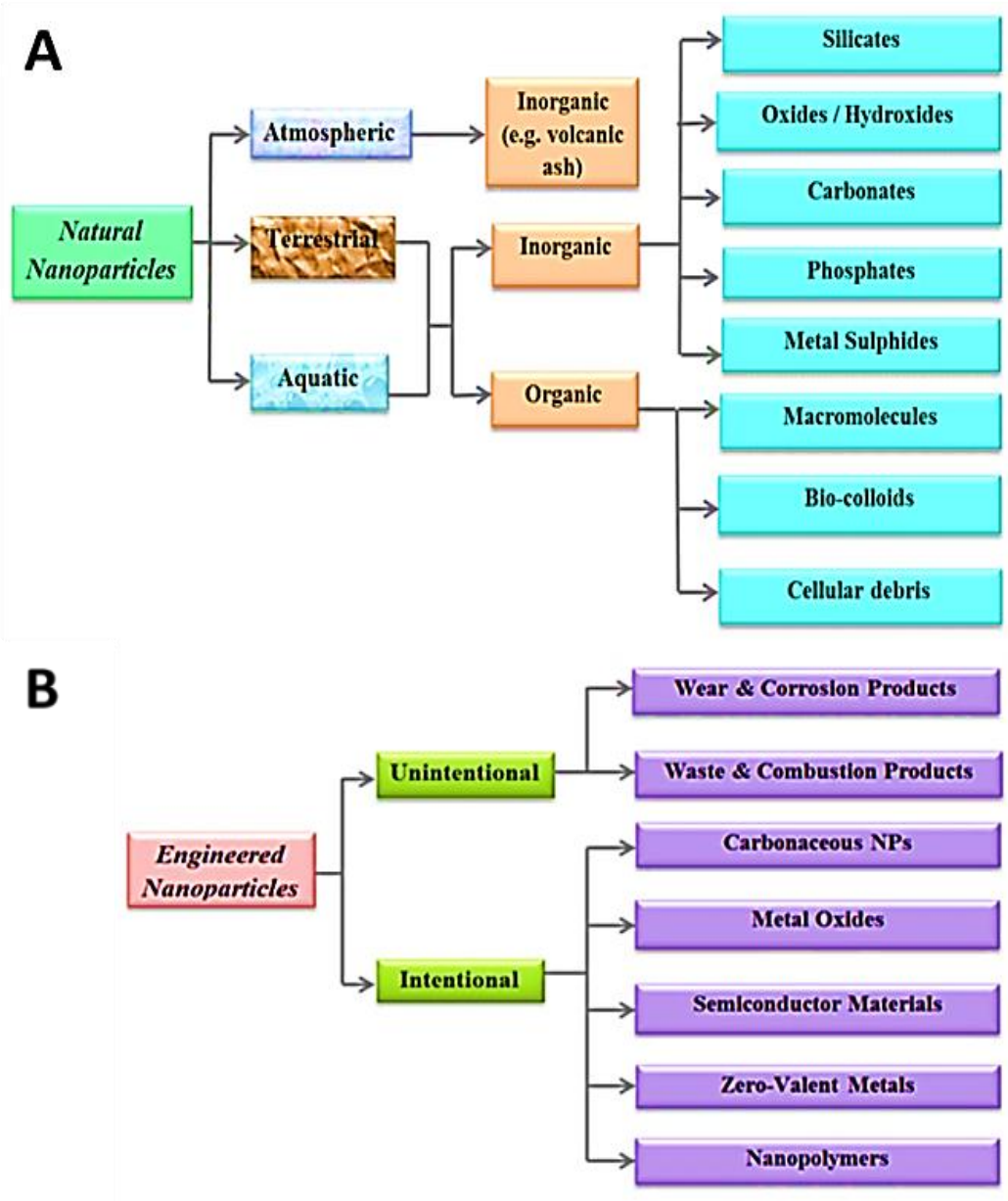

Figure 1.2. A) Natural nanoparticles and B) engineered nanoparticles in the environment (Source: Bhatt and Tripathi, 2011).

1.2.2 Types of engineered nanoparticles

On the basis of core material and structural configuration, engineered nanoparticles can be classified into the following types (Bhatt and Tripathi, 2011; López-Serrano et al., 2014):

1. ***Carbon-based:*** These nanoparticles are made of pure carbon, and are usually present in the form of hollow spheres, or tubes. They can be classified into fullerenes (compounds containing at least 60 carbon atoms) and carbon nanotubes (CNTs). Cylindrical shaped carbon nanoparticles are referred to as nanotubes, while spherical ones are called fullerenes.

2. ***Metal-based:*** These nanoparticles belong to the second class of ENPs containing metal, particularly transient metal oxides. The main component is metal and this group includes metal oxides such as titanium dioxide (TiO_2) zinc oxide (ZnO) and copper oxide (CuO), and binary oxides such as lithium cobalt dioxide ($LiCoO_2$), and indium tin oxide (InSnO) that combine the special properties of these elements with the high reactivity of nanoparticles.

3. ***Quantum dots (QDs):*** The third class of ENPs, also known as quantum dots (QDs), constitute nanometre sized semi-conductor nanocrystals, with size ranges between 2–10 nm. QDs possess a reactive core consisting of a metal or semi-conductor that controls their optical and electrical properties such as cadmium selenide (CdSe), indium phosphide (InP), or zinc selenide (ZnSe) (Klaine et al., 2008; Logothetidis, 2006).

4. ***Elemental metallic NPs / zero-valent metals:*** As the name indicates, the fourth class of ENPs includes zero-valent metals, and are prepared by reduction of metal salts. This includes some inorganic NPs mainly composed of noble elements (Au, Ag), but also some transition metals with many applications.

5. ***Organic polymers / dendrimers:*** The fifth class of ENPs is made by dendrimers, which are complex, multifunctional polymers with 1–10 nm diameter. They assume highly asymmetric shapes and increase in

branching as they adopt a globular structure. The presence of numerous chain-ends confers high solubility and miscibility to dendrimers. Physico-chemical and biological properties of dendrimers are comparable to traditional polymers, and their size, topology, flexibility, and molecular weight can be controlled.

1.2.3 Production of engineered nanoparticles

ENPs may be produced by reducing macroscopic materials to a nanometric scale or from the groups of atoms and molecules with structured organization (Aitken et al., 2004). Two distinct methods such as top-down and bottom-up are used for the synthesis of nanoparticles (Bhatt and Tripathi, 2011). Top-down methods involve well-organised assemblies or ENPs are directly produced from the bulk materials through the generation of isolated atoms using various techniques (Niemeyer, 2001). Top-down strategies generally involve physical methods such as repeated quenching, photolithography and milling (Gao et al., 2004a). In contrast, bottom-up generally involve molecular components as starting materials that are further linked by nucleation, chemical reactions and growth processes. This approach promotes the formation of more complex clusters (Ju-Nam and Lead, 2008).

Owing to the relative novelty of ENPs, their use has been accelerated in a wide variety of applications and consumer products and a dramatic increase is expected in future. Worldwide production of ENPs is expected to grow to half a million tonnes with the number of ENP-containing consumer products reaching 3,400 by 2020 (Rocha et al., 2015).

1.2.4 Physico-chemical properties of nanoparticles

The most important feature of nanoparticles are their ultra-small size, large surface area to mass ratio, and high reactivity that differentiate them from the material of the same composition on a micro scale or other larger size, commonly stated as bulk material (Fabrega et al., 2011). Two primary characteristics of nanoparticles, surface properties and quantum effects, determine their chemical

reactivity, density, resistivity, magnetisation, dielectric constant and the number of atoms at the surface (Poole and Owens, 2005). The size and surface area may be further illustrated in a way that one carbon microparticle with a diameter of 60 μm has a mass of 0.3 μg and a surface area of 0.01 mm^2. The same mass of carbon (0.3 μg) converted to nanoscale with each particle having a diameter of 60 nm has a surface area of 11.3 mm^2 and consists of 1 billion nanoparticles (Figure 1.3). Therefore, a larger surface area and enhanced reactivity of ENPs helps to create better catalysts e.g., catalytic converter in a car that reduces the toxicity of the engine exhaust fumes. A variety of materials are in use today, such as nanoengineered batteries, fuel cells, and catalysts to produce cleaner, safer, and more affordable modes of generating and storing energy. Other applications include nanostructured membranes and materials for water treatment and desalination plants. This also helps support the "functionalization" of nanoscale material surfaces for applications ranging from drug delivery to clothing insulation (https://www.nano.gov/nanotech-101/special).

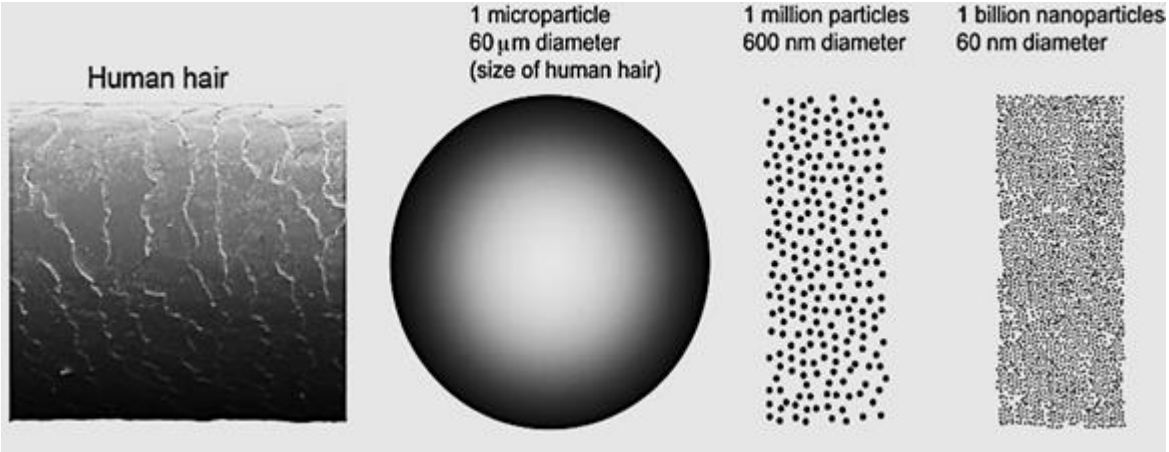

Figure 1.3. An example explaining nano vs bulk particles: a bulk/microparticle of 60 μm diameter, about the size of a human hair - shown in the left at scale, and the number of nanoparticles with a diameter of 600 nm and 60 nm having the same mass as one microparticle of 60 μm diameter are comapred (Buzea et al., 2007).

Quantum effect phenomena rules the behaviour and properties of particles when particles are created with dimensions of about 1–100 nm (Aznan and Johan, 2012; Daniel and Astruc, 2004; Kusic et al., 2011). Properties of materials are

size-dependent at this scale. Thus, when particle size is made to be nanoscale, properties such as melting point, fluorescence, electrical conductivity, magnetic permeability, and chemical reactivity change as a function of particle size. For example, gold can appear red or purple at the nanoscale, the motion of the gold's electrons is confined, enabling it to react differently with light compared to larger-scale gold particles. This property of gold nanoparticles allows them to be used in nano-medicine (https://www.nano.gov/nanotech-101/special).

1.2.5 Applications of engineered nanoparticles

A majority of biological processes happen at minute scale that encouraged scientists to believe that particles at nano-scale can enable new routes of biological interactions that can enhance their action in medicinal imaging, computing, printing, chemical catalysis, materials synthesis, and many other fields (https://www.nano.gov/you/nanotechnology-benefits). The salient characteristics of nanoparticles, such as increased surface area and quantum effect phenomenon, provided impetus to the science of nanotechnology which has become a fast growing scientific field resulting in its wide application in biomedicine (Barnett et al., 2007; Dong and Feng, 2007; Salata, 2004), electronic devices (Kachynski et al., 2008), cosmetics (Lens, 2009; Müller et al., 2002), renewable energies (Pavasupree et al., 2006; Wei et al., 2008), and environmental remediation (Tungittiplakorn et al., 2004; Zhang, 2003). Their application has also been recognised in waste management, biodegradation of waste material, and air pollution control. Some of these applications that have already been proposed and are in use are listed in Table 1.1.

1.2.6 Nanoparticles used in the present study: Nano silver

The present study focussed mainly on metallic nanoparticles, mainly silver nanoparticles (n-Ag) and titanium dioxide nanoparticle (n-TiO_2), and later a comparison was also made between the nanoparticles and their bulk counterparts using oxides of zinc and copper. Metal oxide nanoparticles were chosen because of their toxicity and occurrence in consumer products, large scale industrial

Table 1.1. Commonly used nanoparticles and their applications (Bhatt and Tripathi, 2011; López-Serrano et al., 2014).

Class of ENPs	Application
Carbonaceous compounds	
1. Carbon nanotubes (CNTs) and their derivatives	Environmental applications e.g., adsorption of pollutants from contaminated sites (Jang et al., 2001) increasing growing rate of plants (Khodakovskaya et al., 2009; Upadhyayula et al., 2009); alternative energy storage media (Upadhyayula et al., 2009): as an advanced construction material (Stone et al., 2010); medical use (Upadhyayula et al., 2009)
2. Fullerenes	Medical purpose e.g., antiviral activity and sorption of organic compounds (Friedman et al., 1993; Mueller and Nowack, 2008)
3. Nanowires	Early detection of cancers (Choi et al., 2010)
Metals and metal oxides	
1. TiO_2	UV radiation filter in cosmetics, skin care products and sunscreens (Peters et al., 2011); construction, semiconductors, paints and coatings (Auffan et al., 2009; Berra et al., 2012); Plant growth promoter (Zheng et al., 2005)
2. ZnO	Cosmetics and skin care products; cereals (Peters et al., 2011)
3. CeO_2	Combustion catalyst in diesel fuels, gas sensors, solar cells, oxygen pumps (Zhou et al., 2006)
CuO and Al_2O_3	Improved thermal conductivity (da Silva et al., 2011); catalyst (Cho et al., 2006)
Iron oxides	Biomedical applications for its super paramagnetic properties (Ding et al., 2010); sensors for microbial detection (Perez et al., 2003)
Semi-conductor devices	
Quantum dots (QDs), CdSe, CdS, CdSe/ZnS	Medical diagnosis (Schirhagl et al., 2012); Imaging and targeted therapeutics (Gupta and Gupta, 2005)
Zero-valent metals	
1. Zero-valent iron	Detoxification of organic pollutants (Sadeghi et al., 2010)
2. Nanoparticulate- silver	In wound dressings, socks, and other textiles; air filters, toothpastes, baby-products, vacuum cleaners, and washing machines for antimicrobial properties (Benn and Westerhoff, 2008; da Silva et al., 2011); sensors (Benn and Westerhoff, 2008)
3. Colloidal elemental gold	Sensors, surface plasmon resonance (Sadeghi et al., 2010); proteomic studies (Wu and Hu, 2007)
Polymers	
Dendrimers	Drug delivery and tumor treatment (Roney et al., 2005)

production and industrial pollutants (Abdel-Khalek et al., 2015; Melegari et al., 2013).

n-Ag is produced in huge amounts because of the unique physico-chemical properties, low manufacturing costs and extensive use in everyday life (Fabrega et al., 2011; Gomes et al., 2013a; Gomes et al., 2013b). They are used in numerous industries including in inks (Perelaer et al., 2009; Tay and Edirisinghe, 2002), cosmetics, as bacteriocides in fabrics and other consumer products (Jeon et al., 2003; Kim et al., 2007), microelectronics (Wu et al., 2006), and medical imaging (Jain et al., 2008). Based on usage the predicted concentrations of n-Ag are 0.088–2.16 ng/l in European and North American surface waters (Gottschalk et al., 2009). The exceptional broad spectrum bacteriocidal activity of n-Ag has made it extremely popular and it is used in a diverse range of consumer products including plastics, soaps, pastes, metals, and textiles (Frattini et al., 2005; Gomes et al., 2012). Accoring to the Woodrow Wilson database there are 435 products on the market containing n-Ag, with 32.4% in creams and cosmetic items, 18% in textiles and clothing, 16.4% in household items, 12.3% in air and water filters, 8.2% in detergents, 4.1% in health supplements and 8.6% in other products (Fabrega et al., 2011; Vance et al., 2015). Their bactericidal action makes n-Ag the largest and fastest growing class of nanoparticles in product applications. Silver is naturally present in the environment but exposure to high concentrations can be harmful. Ionic silver is a known environmental stressor due to its persistence and accumulation in the environment (water, sediments and organisms) as well as high toxicity towards aquatic organisms, even at low levels (µg/l range) (Fabrega et al., 2011; Luoma, 2008). Even though n-Ag is one of the most studied nanoparticles, the toxicity mechanisms are still not fully clear (Vale et al., 2016). In aquatic organisms (e.g., fish) the effects of Ag are well documented but less is known about the mechanism by which the nano form of Ag exerts toxicity to organisms such as invertebrate species (Gomes et al., 2013b). Some assume that intact n-Ag particles are responsible for the induction of toxic responses in the organisms, while others assign the n-Ag toxicity to the release of Ag^+ ions to the media (Schultz et al., 2014).

It has been considered for n-Ag that two distinct routes are followed that could induce the biochemical response of nanoparticles in the organisms. The first is the effect produced by the nanoparticles itself, and the second by the presence of both n-Ag and dissolved Ag^+. In the first case the responses are unique and

produced due to the presence of n-Ag which does not occur in matched Ag^+ exposures. The response of n-Ag alone can be produced by using n-Ag with low dissolution rates, so that the leached Ag^+ in the media remains insufficient to induce toxicity to the organisms. Alternatively, end points specific to n-Ag, such as internalization of nanoparticles, or specific cytotoxicity and genotoxicity can be measured.

Prior to the interest in nanoparticles, the silver ion was considered the most toxic form of silver in water (Ratte, 1999). As with all metals, the chemistry of the surrounding environment affects association of silver ions with various ligands, in turn influencing bioavailability and toxicity (Adams and Kramer, 1998; Erickson et al., 1998; Luoma et al., 1995). It is well known that silver ions have the propensity to bioconcentrate in organisms, since the chemical properties of the Ag ions make them compatible for uptake via cell membrane ion transporters, similar to those regulating sodium and copper ion transport into cells (Luoma, 2008). N-Ag may be discharged to the environment by several routes, including synthesis, during manufacturing and incorporation of the nanoparticles into goods, the usage of the goods, and during recycling or disposal of goods (Fabrega et al., 2011). Even though a wide majority of studies have been conducted on the effects of silver nanoparticles, it is not yet possible to establish if the n-Ag potential toxicity is due to its particulate or ionic form; however, it is clear that n-Ag poses a high risk to aquatic life when present in freshwater systems.

1.2.7 Nanoparticles used in the present study: Nano titanium dioxide

The most widely produced nanoparticle in the world, with an expected production of 201,000 tons during 2015 is n-TiO_2 (USEPA, 2011; Vale et al., 2016). It exists in three main mineral forms, anatase, rutile and brookite, which differ in crystal structures (Arami et al., 2007; Li et al., 2004), of which the first two are usually considered the most important in the environment. Each of these forms presents different properties and therefore different applications and environmental impacts. The anatase form is considered more stable than the rutile at particle diameters below 14 nm (Ju-Nam and Lead, 2008). N-TiO_2 is extensively used in pharmaceutical products, sunscreens and cosmetics as a UV

absorbent. The anatase phase is used in catalysis and photocatalysis applications, because it possesses high photocatalytic activity. The rutile phase is used in paints, papers, inks, and toothpastes as a white pigment. It is also used in plastics and as a pigment and thickener in other applications (Mueller and Nowack, 2008). Other compounds such as silica can also be added as a surface coating to n-TiO_2 to reduce its photoactivity to protect human skin, plastics and other objects from direct exposure to UV radiation (USEPA, 2009). Other uses of n-TiO_2 are in conductive coatings, electrocatalytic processes, as absorption materials in solar cells (Li et al., 2003), light emitting diodes, disinfectant sprays, sporting goods, water treatment agents and antifouling paints (USEPA, 2009).

Nanoparticles exert toxicity through three main routes: due to physical stress associated with their size, their photocatalytic activity, and their capacity to adsorb xenobiotics acting as a carrier. It can get internalised in different organs of the organism causing tissue damage and stress. On exposure to natural and/or UV light radiation photocatalytic nanoparticles like n-TiO_2 can generate radical species that can cause toxic effects. It is extensively documented that dispersed n-TiO_2 interacts with various elements e.g., Cd, Zn, Pb, Cu, Ni and As (Engates and Shipley, 2011; Gao et al., 2004b; Vale et al., 2014), and affects their bioavailability and toxicity to organisms by changing their speciation in the media, also if n-TiO_2 is already present in the tissue it may increase bioaccumulation of other xenobiotics (Tan and Wang, 2014). Irrespective of the route of internalization of nanoparticles, the toxicity is usually associated with the generation of reactive oxygen species (ROS) and antioxidant enzymes activity are the most commonly related biochemical endpoints.

Considering the production of n-TiO_2, 5,000 metric tonnes were produced commercially between the years 2006-2010, and more than 10,000 metric tonnes per year between 2011 and 2014 (UNEP, 2007). Its production is likely to increase to 2.5 million metric tonnes by 2025 (Robichaud et al., 2009). N-TiO_2 generates ROS at its surfaces because it is photo-inducible and redox active. It is released into aquatic ecosystems through wastewater treatment plant effluents, from exterior facades or accidents during manufacture, use and transport (Salieri et al., 2015).

1.2.8 Nanoparticles used in the present study: Nano zinc oxide

Zinc oxide nanoparticles (n-ZnO) are one of the most used nanoparticles with 30,000 metric tons of estimated production per year (Vale et al., 2016). The predicted environmental concentrations for n-ZnO in surface waters range from 0.008–0.055 µg/l in Europe and 0.001–0.003 µg/l in the US (Gottschalk et al., 2009; Gottschalk et al., 2013). The global production of nanoparticles for sunscreen products alone was estimated to be approximately 1,000 tonnes for 2003/2004, consisting principally of TiO_2 and ZnO particles (Borm et al., 2006). They have unique optical and electrical properties and are components of many commercial products, including personal care products (cosmetics and sunscreens), due to their excellent UV absorption and reflective properties. It is also used in plastics, ceramics, glass, cement, rubber, lubricants, paints, pigments, food cereals, batteries and fire retardants (Chang et al., 2012; Ma and Lin, 2013; Trevisan et al., 2014). It is also very effective as a photocatalyst and has been shown to be effective in a range of environmental control technologies, from remediation of environmental pollutants to medical disinfection (Chang et al., 2012). Such widespread and expanding production and use of n-ZnO increases the potential for their release to the environment. Current estimates of n-ZnO concentrations in the UK environment range from less than 100 µg/l (in water) to a few mg/kg (in soil) (Boxall et al., 2007), and a more recent study by Gottschalk et al. (2009) mentioned modelled n-ZnO concentrations of 10 ng/l in natural surface water and 430 ng/l in treated wastewater in Europe. Environmental levels of n-ZnO are expected to increase continually given the widespread application of these nanoparticles (Daughton and Ternes, 1999).

Even though it is an essential microelement, ionic zinc is known to be toxic at higher concentrations to aquatic organisms (Brun et al., 2014; Mortimer et al., 2010). The toxicity of n-ZnO, just like other metal oxide nanoparticles, is related to the ionic dissociation (Zn^{2+}) in water, generation of ROS through media-surface interactions and interaction with biological targets, for instance destabilization of lipid membranes and damage to proteins and DNA. In several freshwater organisms oxidative stress was induced by both n-ZnO and/or released Zn^{2+}. N-ZnO adsorbs several elements like As, Al, Mo, Hg, Pb, Cu, Ni and Cd that changes their speciation in the media and bioavailability (Gagne et al., 2013b;

Ghiloufi, 2013; Hua et al., 2012). On internalization in the organism, these NP-metal complexes may undergo dissociation, and dissolution of nanoparticles due to pH changes that may result in the release of the adsorbed trace elements. However, information on the ecotoxicological effects of n-ZnO has been very limited across all taxa and further studies are needed to explain the mechanisms of n-ZnO toxicity from the cellular level to the organism or population levels (Ma and Lin, 2013; Vale et al., 2016).

1.2.9 Nanoparticles used in the present study: Nano copper oxide

Copper is an essential metal with a well-established role as a co-factor in many enzyme systems and other proteins (e.g., cytochrome oxidase, superoxide dismutase), and is involved in several biological processes required for growth, development and maintenance. However, copper can be extremely toxic if present in high concentrations or if organisms are exposed chronically to low levels in the environment (Gaetke and Chow, 2003). It is a widely used metal oxide that is biologically active, (Moschini et al., 2013), produces DNA damage and cell death compared to other micro-sized particles, n-TiO_2 and n-ZnO (Ahamed et al., 2010).

The inherent toxicity of copper is a consequence of the propensity of free Cu ions to participate in the formation of ROS (and hence induction of oxidative stress) through Fenton and Haber-Weiss reactions (Bebianno et al., 2004; Regoli and Principato, 1995). Additionally, Cu has a high affinity for thiol groups and, thus being capable of binding to cysteine and leading to protein inactivation. The adverse effects of copper bioaccumulation in aquatic organisms have been extensively investigated (Maria and Bebianno, 2011; Regoli and Principato, 1995). They have several industrial and commercial applications associated with their antimicrobial properties, elevated thermal and electrical conductivity, and improvements in thermal viscosity e.g., air and liquid filtration, coatings of integrated circuits and batteries, wood preservation, inks, skin products and textiles (Buffet et al., 2011; Chang et al., 2012; Griffitt et al., 2009). Aside from the antibacterial properties of n-CuO (Ruparelia et al., 2008; Yoon et al., 2007), they are also cytotoxic and genotoxic. ROS-derived oxidative stress (Buffet et al., 2011; Fahmy and Cormier, 2009; Gomes et al., 2011; Ivask et al., 2010), DNA

damage and oxidative lesions (Ahamed et al., 2010; Karlsson et al., 2008) were observed not only in human cell cultures but also in freshwater and marine organisms (Gomes et al., 2012). In spite of the limited data available on the cytotoxicity, genotoxicity and ROS generation in freshwater organisms, conclusions can be made that n-CuO induces ROS toxicity mediated by ionic Cu.

1.2.10 Legislation and standards related to nanoparticles

At present there are no Kuwait EPA published standards for the production, use and/or discharge of nanoparticles to the environment. However, there are international standards such as ISO 14040 series (ISO, 2006a, 2006b) and European Commission (UNEP, 2011) which adopted Life Cycle Assessment (LCA) methods to regulate environmental as well as health effects of ENPs (Hischier, 2014; Salieri et al., 2015). In addition, numerous other standard setting organizations such as ASTM (American Society for Testing and Materials), ISO (International Standardization Organization), IECTC (International Electrotechnical Commission Technical Committee) also set up technical committee to deal with the problems originating from the advancement of nanotechnologies. An active Federal Government agency of the US, NIST (The National Institute of Standards and Technology) is actively involved in the nanotechnology standard development by participating in ASTM Committee E56 on Nanotechnology. There is a 'Nanotechnology Standards Database' hosted by the ANSI (American National Standards Institute) that invites private sectors and government experts to formulate nanotechnology standards (ISO TC 229, *Nanotechnologies*) with strong scientific basis to be adopted universally (ISO TC 229, *Nanotechnologies*). A working group under ISO TC 229 defined Health, Safety and Environmental Aspects of Nanotechnologies (Table 1.2).

As a response to the discussions regarding potential unintended hazards of ENPs to humans and the environment, the Organization for Economic Cooperation and Development (OECD) has launched a ***strategic programme on the safety evaluation and risk assessment of manufactured nanoparticles.*** The

Table 1.2. Available standards for nanoparticles (http://www.nano.gov/you/standards).

Type of Standard	Identifier	Title
Terminology	ASTM E2909-13	Standard Guide for Investigation/Study/Assay Tab-Delimited Format for Nanotechnologies (ISA-TAB-Nano): Standard File Format for the Submission and Exchange of Data on Nanomaterials and Characterizations
	ISO/TS 80004-1:2010	Nanotechnologies—Vocabulary—Part 1: Core terms
	ISO/TR 14786	Nanotechnologies—Considerations for the development of chemical nomenclature for selected nano-objects
	ISO/TS 80004-3:2010	Nanotechnologies—Vocabulary—Part 3: Carbon nano-objects
Measurement	ASTM E2578-07(2012)	Standard Practice for Calculation of Mean Sizes/Diameters and Standard Deviations of Particle Size Distributions
	IEEE 1620-2004	Standard Test Methods for the Characterization of Organic Transistors and Materials
	ASTM E2864-13	Standard Test Method for Measurement of Airborne Metal and Metal Oxide Nanoparticle Surface Area Concentration in Inhalation Exposure Chambers using Krypton Gas Adsorption
	ISO/TR 13014:2012	Nanotechnologies—Guidance on physico-chemical characterization of engineered nanoscale materials for toxicologic assessment
	ISO/TS 14101:2012	Surface characterization of gold nanoparticles for nanomaterial specific toxicity screening: FT-IR method
EHS Effects	ASTM E2535-07(2013)	Standard Guide for Handling Unbound Engineered Nanoscale Particles in Occupational Settings
	ASTM WK 34427	New Guide for Nanotechnology Environment, Health and Safety (EHS) Education and Training (*under development*)
	ISO/TR 13121:2011	Nanotechnologies—Nanomaterial risk evaluation
	ISO 10808:2010	Nanotechnologies—Characterization of nanoparticles in inhalation exposure chambers for inhalation toxicity testing
	ISO/TS 12901-1:2012	Nanotechnologies—Occupational risk management applied to engineered nanomaterials
	OECD/ENV/JM/MONO(2012)40	Guidance on sample preparation and dosimetry for the safety testing of manufactured nanomaterials
	OECD/ENV/JM/MONO (2015)20	Analysis of the Survey on Available Methods and Models for Assessing Exposure to Manufactured Nanomaterials
	OECD/ENV/JM/MONO(2009)21	Preliminary Review of OECD Test Guidelines for their Applicability to Manufactured Nanomaterials
	OECD/ENV/JM/MONO (2014)1	Ecotoxicology and environmental fate of manufactured nanomaterials: Test guidelines

objective was to support accountable development of nanotechnology and for that OECD's *Working Party on Manufactured Nanoparticles* (WPMN) was established in 2006. The OECD programme has focused in generating appropriate methods and strategies to ensure potential safety issues, through establishing an OECD database on manufactured nanoparticles, testing specific nanoparticles for their health and safety evaluation, facilitating international co-operation on risk assessment strategies, developing guidance on exposure measurement and exposure mitigation, and promoting the environmentally sustainable use of nanotechnology.

1.2.11 Problems and progress in nanoparticles toxicity testing

Traditionally in aquatic testing the exposure dose is calculated on steady mass concentrations of the test substance over fixed exposure times (i.e., concentration x exposure time = dose). Measurements of the concentration of the chemical compound at the beginning and the end of the experiment are the requirement of standard aquatic toxicity testing. Thus, measuring the NM concentration at the beginning and end of an experiment is suggested as a minimum frequency. However, with ENPs the two-dimensional approach may not be applicable *in toto* because of the behaviour of the ENPs in exposure medium (Baun et al., 2009). Their agglomeration and aggregation and deposition to the benthos may cause more pronounced effect on benthic species than on pelagic species.

Therefore, characterizing changes to the NM, such as agglomeration or dissolution rates in the defined test media, and during the tests when the organisms are present may be critical to understanding the exposure and thus the subsequent toxic effect. However, characterization of nanoparticle and its accurate and precise detection of physical and chemical state in a test medium is still a challenge. Available standardized methods are expensive, time consuming and are not free from providing vague information like electron microscopy to identify nanoparticles was found to be an artefact by several authors (Edgington et al., 2014; Petersen et al., 2014). There are some emerging approaches but at this stage, far from being standardized and widely available, eg., single-particle

inductively coupled plasma– mass spectrometry (spICP-MS), for providing size distribution of the ENPs in the tissue of interest (Gray et al., 2013). However, such methods are limited to metal or metal oxide particles that will survive the chemical digestion processes needed to make a liquid sample for ICP-MS, and the detection of particles <20 nm is problematic with this method for some elements.

The OECD working group encouraged international co-operation in determining human health and environmental safety of engineered nanoparticles and worked for understanding of the applicability of the OECD test guidelines (TGs), designed for chemicals to ENPs. Firm proposals on the modifications with their justification are now available for methods required for the assessment of ENPs in the scope of regulation. They cover the testing of the green algae *Raphidocelis subcapitata* (OECD TG 201, 2011), the daphnid *Daphnia magna* (OECD TG 202, 2004), the fish *Danio rerio* (OECD TG 210, 1992), the sediment organism *Lumbriculus variegatus* (OECD TG 225, 2007), soil microflora (OECD TG 216, 2000; OECD TG 217, 2000) and terrestrial invertebrates *Enchytraeus crypticus* and *Eisenia fetida* (OECD TG 220, 2004; OECD TG 222, 2004). The proposed adaptations include general topics and were developed using Ag and TiO_2 ENPs. With the materials applied, two main variants of ENPs (ion releasing vs. inert ENPs) were addressed. As the modifications of the test guidelines refer to general test parameters (e.g., test duration or measuring principle) it is assumed that the described approaches and modifications will be suitable for the testing of further ENPs with other chemical compositions. Nevertheless, currently no compilation of proposed nano-specific test modifications on OECD TGs for ecotoxicity is available and therefore, the adaption of the test guidelines is hampered.

1.3 Release of Nanoparticles into the Environment

There is a rapid increase in the production of ENPs with approximately 3,400 consumer products in the market by the year 2020 (Rocha et al., 2015). Consequently the release of ENPs in the environment is also expected to increase. The concern for the regulatory authorities is because of the persistence of some ENPs in the environment due to their stability affected by capping or fixing agents

(Handy et al., 2008b). Their release into the aquatic environment is either direct through industrial and domestic discharges or indirectly via aerial deposition, and run-off (Baker et al., 2014; Moore, 2006). The intentional contamination of a water body may be due to the use of nanoparticles for remediation of contaminated soil and water (Biswas and Wu, 2005; Klaine et al., 2008). However, to determine the potential risk to the environment requires information on exposure levels that cause possible ecotoxicological effects. To generate such information, modelling studies and actual release studies have been conducted. The modelling approach to predict quantitative release of ENPs in environmental media was first used by Boxall et al. (2007). Several algorithms were developed for ENP concentrations in water, biosolids and soils. Subsequently, with further availability of quantitative production data and use, the models were refined using material flow analysis and considering best case and worst case scenarios (Mueller and Nowack, 2008). Gottschalk and his group used a probabilistic material flow analysis approach (Gottschalk et al., 2010; Gottschalk et al., 2009) that builds on Monte Carlo computer simulations and predicted environmental concentrations (PEC) for various ENPs. The environmental concentrations were calculated as probabilistic density functions and were compared to data from ecotoxicological studies. The simulated mode (the most frequent value) for surface waters was 21 ng/l for n-TiO_2, 13 ng/l for n-ZnO, and 0.7 ng/l for n-Ag. These simulated values were also validated by comparing with measured data reported by Kiser et al. (2009), where n-TiO_2 in sewage treatment plant (STP) effluents ranged from 5-15 µg/l. In runoff water that contacted painted facades the concentration of n-TiO_2 was reported to be as high as 3.5×10^8 particles/l (Kägi et al., 2008; Ward and Kach, 2009).

The measured values for n-Ag in surface water were higher by a factor of 10-100 than that of the simulated values (Blaser et al., 2008). Two studies conducted n-Ag release from socks when immersed in liquid phase. Benn and Westerhoff (2008) referred to the leaching of n-Ag from socks to distilled water whereas Geranio et al. (2009) investigated the release of n-Ag under real washing procedures. The amount of Ag leached from the socks ranged from 0.3-377 µg/g (Geranio et al., 2009), which is much higher than the range 1-68 µg/g observed by Benn and Westerhoff (2008). Releases of up to 46 µg/g Ag per product were seen

in another study (Benn et al., 2010), where n-Ag emissions into tap water from different consumer products as opposed to the “real-world” experimental settings was investigated using a washing protocol. Available predicted and modelled concentrations of various ENPs used in the present study are given below (Table 1.3, 1.4 and 1.5).

1.3.1 Behaviour of ENPs in the environment

The fate of ENPs in the environment greatly depends on their transformation i.e. chemical transformation dissolution and/ or transport characteristics (e.g., agglomeration/ aggregation, adsorption or sedimentation) (Lowry et al., 2012). The major processes affecting the behaviour of ENPs in aquatic systems are agglomeration and sedimentation and the results suggest they are mainly controlled by the combined effects of ionic strength, pH, the presence and concentration of organic matter and the salt composition (Christian et al., 2008; Phenrat et al., 2007). To predict the behaviour of ENPs in aquatic systems different experimental approaches have been used. The effects of different physical and chemical factors (i.e. pH, electrolyte, organic matter, etc.) should be considered individually in order to identify the main factors affecting ENPs stability. Another realistic but more complex approach investigates ENP behaviour in natural waters under environmentally-relevant conditions (Baalousha et al., 2013; Romanello and de Cortalezzi, 2013). The presence of organic matter, role of pH, and electrolyte composition in the aggregation or stabilization of ENPs has been confirmed by these studies. However, few studies are available on the stability and structure of the resulting aggregates and their potential for disaggregation. An important criterion that can significantly influence aggregate behaviour and fate of ENPs is to evaluate the aggregate structure (i.e. whether they are loosely bound or densely packed).

From a toxicological point of view the important characteristics of nanoparticles are their size, surface area, surface chemistry and charge, crystallinity, shape, solubility and agglomeration/aggregation state. In the nanoparticle scale, the reactivity generally

Table 1.3. Predicted environmental concentrations for n-Ag (ng/l), shown as mode (most frequent value) for Europe and the US for different environmental compartments. STP = Sewage treatment plant. Source: (Fabrega et al., 2011).

Environmental compartment	**Predicted environmental concentration**	
	Europe	U.S.
Soil	22.7	8.3
Sludge treated soil	1581	662
Surface water	0.7	0.1
STP effluent	42.5	21
STP sludge	1.7	1.6
Sediment	952	195
Air	0.008	0.002

Table 1.4. Modelled concentrations of n-TiO_2 released into the environmental compartments in different geographic areas (Source: Menard et al., 2011). STP = Sewage treatment plant. [a]Mueller and Nowack (2008) [b]Gottschalk et al., (2009)

Environmental compartment	**Predicted environmental concentration**		
	Switzerland	Europe	U.S.
Water (µg/l)	0.7-16[a]	0.012-0.057[b]	0.002-0.010[b]
	0.016-0.085[b]		
Soil (µg/kg)	0.4-4.8[a]	1.01-4.45[b]	0.43-2.3[b]
	0.21-1.04[b]		
Sludge treated soil (µg/kg)	NA	70.6-310[b]	34.5-170[b]
Sediment (µg/kg)	426-2382[b]	273-1409[b]	44-251[b]
Air (µg/m^3)	0.0015-0.042[a]	0.0005[b]	0.0005[b]
	0.0007-0.003[b]	NA	NA
STP effluent (µg/l)	3.50-16.3[b]	2.50-10.8[b]	1.37-6.70[b]
STP sludge (µg/kg)	172-802[b]	100-433[b]	107-523[b]

Table 1.5. Modelled concentrations of ZnO nanoparticles released into environmental compartments in different geographic areas. STP = Sewage treatment plant (Gottschalk et al., 2009).

Enviromental Compartment	**Predicted environmental concentration**		
	Europe	U.S.	Switzerland
Surface water (µg/l)	0.010	0.001	0.013
STP effluent (µg/l)	0.432	0.3	0.441
STP sludge (µg/kg)	17.1	23.2	21.4
Sediment (µg/kg)	2.90	0.51	3.33
Soil (µg/kg)	0.093	0.050	0.032
Sludge treated soil (µg/kg)	3.25	1.99	NA
Air (µg/m^3)	<0.0005	<0.0005	<0.0005

increases with decreasing size. The two main pathways of nanoparticle uptake in the cell are active uptake by endocytosis, and passive uptake by free diffusion. It has been reported that macrophages effectively cleared aggregated micron-sized (3-6 μm) but not nano-sized (20 nm) TiO_2 particles (Geiser et al., 2008). Thus, aggregation may influence the process of uptake by phagocytosis and the reactive surface area of nanoparticles (Wigginton et al., 2007). Nevertheless, knowledge in this area is still lacking, partly because this type of investigation provides a significant analytical challenge (Chekli et al., 2015).

1.3.2 Transformation of ENPs in aquatic systems

The aquatic compartment can be contaminated with ENPs either by direct contact with ENP-containing paints on boats, or by deposition from air. In addition, effluents from waste water treatment plants can be a major source of contamination. On reaching a water body the particles come in contact with dynamic physical and chemical environment that can cause a change in their original physicochemical properties. The particles may undergo dissolution, aggregation and sedimentation in the water body depending on their physicochemical properties and the passage from their point of release.

In the aquatic environment, inorganic and organic colloidal particles originated from both natural and anthropogenic sources are abundant which can interact with nanoparticles, thereby their forms and properties are changed with time (dynamic speciation). These changes affect their bioavailability. Therefore, the speciation and reaction rates of ENPs are governed by their physicochemical properties and the environmental compartment in which they are present. In order to make relevant prediction of ENPs fate and risks it is important to consider their dynamic speciation. However, this critical issue has not received much attention possibly because of limited available information (Vale et al., 2016).

One of the main transformations of metallic nanoparticles (n-ZnO, n-CuO, and n-Ag) is the process of dissolution primarily because of the formation of partially soluble metal oxide (Domingos et al., 2013b; Heinlaan et al., 2008; Wang et al., 2015). Secondly, the oxidation of the particle constituents (Dale et al., 2013; Derfus et al., 2004; Lok et al., 2007; Ma et al., 2014; Wang et al., 2013).

Thirdly, the constituent metal in metallic ENPs may form complexes with naturally occurring colloidal material in the environmental compartment or manufactured stabilizers embedded in the matrix of ENPs (Domingos et al., 2013a, 2015). In aquatic environments, the processes of sulfidation can suppress oxidation of metallic ENPs that can reduce dissolution (Dale et al., 2013; Thalmann et al., 2014; Wang et al., 2013). The process of dissolution in aquatic environments reduces persistence of metallic ENPs but the release of toxic cations increases the toxicity. Already existing models for metal speciation and toxicity can be used for predicting the impact of metallic ENPs based on the knowledge of their complete dissolution in the aquatic compartment.

Another environmental factor that can affect transformation of ENPs is photoreactions. Photoreaction can influence the nanoparticle coatings, oxidation state, generation of ROS, and persistence. This photoactive characteristic is the function of most widely used ENPs of TiO_2 and ZnO (Hund-Rinke and Simon, 2006; Zhang et al., 2007).

Transformation of ENPs by aggregation is one of the most important factors in aquatic environment. Aggregation is caused by interaction of ENPs with naturally occurring bio- or geo-macromolecules that alters size and surface chemistry. Organic matter present in water provides both charge and steric stabilization of ENPs and in the presence of multiple charged cations and anions results in bridging flocc (Domingos et al., 2010; Domingos et al., 2009). It is difficult to predict the effect of organic matter, since their concentration in aquatic environment is several fold higher than ENPs that may impact substantially on the properties of ENPs. Though aggregation with both organic and inorganic matter in aquatic environment is important, to the best of authors knowledge, no relevant toxicity studies are available.

The two modifications of ENPs, dissolution and aggregation in aquatic environment are dynamic processes that can bring about changes in available surface area and reactivity (Hotze et al., 2010). The size is the main reason of ENPs preparation since it enhances surface area and reactivity. The bioavailability to the organisms is also affected by the size, therefore, aggregation results in ENPs becoming too large for direct transport across the cell wall and/or

membrane and uptake may be prevented. On the other hand, the dissolution process reduces sizes that would facilitate cellular transport. In the natural environment the two processes are not in equilibrium and require real-time kinetic measurement studies. There are many methodological limitations since the dissolution rate may be fast or not attain the equilibrium and the aggregation rate can be fast or the aggregates size distribution may not reach equilibrium within the experimental time frame.

1.4 Biological Uptake and Toxicity of Nanoparticles

Nanoparticles may be taken up by organisms in different ways - either physical or chemical (Bhatt and Tripathi, 2011; Nel et al., 2009). Physical mechanisms are mainly attributed to particle size and surface properties that result in disruption of membrane (Hussain et al., 2005; Leroueil et al., 2008) and transport processes (Øvrevik et al., 2004). Nanoparticles may adhere to a cell, block the cell-membrane pores that allow selective permeability of substances, and consequently affect membrane function. Nanoparticles can also make entry into the cell via endocytosis, through the process of diffusion, or by ion transport systems (Figure 1.4). Chemical mechanisms mainly include the production of (ROS), dissolution and release of toxic ions, disturbance of the electron/ion cell membrane transport activity, oxidative damage through catalysis and lipid peroxidation (Elsaesser and Howard, 2012).

The major routes of nanoparticle uptake in terrestrial organisms are by inhalation or ingestion (Brigger et al., 2002; Colvin, 2003, 2004; Dowling, 2004; Howard, 2004; Moore and Allen, 2002). In aquatic organisms, nanoparticles may cross the external surface epithelia and may also have direct passage across gills. The internalization of C60-fullerenes in fish was through these routes (Oberdorster, 2004). However, the internalization of nanoparticles at the cellular level will occur via endocytosis as supported by studies conducted by Moore (2006), where the uptake of polyester nanoparticles in mussels occurred via lysosomes and endosomes.

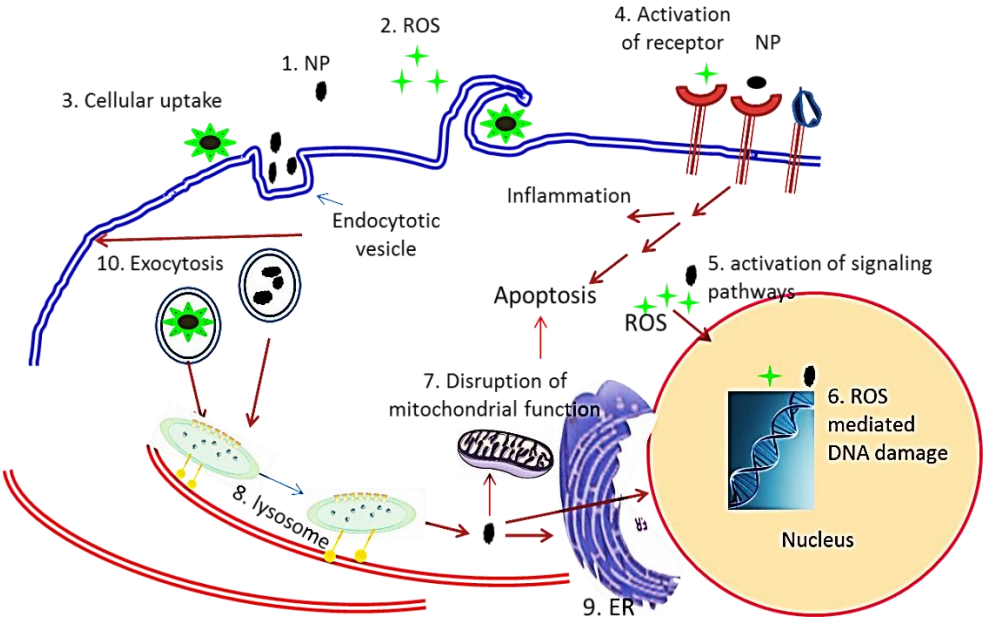

Figure 1.4. Cellular uptake of nanoparticles and interactions with subcellular components (1. NP, nanoparticle; 2. ROS, reactive oxygen species 3. cellular uptake via endocytosis; 4. activation of receptor sites; 5. ROS-mediated activation of signalling pathway 6. ROS-mediated DNA damage 7. disruption of mitochondria 8. lysomal reactions; 9. effect on endoplasmic reticulum; 10. ejection from cell by exocytosis) (Shang et al., 2014).

In plants, algae, and fungi the cell wall acts as a barrier to nanoparticles; however, pores (of size 5 to 20 nm) allow their entry (Zemke-White et al., 2000). It has been suggested that nanoparticle interactions with the cell results in the formation of new and bigger pores thus increasing their internalization through the cell wall (Navarro et al., 2008). Once inside the cell, nanoparticles enter and bind with the cell organelles through endocytotic pathways, and interfere with various metabolic processes (Bhatt and Tripathi, 2011; Moore, 2006). Potential uptake methods and effects of nanoparticles on various marine organisms are presented in Table 1.6.

1.4.1 Oxidative stress and nanoparticles

Metals can potentially increase ROS production at the cellular level (Regoli et al., 2002a; Regoli et al., 2002b; Viarengo et al., 2007). ROS are produc

Table 1.6. Potential uptake methods and effects of nanoparticles on various marine organisms. Source: (Baker et al., 2014)

Organism	Uptake method and effect	References
Bacteria	Gram-positive bacteria more susceptible than gram-negative. Nanoparticles may affect the composition, but not function, of biofilms, but accumulation may make them available to predatory grazers.	(Bradford et al., 2009; Doiron et al., 2012; Fabrega et al., 2009a; Fabrega et al., 2011; Fabrega et al., 2009b; Suresh et al., 2010)
Algae	Nanoparticles may adsorb to cell surfaces. Ions dissolved from nanoparticles can interfere with nutrient uptake and therefore limit growth. Negative charge in diatom casings (frustulae) may attract dissolved cations.	(Angel et al., 2013; Manzo et al., 2013; Matranga and Corsi, 2012; Miao et al., 2009; Miller et al., 2010; Peng et al., 2011; Turner et al., 2012; Wong et al., 2010)
Arthropods	Nanoparticle adsorption to nauplii limits movement; nauplii may feed on nanoparticles. Zn^{2+} dissolution a driver of sub-lethal toxicity.	(Fabrega et al., 2011; Hanna et al., 2013; Larner et al., 2012; Wong et al., 2010)
Annelids	Ingestion of sedimented nanoparticles by deposit feeders, and of floating nanoparticles by filter feeders. Sub-lethal effects even seen with insoluble nanoparticles.	(Buffet et al., 2011; Galloway et al., 2010; Garcia-Aonso et al., 2011; Mermillod-Blondin and Rosenberg, 2006)
Bivalves	Nanoparticles trapped by exopolymeric substances; some stay trapped in gills, some transferred to the digestive system, most rejected into pseudofaeces. May use some dissolved Zn^{2+} as a micro-nutrient. Oxidative stress noted even with insoluble nanoparticles.	(Barmo et al., 2013; Buffet et al., 2011; Canesi et al., 2012; Canesi et al., 2010a; Canesi et al., 2010b; Garcia-Negrete et al., 2013; Gomes et al., 2013a; Gomes et al., 2013b; Gomes et al., 2012; Hull et al., 2013; Kach and Ward, 2008; Kadar et al., 2011; Koehler et al., 2008; Li et al., 2008; McCarthy et al., 2013; Montes et al., 2012; Ringwood et al., 2010; Tedesco et al., 2010a, b; Tedesco et al., 2008; Ward and Shumway, 2004; Wegner et al., 2012; Zuykov et al., 2011a; Zuykov et al., 2011b)
Gastropods	Adsorption to body may cause oxidative stress in situ. Not significant accumulators in mesocosm studies.	(Kach and Ward, 2008; Li et al., 2013; Zhu et al., 2011)
Echinoderms	Down-regulation of immune system in adults; ion dissolution may cause larval mortality in chronic exposures.	(Fairbairn et al., 2011; Falugi et al., 2012; Oral et al., 2010; Radenac et al., 2001)
Stenohaline fish	May lodge in gill mucus and dissolve. Nanoparticles accumulate in the gut following drinking; carbonate production may biotransform and release dissolved ions.	(Alishahi et al., 2011; Griffitt et al., 2012; Marshall, 2002; Perry et al., 2011; Scown et al., 2010; Tian and Yu, 2011; Wilson et al., 2009; Wong et al., 2010)
Euryhaline and diadromous fish	Ion dissolution interferes with change in gill function moving from full marine fresh water. Encourages stress-related drinking, leading to uptake of nanoparticles in the gut.	(Joo et al., 2013; Reilly et al., 2011; Scown et al., 2010; Shaw et al., 2012; Shaw and Handy, 2011)

-ed directly as by- products of many biochemical processes and may result in DNA damage, lipid peroxidation and depletion of protein sulphydryls (Valko et al., 2005). When ROS levels exceed an organism's antioxidant defence, the cells go into oxidative stress which can detrimentally affect membrane lipid peroxidation, lysosomal membrane stability, filtration rate, gill structure, and ion uptake. Lipid peroxidation is considered a major mechanism by which oxyradicals can cause tissue damage leading to impaired cellular function and alteration in membrane physico-chemical properties, which can disrupt vital functions in the body (de Almeida et al., 2007). The antioxidant defence system includes antioxidant enzymes including glutathione-s-transferase and catalase. The activity of these antioxidant enzymes increases or decreases depending on the level of cellular oxidative stress. They are mainly evaluated in target organs such as gills and digestive glands (Cheung et al., 2001; Lam et al., 2004).

As reported in previous sections, ENPs induce oxidative stress in biological entities by generating ROS. The potential for oxygen free radicals and other ROS to damage tissues and cellular components is called oxidative stress. ROS or a free radical can be defined as an unstable atom or molecule that contains one or more unpaired electrons. ROS are considered the main underlying chemical process in nanotoxicology, leading to secondary processes that can ultimately cause cell damage and even cell death. Moreover, ROS is one of the main factors involved in inflammatory processes and can also have direct impacts on cell integrity (Elsaesser and Howard, 2012).

ROS include the superoxide anion (O^{2-}), hydrogen peroxide (H_2O_2), and hydroxyl radicals (OH^-) that are capable of reacting with and damaging DNA, proteins and lipids. In order to gain stability, ROS attempt to gain electrons from other molecules by electrophilic attack, in the process creating new radical species and thus triggering a chain oxidation reaction. The univalent reduction of an oxygen molecule continuously results in the formation of ROS during the oxidative metabolism of cells (Halliwell and Gutteridge, 1986). Anthropogenic chemicals/ pollutants may also exert harmful effects by catalyzing ROS production (Winston and Di Giulio, 1991). However, living cells are not defenceless and they have protective mechanisms that have evolved to prevent, limit and or repair oxidative damage from ROS. If an imbalance occurs between

the oxidants and the antioxidants, oxidative stress arises and the ROS can cause tissue damage, interfere with informative molecules which may lead to impaired cellular functions, protein oxidation or disruption of cell membranes, and finally, disrupt vital functions (Figure 1.5) (Baker et al., 2014; Manduzio et al., 2005).

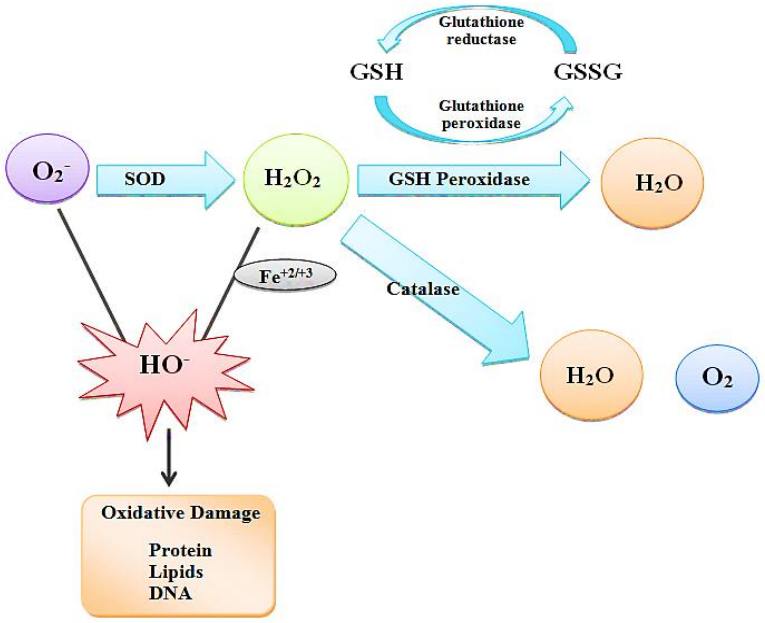

Figure 1.5. Imbalance between the production of pro-oxidants and anti-oxidants in cells produces free radicals like activated oxygen (O_2) and ROS, thus leading to serious cellular damage and oxidative stress. O_2^- = oxygen radical; SOD = superoxide dismutase; H_2O_2 = hydrogen peroxide; GSH = reduced glutathione; GSSG = oxidised glutathione; H_2O = water; Fe = iron; O_2 = molecular oxygen; HO^- = hydroxyl radical; DNA = deoxyribonucleic acid (Wakamatsu et al., 2008).

1.4.2 Invertebrate immunity

To cope with oxidative damage the invertebrate immune system relies on three basic mechanisms of immune defence: physicochemical barriers, cellular defences and humoral mechanisms, with cells and humoral responses acting in a coordinated way for efficient elimination of potential pathogens (Adamo, 2012).

The common defence mechanisms used by most invertebrates are phagocytosis, production of ROS and nitrogen radicals, synthesis and secretion of antibacterial and antifungal proteins, cytokine-like proteins, hydrolytic enzymes,

agglutination and nodule formation, encapsulation of foreign objects, activation of enzymatic cascades that regulate melanization and coagulation of haemolymph. These functions are generally carried out by free circulating cells (haemocytes, coelomocytes) in the blood (haemolymph, coelomic cavity) (Canesi and Prochàzovà, 2013).

Molluscs also possess a natural immunity formed by anatomical and chemical protective barriers that prevent damage of the underlying tissues, body fluid losses and the infections of pathogenic microorganisms and parasites. The main physical barrier is the shell and mucus layer which covers the soft body. Glinski and Jarosz (1997) while studying the molluscan immune defences suggested that the integrity of body coverings is supported by blood clotting and wound healing. The internal defence mechanisms of molluscs involve such cellular reactions as: phagocytosis, nodule formation, encapsulation, pearl formation, atrophy, and necrosis and tissue liquefaction. Granular haemocytes are the most numerous cell type of molluscan blood active in cellular defence and are considered mature cells being capable of phagocytosis, ROS and NO production, release of hydrolytic enzymes and antimicrobial peptides (Canesi et al., 2012). Numerous and large intruders are eliminated by nodule formation or encapsulation, either cellular or humoral. Humoral components of molluscan immunity are formed by lysozyme activity, lectins and the phenyloxidase system (Glinski and Jarosz, 1997).

1.4.3 Mechanisms of cellular defence and biomarkers of oxidative stress in invertebrates

The cellular defence mechanism in invertebrates involves the detoxification of endogenous and exogenous organic compounds by a suite of enzymes mainly enclosed in phase I (or functional reactions) and phase II (or conjugative reactions) of biotransformation (Livingstone, 1991). Molecular biomarkers are used to test the oxidative damage of biomolecules and various aspects of oxidative stress by free radicals. In addition to using primary and secondary products of free radical damage, biomarkers can monitor the status of various antioxidant defence mechanisms against free radicals.

1.4.4 Antioxidant enzymes

In living organisms the antioxidant defence system is divided into enzymatic antioxidants, like superoxide dismutase, catalase, glutathione peroxidase, and nonenzymatic antioxidants, like glutathione, vitamin E, ascorbate, β-carotene, and urate (De Zwart et al., 1999; Valavanidis et al., 2006). In the present study the antioxidant enzymes catalase, glutathione peroxidase, glutathione reductase and glutathione-S-transferase were studied along with metallothionein as metal scavenging protein and lipid peroxidation. The assessment of these antioxidant enzymes in mussels has been shown to be a suitable tool to monitor environmental pollutants (Box et al., 2007; Cheung et al., 2002; Lionetto et al., 2003; Regoli and Principato, 1995). A brief description of the antioxidant enzymes is given below.

1.4.5 Glutathione S-transferases

Glutathione S-transferases (GSTs) are the enzymes of phase II reaction, induced in the cell for biotransformation and excretion of lipophilic xenobiotic molecules such as oils and hydrocarbons polychlorinated biphenyls, polycyclic aromatic hydrocarbons and organochlorine and organo phosphorus pesticides (Damiens et al., 2007; Narbonne et al., 1991; Trisciani et al., 2012). GSTs enable the excretion of organic compounds by the enhancement of their polarity through conjugation with glutathione (Fitzpatrick et al., 1995). This enzyme family is also involved in the metabolism of glutathione which plays important role in non-enzymatic antioxidant system and fights against oxidative stress (Prohaska, 1980; Valavanidis et al., 2006). GST is the key enzyme of cellular defence and used as a biomarker of stress (Benali et al., 2015; Schmidt et al., 2013). It has been used as an indicator of increased phase II biotransformation reactions in mussels (Gowland et al., 2002; Moreira and Guilhermino, 2005; Richardson et al., 2008; Rocher et al., 2006).

1.4.6 Superoxide dismutase

Superoxide dismutase **(**SOD) is another important antioxidant enzyme ubiquitously present in nearly all the cells exposed to oxygen. SOD catalyzes the dismutation of superoxide anion radicals to hydrogen peroxide and molecular oxygen. In the SOD mediated catalytic reaction of superoxide anion the oxidation state of the metal cation oscillates between n and n+1 as shown below:

$$M^{(n+1)+}\text{-SOD} + O_2^{-} \rightarrow M^{n+}\text{-SOD} + O_2$$
$$M^{n+}\text{-SOD} + O_2^{-} + 2H^{+} \rightarrow M^{(n+1)+}\text{-SOD} + H_2O_2$$

Where M = Cu (n=1); Mn (n=2); Fe (n=2); Ni (n=2)

There are three major families of superoxide dismutase, depending on the metal cofactor: Cu/Zn (which binds both copper and zinc), Fe and Mn types (which bind either iron or manganese), and the Ni type, which binds nickel. SOD activity is positively correlated with metal concentration in the surrounding medium (Fonseca et al., 2011) and measured as index of antioxidant status of the organism (Avci et al., 2005).

1.4.7 Catalase

Catalase (CAT) is a major intracellular antioxidant enzyme which is present in all living organisms to provide defence systems against radicals produced by oxidizing ambient pollutants causing oxidative stress (Damiens et al., 2004; Roméo et al., 2003). It is a peroxisomal hydroperoxidase playing an essential role in the degradation of H_2O_2 to H_2O and O_2. H_2O_2 is a precursor of the hydroxyl radical that is reactive with O_2 causing DNA damage (Halliwell, 1999). CAT is remarkable in its function; its turnover rate is very high, with a single enzyme molecule converting millions of H_2O_2 molecules per second. It is active at a broad pH range from pH 4 to pH 11 depending on the species. The abundance of CAT and its ability to function at a broad pH range (pH 4 to 11 depending on the species explains its important role in antioxidant defence in aquatic molluscs

(Benali et al., 2015; Pampanin et al., 2005; Valavanidis et al., 2006). The reaction of catalase in the decomposition of H_2O_2 in living tissue is as follows:

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

The reaction is believed to occur in two stages:

$$H_2O_2 + Fe^{(III)}\text{-}E \rightarrow H_2O + O{=}Fe^{(IV)}\text{-}E^{(+)}$$
$$H_2O_2 + O{=}Fe^{(IV)}\text{-}E^{(+)} \rightarrow H_2O + Fe^{(III)}\text{-}E + O_2$$

Where, Fe represents the iron centre of the haem group attached to the enzyme.

1.4.8 Glutathione peroxidase

Glutathione peroxidase (GPx) is an important enzyme of the peroxidases group that reacts both with inorganic peroxides (H_2O_2) as well as with organic peroxides (lipid hydroperoxides) generated in the cell in association with glutathione oxidation. Lipid hydroperoxides are converted to alcohols and H_2O_2 to water; thereby protecting the cell from oxidative damage. Alterations in GPx levels under environmental exposure (Gomes et al., 2012) reflect that cysteine thiol groups present in enzyme proteins that react with oxidizing species and contribute to antioxidant defence are modified (Hansen et al., 2009). There are several isoforms of GPx, out of which GPx1 is most abundant in the cytoplasm which acts on free H_2O_2, whereas GPx4 acts on lipid hydroperoxides. The main reaction that glutathione peroxidase catalyzes is:

$$2GSH + H_2O_2 \rightarrow GS{-}SG + 2H_2O$$

Where GSH represents reduced glutathione monomer, and GS–SG represents glutathione disulphide. The mechanism involves oxidation of the selenol of a selenocysteine residue by H_2O_2 producing derivative with a selenenic acid (RSeOH) group. The selenenic acid is then converted back to the selenol by a two-step process utilizing GSH to form the GS-SeR and water. The GS-SeR intermediate is converted back to selenol by utilizing another GSH

molecule releasing GS-SG as the by-product. A simplified representation is shown below:

$$RSeH + H_2O_2 \rightarrow RSeOH + H_2O$$
$$RSeOH + GSH \rightarrow GS\text{-}SeR + H_2O$$
$$GS\text{-}SeR + GSH \rightarrow GS\text{-}SG + RSeH$$

1.4.9 Glutathione reductase

Glutathione reductase (GR) is widely distributed in all organisms that generate reduced glutathione (GSH) in the cell from its oxidized form glutathione disulphide (GSSG), which is essential for the maintenance of cellular homeostasis and the scavenging of nucleophilic compounds (Manduzio et al., 2005). Alterations in GR activity can cause a disturbance to the reduction of GSSG under oxidative conditions (Mitozo et al., 2011; Trevisan et al., 2014). The reduction of GSSG to the sulfhydryl form GSH is an energy dependent reaction and requires one mole of NADPH for every mole of oxidized glutathione (GSSG) to reduce it to GSH. The enzyme forms an FAD-bound homodimer. The reaction equation is as given below:

$$GS\text{–}SG + NADPH + H^+ \rightarrow 2\ GSH + NADP^+$$

1.4.10 Metallothioneins

Metallothioneins (MTs) are low molecular weight proteins that are rich in cysteine and possess a high affinity to bind metals and represent important cellular defences against toxic effects caused by metals (Benali et al., 2015; Viarengo et al., 1997). Metallothioneins in mussels have been reported to be involved in metal homeostasis and over-expressed in organisms experiencing high metal concentrations in their environment (Viarengo and Nott, 1993). MTs have also been reported to be part of the antioxidant defence system of the cells through the scavenging of ROS (Fernandez et al., 2012; Viarengo et al., 1999; Viarengo et al.,

2000). The level of metallothionein-like proteins has been used as a biomarker for metal contamination in the aquatic environment.

1.4.11 Lipid peroxidation

Lipid peroxidation is a process of oxidative degradation of lipid-rich membranes caused by the reactive oxygen species (ROS) (Elsaesser and Howard, 2012). In the cell, ROS interact with polyunsaturated fatty acids and produce an unstable fatty acid radical which quickly interacts with molecular oxygen to produce lipid peroxide and another unstable peroxy fatty acid radical. The peroxy fatty acid radical reacts with free fatty acids to produce lipid peroxide and a lipid radical thus a cyclic oxidation process is initiated. The mechanism of LPO involves initiation, propagation and termination and is explained in Figure 1.6.

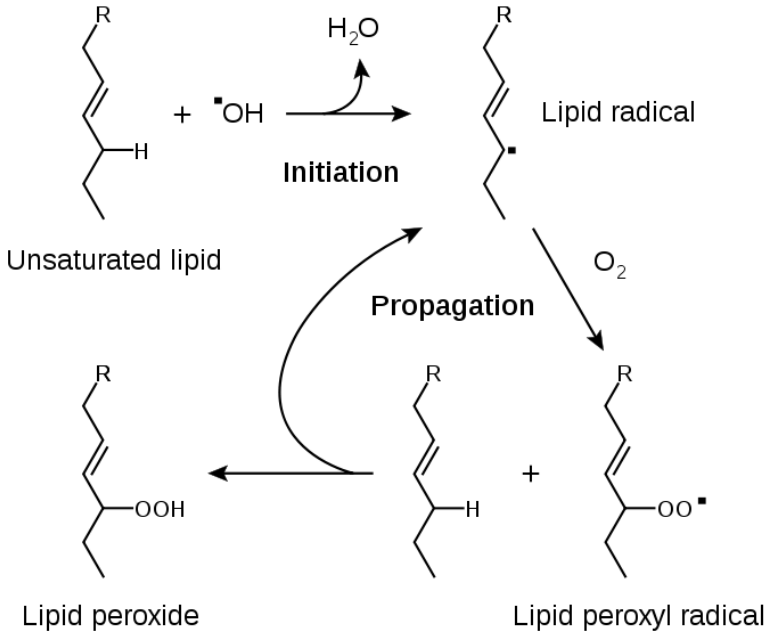

Figure 1.6. Mechanism of lipid peroxidation

Initiation is the step in which a fatty acid radical is produced by interacting with ROS, such as OH· and HOO·, which combines with a hydrogen atom to make water and a fatty acid radical. In the propagation step, the fatty acid radical reacts readily with molecular oxygen, thereby creating a peroxyl-fatty acid radical. This

radical being an unstable species reacts with another free fatty acid, producing a different fatty acid radical and lipid peroxide, or cyclic peroxide if it had reacted with itself and this cycle continues. In the termination step, when a radical reacts with a non-radical, it always produces another radical, which is why the process is called a "chain reaction mechanism". In living organisms different antioxidant molecules like vitamin E and antioxidant enzymes tend to catch free radicals that speed up termination to protect the cell membrane. The most common method of detection of cellular damage is the measurement of the end product of lipid peroxidation, malonaldehyde by reacting with thiobarbituric acid (TBA) (Buffet et al., 2013a).

1.5 Toxicity of Nanoparticles to Aquatic Organisms

For aquatic invertebrates, the ecotoxicological potential of ENPs depends on their inherent characteristics (Schaumann et al., 2015) stemming from particle size, shape, and surface area (Crane et al., 2008; Navarro et al., 2008). Though detailed toxicity mechanisms caused by ENPs are yet to be elucidated, a few mechanisms like ROS production, protein destabilization and oxidation, damage to membrane integrity and nucleic acids, release of harmful and toxic components, and interruption of energy transduction are likely involved (Klaine et al., 2008). Cell membranes are potential ENP targets. ENPs induce heat shock responses in *Escherichia coli* by weakening the membrane (Hwang et al., 2007). It has also been suggested that ENPs are probably involved in tumour formation through DNA damage in lungs (Bhatt and Tripathi, 2011).

ENPs stimulate ROS production that can potentially cause damage in every cell component. Oxidation of the double bonds of fatty acids by ROS in cell membranes results in increased permeability rendering the cell more susceptible to osmotic stress. Toxicity reports are available for n-TiO_2 to fish, invertebrates and algae (Menard et al., 2011). n-TiO_2 with photocatalytic properties have been shown to generate generate ROS upon exposure to UV radiation (Kus et al., 2006; u Zhao et al., 2007) and even in the absence of UV. This has also been confirmed in rainbow trout (*Oncorhrynchus mykiss*), where n-TiO_2 caused inflammatory injury resulting in respiratory distress (Reijnders, 2008). The damaging effects of

n-TiO_2 on bacteria were enhanced in the presence of sunlight or UV light (Adams et al., 2006) and toxic effects have also been observed in algae (Kahru and Dubourguier, 2010; Kim et al., 2007; Klaine et al., 2008). ROS production can lead to DNA cross-linking, strand breaks, and adducts of the bases or sugars (Cabiscol et al., 2010).

Inorganic nanoparticles are known to release harmful components, such as metal ions, which cause toxicity (Klaine et al., 2008); however, the rate of dissolution depends on the size and chemical nature of the nanoparticles, temperature of the surrounding environment, salinity and the pH (Hardman, 2006; Meulenkamp, 1998). The metals released from ENPs cause damage at various levels in the cell due to long retention time. n-Ag can release silver ions into the environment which are bioavailable and highly toxic to marine organisms ranging from prokaryotes right through to higher vertebrates (Bianchini et al., 2002; Bilberg et al., 2010; Choi et al., 2009; Choi and Hu, 2008; Erickson et al., 1998; Fabrega et al., 2009a; Fabrega et al., 2009b; Griffitt et al., 2009; Miao et al., 2009; Navarro et al., 2008; Scown et al., 2010; Yeo and Yoon, 2009) and toxicity was observed in most cases. Due to the chemical properties of silver ions, uptake through the cell membrane ion transporters is possible (Luoma, 2008). N-Ag can also enter the cell through endocytosis as shown in the mussel *Mytilus edulis* (Moore, 2006). Ionic silver interact with the thiol groups of vital enzymes resulting in their inhibition (Matsumura et al., 2003). Silver ions are inhibitory to certain respiratory enzymes and induce ROS production (Kim et al., 2007). Further, silver ions also have a tendency to bind with phosphorus- and sulphur-containing antioxidant molecules (Pappa et al., 2007), thus depleting their concentration and weakening the cells' antioxidant defence system (Hussain et al., 2005). Reports show the involvement of silver ions in affecting DNA replication and cell membrane permeability (Feng et al., 2000). These nanoparticles attack the respiratory chain and affect the cell division ultimately leading to cell death (Rai et al., 2009).

The toxicity of metallic nanoparticles is mediated by three distinct mechanisms; i) release of toxic metal ions from nanoparticles into the aqueous phase, ii) production of toxic chemical radicals or reactive oxygen species by

surface interactions, and iii) a direct interaction of particles with macromolecules like DNA or surface reactivity of particles with membrane causing disruption of biological targets (Brunner et al., 2006). In aquatic systems, n-ZnO exert toxic effects though all the three possible mechanisms since it gets partially dissolved in water and Zn^{2+} contributes substantially to the cytotoxicity along with direct effect of particulate species (Brunner et al., 2006; Heinlaan et al., 2008). In addition to that ZnO promotes generation of ROS under irradiation with energy at or above its energy band gap due to its photochemical reaction and thereby causes a significant toxicity to exposed organisms (Ma et al., 2009; Ma et al., 2011; Navarro et al., 2008). Several reports are available on the toxicity of ZnO-NPs on freshwater crustaceans such as *Daphnia magna* (Blinova et al., 2010; Heinlaan et al., 2008; Wiench et al., 2009; Zhu et al., 2009) and *Thamnocephalus platyurus* (Blinova et al., 2010; Heinlaan et al., 2008). The toxicity of n-ZnO (50-70 nm) determined as 48h LC_{50} values reported by Heinlaan et al. (2008) was 3.2 mg/l and 0.18 mg/l to *D. magna* and *T. platyurus*. In another study Blinova et al. (2010) found 48 hours an EC_{50} of 2.6 mg/l for *D. magna* and 24 hours LC_{50} of 0.14 mg/l for *T. platyurus*. In both studies n-ZnO toxicity was attributed to Zn^{2+} as suggested by recombinant Zn-sensor bacteria. In the later study, n-ZnO toxicity in natural waters with dissolved organic carbon content 13.2-34.5 mg/l was compared with artificial Milli Q water that does not contain organic matter, and the results suggested that organic matter within the natural river water did not significantly affect n-ZnO toxicity. Similar conclusions were drawn by studies with Zn sensor bacteria which indicated small differences in bioavailable Zn^{2+} concentrations in artificial and natural waters. Comparison of nanoscale (<200 nm) and bulk, non-nanoscale ZnO (<1000 nm) in several different formulations to *D. magna* in three different test media revealed 48 hours EC_{50}s close to 1 mg/l (Wiench et al., 2009). The toxicity of ZnO was found independent of the particle size, or coating of particles or the type of medium or applied dispersions procedures like stirring, ultrasonication, or a combination of both. Chronic effects of n-ZnO on growth and reproduction in *D. magna* have not been reported. However, marine amphipod *Corophium volutator* chronically exposed to waterborne n-ZnO (35 ± 10 nm) at 1 mg/l registered a significant change in survival, growth, and reproduction and the toxicity was not only due to the solubilized Zn^{2+} (Fabrega et al., 2011; Ma and Lin, 2013).

Copper is one of numerous indispensable elements required to maintain organismal homeostasis (Galhardi et al., 2005). Cu ions, once they exceed the physiological tolerance range *in vivo*, may cause toxicity (Galhardi et al., 2004). Therefore, the toxicology of n-CuO and the possible health effects are a cause for concern. Research into n-CuO toxicity has mostly focused on aqueous environments. The most common experiment models, whose growth and toxicity are treated as environmental relevance indicators are algae and zebrafish. The toxicity of n-CuO to the chlorophyte *Pseudokirchneriella subcapitata* was compared (Aruoja et al., 2009) with bulk formulation of the metal oxide as a control and the data revealed that low concentrations of n-CuO (EC_{50} = 0.71 mg Cu/L) were more soluble and more toxic than the controls (EC_{50} = 11.55 mg Cu/L). The results showed that the solubility of the Cu ions determined the toxicities of bulk and n-CuO to *P. subcapitata* as observed in zebra fish (Griffitt et al., 2007; Grosell et al., 2007).

Exposure to n-CuO decreased the chlorophyll content of duckweed and the toxicity was three to four times higher than that exposed to its ionic form (Shi et al., 2011). With higher uptake of n-CuO the release of copper ion was also increased. However, Griffitt et al. (2007) tested the responses of fish exposed to n-CuO and soluble Cu and it was concluded that the effects on gill morphology and global gene expression pattern were not solely due to the dissolution of n-Cu. In some other studies, n-CuO suspensions caused damage in gill lamellae and inhibited epithelial cell proliferation as well as chloride cell number and diameter (Griffitt et al., 2007; Pelgrom et al., 1995). It is considered that the effect was due to aggregation of n-CuO in the digestive gland of mussel (Gomes et al., 2012). Adverse effects of n-CuO on bacteria revealed that dissolved Cu^{2+} induced toxic effects by triggering ROS production that caused DNA damage (Bondarenko et al., 2012; Chang et al., 2012).

In aquatic invertebrates the success of early life stages is highly affected by adverse environmental conditions. The presence of nanoparticles in the exposure medium has shown developmental abnormalities in aquatic organisms (Browning et al., 2013; Massarsky et al., 2013; Ribeiro et al., 2014). Studies on rainbow trout exposed to silver nanoparticles at different life stages revealed that

the fish were more sensitive to colloidal silver nanoparticles during the early life stage than in later stages. The author also suggested that the release of colloidal silver nanoparticles into the environment or their direct application as an antifungal/bacterial medication during the hatching period of eggs or larval stages should be avoided as they are very toxic/toxic to the eleutheroembryo-larva stages and juvenile stage of rainbow trout (Johari et al., 2013). Similar responses were observed where eggs, larvae, juveniles and adults of *Platynereis dumerilii* were exposed to different formulations of silver (Garcia-Alonso et al., 2014). The study suggested, mortality and abnormal development rate increased with younger life stages, while adults and juvenile were the most tolerant life stages and fertilized eggs were highly sensitive to $AgNO_3$, citrate coated n-Ag and humic acid n-Ag. Exposures to humic acid –Ag nanoparticles triggered the highest acute toxicity responses in *P. dumerilii* and in most cases both AgNPs were more toxic than $AgNO_3$. Uptake rate of humic acid-AgNPs in adult worms was also higher than from other Ag forms, consistent with toxicity to other life stages. A study on the zebrafish embryos with nano silver and also reported that the earlier developmental stage embryos were more sensitive to developmental abnormalities and the effects were dose-dependent (Browning et al., 2013). Distinctive phenotypes (head abnormality and no eyes) observed in cleavage and early gastrula stage embryos treated with the nanoparticles, shows the stage-specific effects of the nanoparticles. The study also suggests that the embryonic phenotypes strikingly depend upon the sizes of n-Ag and embryonic developmental stages. The effects of n-Ag and silver ions on zebrafish development suggested that Ag+ was more toxic than n-Ag but both lead to death and delayed hatching in surviving embryos (Massarsky et al., 2013).

1.5.1 Bivalve molluscs as test organisms

Bivalve molluscs belong to one of the largest invertebrate phyla, the Mollusca (Helm, 2004). The phylum comprises more than 50,000 living species. Molluscs are soft bodied and, in the main, have a prominent shell to protect the inner body mass. They are mostly aquatic and their habitats range from the deep ocean through to moist terrestrial niches. There are six classes of molluscs namely: Amphineura, Monoplacophora, Gastropoda, Pelecypoda (Bivalvia),

Scaphopoda, Cephalopoda. They are a major component of the macrofauna of many aquatic ecosystems (Huber, 2010).

Ecological significance of bivalves

The role of suspension-feeding bivalves in estuarine and marine ecosystems has been extensively documented through research in ecology, physiology, biogeochemistry, mariculture, interdisciplinary marine science, and fisheries science. Suspension-feeding bivalve molluscs consume at the lowest trophic level, feeding largely as herbivores (Duarte et al., 2008). On average an oyster filters 15-55 litres of water/day and releases dissolved ammonia and other nutrients either directly or through microbial decomposition of their pseudofaeces. Bivalves sequester nitrogen and produce protein in muscle tissues, and sequester carbon as $CaCO_3$ in their shells. They are considered as key-stone species controlling phytoplankton density by grazing and also nutrient removal through biodeposits. Considering their significance, bivalve aquaculture for edible species and non-edible species like pearl oyster for bioremediation of polluted sites have been explored (Gallardi, 2014).

1.5.2 Biogeochemical cycling

Benthic suspension feeders are intermediary between benthic and pelagic systems (Dame, 1993). Their functional activity to filter large volumes of water containing particles of different size and nature contributes to nutrient release into the water column and excretion of organic waste that is deposited in the coastal sediment. The nutrient dynamics is influenced either by direct excretion or indirectly through remineralisation of their organic deposits in the sediments by microbial activity (McKindsey et al., 2006). This benthic-pelagic coupling ability of bivalves that affect turbidity and nutrient re-mineralization in water columns influences the primary productivity of the area (Dame and Olenin, 2005). Such processes have generally a positive influence on the overall water quality of a system (Dame, 2005).

Bivalves also contribute to biogeochemical process by trapping carbon in the form of calcium carbonate in their shell. The dead shells are incorporated in various structures in estuarine, coastal, and oceanic systems and contribute to ecosystem complexity and erosion reduction (Coen and Grizzle, 2007). It also contributes to sedimentary carbonate content. This aspect is important especially in relation to global warming and increasing atmospheric CO_2 concentration resulting in ocean acidification and changes in seawater carbonate chemistry. It has been observed that increase in acidity negatively affects the growth, development and survival of bivalves (Fabry et al., 2008; Kurihara, 2008).

1.5.3 Bivalves as filter-feeders

Bivalve molluscs are of considerable ecological importance. As filter feeders they process large volumes of water and thus exert a considerable impact on their environment (Charles et al., 1999). Their filter-feeding ability adds greatly to their ecological significance in that bivalves are important calcium and carbon accumulators, they link primary producers (bacteria and phytoplankton) with higher organisms in aquatic food chains and are responsible in tidal zones for filtration of the water body (Canesi et al., 2012; Rocha et al., 2015). The process of filtering particles from the environment is initiated when water passes across the gills. It has been estimated that one kilogram of bivalves (multiple sizes) will filter 180 litres of water per hour (Lei, 1993). Filter feeding affects both the water column from which food and other suspended particles are removed, and the sediment to which faeces and pseudofaeces are deposited. Pseudofaeces are undigested particles that are rejected by the bivalves. Zebra mussels (*Dreissena* sp.) for instance, are able to reject excess particles in order to regulate ingestion when the concentration of suspended particles is above a threshold level (Lei, 1993). The filtering activity of bivalves is influenced by the size of the organism, phytoplankton concentration, and the size and quality of the food/suspended particles. Abiotic factors such as temperature, salinity and water flow can also affect filtration rates (Rajesh et al., 2001).

1.5.4 Environmental remediation

Bivalves have been explored as a coastal, ocean ecosystem restoration tool especially the pearl oyster. The pearl oysters have been recommended for such studies considering their values for obtaining pearls rather than the meat (Gifford et al., 2005). The authors recommend that different species of pearl oysters can be cultured in a polluted location and oysters with accumulated contaminants can be harvested to clean the area from contaminants. They can be used as the natural filter cleaning tools for the remediation of polluted water and sediment.

1.5.5 Bivalve as "biomonitoring tools"

Bivalves are useful "biomonitor" and ecotoxicology study species due to their propensity to bioaccumulate environmental contaminants (Gagne et al., 2008; Vlahogianni et al., 2007). The most commonly used bivalve species are the mytilids mussels of the genus *Mytilus* (*M. edulis, M. galloprovinciallis, M. trossulus*). Their world-wide distribution, important roles in coastal ecology (Craft et al., 2010), occurrence across regions of different pollution status and relatively sessile nature (Mitchelmore and Chipman, 1998) make them ideal monitor species to assess the impacts of marine pollutants (e.g., Mussel Watch) (Goldberg and Bertine, 2000).

1.5.6 Bivalves as indicators of metal pollution

The use of mytilids has been proposed for routine biomonitoring programmes to evaluate the spatial and temporal presence of pollutants, including metals in coastal environments. Molluscs accumulate metals from their food and the surrounding seawater in concentrations that considerably exceed those found in their natural environment (Viarengo et al., 2007).

Common polluting metals include As, Cd, Cr, Co, Cu, Pb, Hg and Ni. They are natural constituents of aquatic environments either from decaying vegetation, volcanic activity and windblown dust, and are generally found in low concentrations. Anthropogenic activities have increased the levels of metal ions in water systems. Principally, domestic and industrial effluents have increased the

metal load in coastal waters that finally gets deposited into marine sediments (Al-Subiai et al., 2011; Ansari et al., 2004; Rainbow, 2002). Metal toxicity depends on chemical speciation, bioavailability and accumulation in the environment. Various metals give rise to a number of chemical species upon dissolution and on reaction with the anions in seawater at different pH levels (Ansari et al., 2004). It is primarily in the ionic form that metals become bioavailable. Bioavailability refers to a portion of the chemical that is potentially available to an organism for biological uptake. The bioavailability of metals also depends on the behaviour and physiology of the organism (Rand et al., 1995).

Metals exist as dissolved or particulate phases in marine environments. Due to the solubility phenomenon, dissolved metals can be taken up by aquatic organisms across their exposed tissues and through feeding (Chapman et al., 1998). The uptake of metals as particulates may also take place through facilitated diffusion, or via permeable surfaces including the gut and respiratory surfaces (Depledge and Rainbow, 1990; Wang, 2001). The dissolution of metals thus promotes bioavailability, bioaccumulation and toxicity of metals (Bat and Raffaelli, 1998; Bryan and Langston, 1992).

When assessing metal toxicity to marine organisms, it is important to consider the absorption efficiency of the dissolved metals (Wang and Fisher, 1996). Metals bind to soft sediments and are subsequently released from sediment particles depending on salinity and pH (Hatje et al., 2003). Metals present in pore water are readily available to filter feeders whereas those associated directly with sediment are the main exposure route for deposit-feeders (King et al., 2004). Toxic chemicals in water or bound to suspended particulate matter may accumulate in the tissues of filter feeders and may cause biochemical and physiological changes. Once inside the body, the organism must excrete and/or detoxify these metals to avoid possible toxic effects (Rainbow, 2002). Metals are potentially toxic if the concentration is in excess for metabolic requirements and storage capacity (Depledge and Rainbow, 1990).

1.5.7 Toxicity studies of nanoparticles to bivalves

Suspension-feeding invertebrates, particularly bivalve molluscs, may represent a unique target group for nanoparticle toxicology (Moore, 2006). These organisms have highly developed processes, endocytosis and phagocytosis for the cellular internalization of nano and microscale particles that are essential for key physiological functions such as intracellular digestion and cellular immunity. Bivalves can filter large volumes of water, processing microalgae, bacteria, sediments, particulates, and natural nanoparticles, potentially accumulating different chemicals in their tissues. Food particles trapped by the gill sieve move towards the labial palps and the mouth thus entering the gut, and reaching the digestive gland, where digestion occurs. Digestive cells have an extremely developed lysosomal system for intracellular digestion and nutrient accumulation for gametogenesis (Gosling, 1992). In bivalves, the innate immune response is carried out by the blood cells, the haemocytes, and soluble haemolymph factors that operate in a co-ordinated way to provide protection from invading microorganisms (Canesi et al., 2002).

In 2011, when the current study was initiated, there were few reports available on the toxicity of nanoparticles to bivalves. Of those, only data on nanocarbon black (Canesi et al., 2008), quantum dots, CdTe (Gagne et al., 2008), gold nanoparticles (Tedesco et al., 2008), C60 Fullerene (Ringwood et al., 2009), and fluorescent polystyrene beads (Ward and Kach, 2009) were accessible. Subsequently, data has become available for a broader range of nanoparticles used for *in vivo* and *in vitro* toxicity testing. However, little information was available on the toxicity of the nanoparticles chosen for the present study (n-TiO_2 and n-Ag) to terrestrial, sedentary and marine species prior to 2007 (Menard et al., 2011). Several published reports on *in vivo* and *in vitro* exposures with various nanoparticles to mussel haemocytes stated ROS production and oxidative stress (Barmo et al., 2013; Canesi et al., 2010a; Canesi et al., 2010b; Ciacci et al., 2012). The studies showed that nanoparticles induced lysosomal membrane instability and the activity levels of antioxidant enzymes were raised in the haemocytes and digestive glands. There was a range of indicators of oxidative damage induced by ENPs in bivalves, notably the breakdown of antioxidant defence systems

including catalase (CAT), glutathione peroxidase (GPx), glutathione-S-transferase (GST) and superoxide dismutase (SOD) (Ali et al., 2012; Barmo et al., 2013; Gomes et al., 2012; Gomes et al., 2014b; Gomes et al., 2011; Zhu et al., 2011), cytoskeleton disorganization (down- and up-regulation of cytoskeleton proteins) (Gomes et al., 2014a; Gomes et al., 2013b), lipid peroxidation, protein oxidation (increased protein carbonylation or a decrease of thiol-containing proteins) (Tedesco et al., 2010a, b), mitochondrial disruption (Trevisan et al., 2014) and DNA damage (DNA strand breaks) (Ali et al., 2012; Gomes et al., 2013a; Katsumiti et al., 2014; Munari et al., 2014; Rocha et al., 2014).

The bivalve immune system is a sensitive target for ENP toxicity. *Mytilus* haemocytes are the cell type most investigated in both *in vitro* and *in vivo* exposures. Upon exposure and after crossing the epithelium of the digestive gland tubules, haemocytes can uptake ENPs through endocytic pathways or via cell-surface lipid raft associated domains named caveolae (Moore, 2006; Moore et al., 2009). Generally, ENPs induce ROS production that leads to changes in the immune system due to inflammatory processes (reduction in phagocytic activity and haemocyte viability). Changes in phagocytosis activity, cell viability/density, stimulation of lysosomal enzyme release, ROS production, lysosomal membrane stability (LMS) damage, mitochondrial damage, DNA damage and pre-apoptotic processes were observed in haemocytes after exposure to different ENPs, such as nanocarbon black (NCB), C60 fullerene, different nanoparticle oxides (TiO_2, SiO_2, ZnO, CeO_2) and quantum dots (CdTe; CdS/CdTe) (Barmo et al., 2013; Canesi et al., 2008; Canesi et al., 2010a; Canesi et al., 2010b; Ciacci et al., 2012; Couleau et al., 2012; Gagne et al., 2008; Katsumiti et al., 2014; Moore et al., 2009; Rocha et al., 2014).

Considering the genotoxic effects of ENPs, DNA is considered as a highly susceptible cellular target for oxidative damage. Assessing DNA damage after exposure to ENPs is extremely important in nanotoxicological assessment due to the importance of DNA in maintaining cellular homeostasis and transmission of genetic information between generations. Genotoxicity of CuO (31 nm; 10 mg/l) and n-Ag (42 nm; 10 mg/l) are mediated by oxidative stress and both nanoparticles showed lower genotoxic effects than their soluble forms in *M. galloprovincialis* after 15 days of exposure (Gomes et al., 2013a). On the other

hand, no genotoxic effects were observed in *Macoma balthica* after exposure to sediment spiked with n-Ag (20, 80 nm) and n-CuO (<100 nm) (200 mg/g d.w. sed.; 35 days) (Dai et al., 2013). In a mesocosm study, similar genotoxicity of n-CuO (29.5 nm) and soluble Cu was observed in *Scrobicularia plana* after 21 days of exposure at 10 mg/l (Buffet et al., 2013b). DNA damage induced by Cd-based quantum dots was observed in mussel haemocytes after *in vitro* exposure to CdS (4 nm; 10 mg/l; 4 hours) (Munari et al., 2014) and CdS quantum dots (5 nm; 0.001-100 mg/l; 24 hours) (Katsumiti et al., 2014), and *in vivo* exposure to CdTe quantum dots (6 nm; 10 mg/l; 14 days) (Rocha et al., 2014). Furthermore, similar genotoxicity was observed in the freshwater mussel *Elliptio complanata* exposed to CdTe quantum dots (1.6-8 mg/l; 24 hours) (Gagne et al., 2008). ENPs can also alter their genotoxic potential when adsorbed to other pollutants, as shown in *Mytilus* haemocytes exposed to C60 (100-200 nm; 0.1-1 mg/l) and polycyclic aromatic hydrocarbons (PAHs; fluoranthene) where an additive effect was observed after 3 days exposure (Al-Subiai et al., 2012).

The effect of ENPs on developmental toxicity was only investigated for n-TiO_2 and zero-valent nano iron in *M. galloprovincialis* (Balbi et al., 2014; Kadar et al., 2011; Libralato et al., 2013). Exposure to natural light increased the embryo toxicity of n-TiO_2 by increasing the frequency of retarded larvae compared to malformed ones (24 nm; 0-64 mg/l; 48 hours) (Libralato et al., 2013). On the other hand, n-TiO_2 (alone or in combination with Cd^{2+}) did not affect mussel larval development at 100 mg/l (Balbi et al., 2014). However, the gametes and early developmental stages of bivalves are potentially more susceptible to toxic effects of ENPs when compared to later development or adult stages (Kadar et al., 2011).

Behavioural biomarkers, such as burrowing, feeding rate and valve opening, are indicated as important tools to assess ENP toxicity in bivalves (Buffet et al., 2011; Pan et al., 2012; Wegner et al., 2012). The behaviour of bivalves exposed to ENPs depends on the size, composition and concentration of ENPs, mode and time of exposure, and species. Data for these types of biomarkers mainly exists for the clam *S. plana*. Large n-Au induced stronger inhibition of burrowing kinetics in *S. plana* when compared to smaller ones (Pan et al., 2012).

Burrowing was also modified after exposure to CuO and n-^{67}ZnO (Buffet et al., 2012; Buffet et al., 2011). Furthermore, the exposure route is an important approach to assay the behavioural responses in bivalves exposed to ENPs. Dietary exposure reduces the clearance rate in *S. plana* more than waterborne exposure to n-Ag (40-50 nm; 10 mg/l; 14 hours) (Buffet et al., 2013b). In addition, *M. edulis* also reduced their filtering activity after exposure to nanopolystyrene (30 nm; 0.1-0.3 g/l; 8 hours) (Wegner et al., 2012).

In many studies isolated haemocytes were used for cytotoxicity or whole animal exposure was conducted to determine organ injury or immune competence. In most exposure studies the digestive system represented a major target for nanoparticle toxicity. Similar to metals, nanoparticles generally affected oxidative stress in target organs. The intensity of injury was dependent on the concentration and exposure periods. In most cases even short term exposure at very low exposure levels induced similar injury in the exposed organism. A summary of key *in vivo* and *in vitro* studies are given in Table 1.7.

1.5.8 Bivalves used in the present study: *Amiantis umbonella* (Lamarck, 1818)

Amiantis umbonella (phylum; Mollusca, class; Bivalvia, sub-class; Heterodonta, order; Veneroida, superfamily; Veneroidea, family; Veneridae, genus; *Amiantis*, species; *Amiantis umbonella*) occurs on the sandy/ muddy intertidal zones throughout Asia where it is a source of cheap protein. The shell is

Table 1.7. Summary of key *in vivo* and *in vitro* metal nanoparticle studies on bivalve molluscs. Bulk = bulk material; ↑ = increase; ↓ = decrease; HSP = heat shock protein; GST = glutathione-S-transferase; BM = biomarkers; SOD = superoxide dismutase; CAT = catalase; GPx = glutathione peroxidase; MT = metallothionein; LPO = lipid peroxidation; G = gills; DG = digestive gland; M = muscle; H = haemolymph/haemocyte; AChE = acetylcholine esterase; LMS = lysosomal membrane stability; TLR = toll-like receptor; TCDD = 2,3,7,8-tetrachlorodibenzo-*p*-dioxins; EPF = extrapallial fluid; TBARS = thiobarbituric acid reactive substances; LDH = lactate dehydrogenase; CSP = caspase I presume; ACP = acid phosphatase; CI = condition index; PSH = protein thiol; GSH = glutathione; GSSG = glutathione disulphide.

ENP type	Species	Test Conc.	Exposure period	Effects	Reference
Ag, CuO	*Mytilus galloprovincialis*	10 µg/ l	15 days	Genotoxicity mediated by oxidative stress (nanoparticles > bulk).	(Gomes et al., 2013a)
Ag	*M. galloprovincialis*	10 µg/ l	15 days	↑ Oxidative stress; Proteomic analysis show classical (HSP70, GST, actin) and new BMs (Major vault protein, Ras parcial, Precollagen-P).	(Gomes et al., 2013b)
Ag	*M. galloprovincialis*	10 µg/ l	15 days	↑ SOD, ↑ CAT, ↑ GPx, ↑ MTs, ↑ LPO (G). ↑ Oxidative stress (G > DG).	(Gomes et al., 2014b)
CuO	*M. galloprovincialis*	10 µg/ l	15 days	↑ Oxidative stress; ↑ LPO; ↓ AChE; ↑ MT.	(Gomes et al., 2011)
CuO	*M. galloprovincialis*	10 µg/ l	15 days	↑Oxidative stress; ↑ MT; ↑ SOD, ↑ CAT, ↑ GPx, ↑ LPO (7d)	(Gomes et al., 2012)
CuO	*M. galloprovincialis*	10 µg/ l	15 days	↑ Oxidative stress; proteomic analysis show classical (HSPs, actin, GST, ATP synthase) and new BMs (caspase 3/7-1, catL, Zn-finger).	(Gomes et al., 2014a)
C-60, TiO_2, SiO_2	*M. galloprovincialis*	1, 5, 10 µg/ ml	30 minutes - 4 hours	Lysozyme release; ↑ extracellular oxyradical & NO production; no LMS damage; haemocytes are significant target for ENPs	(Canesi et al., 2010a)
NCB, C60 Fullerene, TiO_2, SiO_2	*M. galloprovincialis*	0.05, 0.2, 1, 5 mg/ l	24 hours	↓ LMS; lysosomal lypofuscin; ↑ CAT; oxidative stress; ↑ GST; DG lysosomal system is significant target for ENPs *in vivo*	(Canesi et al., 2010b)
ZnO	*M. galloprovincialis*	0.1-2 mg/l	84 days	Toxicity (small mussel > larger mussel); ↓ growth; ↓ survival; ↑ respiration rate.	(Hanna et al., 2013)
ZnO	*M. galloprovincialis*	0.1-2 mg/l	84 days	Changes in energy budgets (↓ feeding capacity, ↑ maintenance requirements; ↓ life time for gametogenesis. Maintenance was a primary target of toxicant action.	(Muller et al., 2014)
TiO_2	*M. galloprovincialis*	1-100 µg/ l	96 hours	↓ LMS; ↑ antioxidant and immune-related gene (DG); Yphagocytosis,↑ extracellular O^{2-}production, ↑ nitrite, ↑ transcription of antimicrobial peptides (H).	(Barmo et al., 2013)
TiO_2	*M. galloprovincialis*	10 mg/l	96 hours	Toxicity (nanoparticles < bulk): histopathological and histochemical changes. Genotoxicity (bulk = "fresh" = "aged" nanoparticles).	(D'Agata et al., 2014)
TiO_2	*M. galloprovincialis*	0-64 mg/l	48 hours	Light exposure ↑ embryotoxicity.	(Libralato et al., 2013)
TiO_2	*M. galloprovincialis*	100 µg/ l	96 hours	Co-exposure (nanoparticles + Cd^{2+}). No effects in Cd^{2+} accumulation. ↓ LMS;↓ phagocytosis; ↑ NO production, lysozyme release (H). ↑ MT; Synergistic effects on lysozyme and TLR-i genes	(Balbi et al., 2014)

TiO_2	*M. galloprovincialis*	100 µg/ l	96 hours	Co-exposure (nanoparticles + TCDD): ↑ TCDD accumulation, synergistic and antagonistic effects time dependent, cell/tissue and BMs. Trojan horse effects	(Canesi et al., 2014)
TiO_2	*M. galloprovincialis*	0.1-100 mg/l	24 hours	Toxicity (small nanoparticles > large nanoparticles; nanoparticles > bulk). No relationship between crystal structure and cytotoxicity. Similar sensitivity between H and G cells.	(Katsumiti et al., 2014)
CeO_2, SiO_2, TiO_2,	*M. galloprovincialis*	1-10 µg/ l	4 hours	↓ LMS; ↑ total extracellular oxyradical; ZnO: mitochondrial damage, cardiolipin oxidation. TiO_2 and ZnO in the endosomes (30 min); TiO_2 in the nucleus (60 min); ZnO: pre-apoptotic processes.	(Ciacci et al., 2012)
CeO_2, ZnO	*M. galloprovincialis*	1- 10 mg/ l	4 days	Excretion in pseudofaeces	(Montes et al., 2012)
Ag	*M. edulis*	0.7 µg/ l	3.5 hours	Transport of Ag to EPF is not form dependent. Complexation by organic molecules in the EPF. Haemocytes play an important role in Ag translocation to extrapallial cavity.	(Zuykov et al., 2011a)
Ag	*M. edulis*	0.7 µg/ l	3.5 hours	Change shell calcification mechanism; shell nacre showed doughnut shape structures.	(Zuykov et al., 2011b)
CuO	*M. edulis*	400-10^3 µg/l	1 hours	↑ Oxidation and carbonylation of cytoskeleton and enzyme proteins. ↑ pigmented brown cells (DG, G, Mt); ↓ LMS.	(Hu et al., 2014)
TiO_2	*Perna viridis*	2.5 µg/ l	9 days	Co-exposure (nanoparticles þ hypoxia). Hypoxia ↑ nanotoxicity; Synergistic effects.	(Wang et al., 2014)
Ag	*Scrobicularia plana*	10 µg/ l	14 hours	Toxicity (nanoparticles-soluble form); ↑ SOD, ↑ CAT, ↑ GST, ↓ clearance rates (dietary exposure); no changes in TBARS, LDH, CSP 3-like and burrowing test; no MT induction; no neurotoxicity.	(Buffet et al., 2013a)
Ag	*S. plana*	10 µg/ l	21 days	Mesocosms. Oxidative stress (TBARS), detoxification, apoptosis (CSP-3 like) and immunomodulation (lysozyme) (nanoparticle= soluble form); genotoxicity (DG; nanoparticles > soluble form); ↑ LPO; no MT induction; no neurotoxicity; no changes in LDH, ACP and behaviour.	(Buffet et al., 2014b)
CuO	*S. plana*	10 g/l	16 days	No oxidative stress. ↑ GST, ↑ CAT, ↑ SOD; behavioural impairments (burrowing and feeding behaviour); no neurotoxicity	(Buffet et al., 2011)
CuO	*S. plana*	10 µg/ l	21 days	Mesocosms. No oxidative stress. ↑ DNA damage; behaviour changes; ↑ MT;	(Buffet et al.,

				↑CSP3-like; ↑ CAT, ↑ GST. No effects in SOD and LDH;	2013b)
ZnO	*S. plana*	3 mg/Kg	16 days	No oxidative stress. ↑ CAT; ↑ LDH; ↓ feeding rate; no change in GST, SOD and MT; no neurotoxicity.	(Buffet et al., 2012)
Ag, CuO	*Macoma balthica*	200 µg/g	35 days	Toxicokinetics is form dependent (ionic >nanoparticles > micron). No effects on mortality, CI, burrowing behaviour; no genotoxicity.	(Dai et al., 2013)
Ag	*Crassostrea virginica*; embryos	0.0016-16 µg/ l	48 hours	↓ LMS (0.16-16 µg/l); ↑ embryotoxicity (1.6 µg/l); ↑ MT (embryos > adults)	(Ringwood et al., 2010)
Ag, TiO_2	*C. virginica*	1-400 µg/ l	2 hours	Toxicity (nanoparticles- ionic form); ↓ Phagocytosis	(Chalew et al., 2012)
Ag	*C. virginica*	0.02-20 µg/ l	48 hours	Toxicity (DG > G): ↓ LMS, ↑ LPO, ↑ GSH	(McCarthy et al., 2013)
ZnO	*C. gigas*	50 µg/ l-50 mg/l	96 hours	LC_{50} = 37.2 mg L^{-1}; ↓GR activity (G, DG), ↓PSH (G); ↑ LPO (G); mitochondrial damage (G, DG). No effects in immunological functions and biochemical BMs (GSH-t, GSSG, CAT, TrxR).	(Trevisan et al., 2014)
Ag	*C. islandica*	110, 151 ng/l	12 days	Toxicokinetics and tissue distribution is size dependent. Anal excretion route dominate over renal excretion.	(Al-Sid-Cheikh et al., 2013)
TiO_2	*Scapharca subcrenata*	500 µg/ l	35 days	Co-exposure (nanoparticles + Phe). nanoparticles as the carrier to facilitate Phe bioaccumulation. No nanoparticles accumulation in M and G.	(Tian et al., 2014)
Ag	*Elliptio complanata*	0.8-20 µg/ l	48 hours	↑ Oxidative stress; ↑ MT; ↑ protein-ubiquitin; ↑ DNA damage. Part of nanoparticles toxicity attributed to release of Ag+.	(Gagne et al., 2013a)
ZnO	*E. complanata*	2 µg/ l	21 days	Co-exposure (nanoparticles + municipal effluent). ↑ oxidative stress; ↑ Zn, ↑ Fe, ↑ Ni, ↑ As, ↑ Mo, ↑ Cd (DG); Co-exposure change nanoparticles effects on metallome.	Gagné et al., 2013b)
TiO_2	*Driessena polymorpha*	0.1 µg/ l	24 hours	↓ Phagocytosis; ↑ ERK1/2, ↑ p38 phosphorylation (5 and 25 mg/l).	(Couleau et al., 2012)
TiO_2	*Corbicula fluminea*	0.1-1 mg/l	10 days	Co-exposure (n-TiO^{2+} Cd^{2+}): ↓ free Cd levels (FW); ↑CAT; ↑ oxidative stress (O); ↑ tissue damage (DG). No effects in SOD, GST and Cd accumulation.	(Vale et al., 2014)

ovate or ovate-triangular in shape, up to 80 mm in length, with each valve bearing three cardinal teeth. It varies in colour from white to violet with distinctive brown zigzag markings at the umbo (Carpenter, 1997). The reproductive cycle of *A. umbonella* is reported to be controlled by temperature changes (Al-Mohanna et al., 2003). In the summer, when the temperature is high, sexual development is restrained and as the temperature drops from November sexual development resumes with a major spawning event in January (Figure 1.7A and B) (Saeedi et al., 2010).

1.5.9 Bivalves used in the present study: *Asaphis violascens* (Forskal, 1775)

Asaphis violascens (Figure 1.7 C and D) (phylum; Mollusca, class; Bivalvia, sub-class: Heterodonta, order; Veneroida, superfamily; Tellinoidea, family; Psammobiidae, genus; *Asaphis*, species; *Asaphis violascens*) is distributed throughout the Indian Ocean, Red Sea and the Republic of Mauritius (http://www.marinespecies.org/aphia.php?p=taxdetails&id=213508). There is no report about the breeding season of this species. It was considered that in summer months the sexual development in *A. violescens* was also restrained and resume in winter months as observed with *A. umbonella* (Saeedi et al., 2010). Therefore, the sampling of both species of bivalves was done in the same months.

1.6 Objectives and Research Questions

Despite the growing literature on the toxicity of ENPs to bivalve molluscs, their mode of action and specific biomarkers for monitoring of water pollution needs further clarification, particularly in less well studied species. In this thesis the in vivo toxicity of four extensively used inorganic nanoparticles (silver, titanium dioxide, copper oxide and zinc oxide) was determined against two understudied marine bivalves species, *Amiantis umbonella* and *Asaphis violascens*. The bivalves were treated with selected nanoparticles and examined for their functional activity as measured by monitoring clearance rate (CR) of particulate matter from the water along with selected biomarkers of oxidative stress in gills and digestive glands.

Figure 1.7. The clam species *Amiantis umbonella* (Lamarck) (A & B) and the cockle *Asaphis violascens* (Forskal, 1775) (C & D)

The study objectives were thus:

1. To establish the bivalves *Amiantis umbonella* and *Asaphis violascens* as laboratory subjects to study the effects of engineered nanoparticles;
2. To determine a baseline health condition index and clearance rate as a measure of functional activity in the bivalves;
3. To investigate the toxic effects of metal nanoparticles on bivalves functional activity;
4. To assess the levels of lipid peroxidation and other biomarkers of oxidative stress in nanoparticle exposed bivalves.

Chapter 2. Effect of Nanoparticles on the Functional Activity of Bivalves

2.1 Introduction

Benthic filter feeding invertebrates are key components of coastal ecosystems where they sustain their life by removing suspended food particles from the water (Riisgård and Larsen, 2000; Riisgård et al., 2000). Toxicants impact the growth and reproduction of these individuals by affecting the biogeochemical influxes such as the nutrient cycle (Hanna et al., 2013). In bivalves, the complete feeding process involves three steps (Saraiva et al., 2011) as: 1) filtration, 2) sorting and ingestion, and 3) digestion and absorption (assimilation). Suspension feeding can only be an energetically feasible mechanism when the cost of pumping does not exceed the energy gained, and it is often a marginal proposition (Järnegren and Altin, 2006).

Considering their significant role in coastal ecosystems, bivalve molluscs are the most studied filter feeding organisms. They process large volumes of water, trap suspended particulate material and excrete nutrients; thus exerting a considerable impact on their environment (Charles et al., 1999). The mechanisms of water pumping and particle retention have been reviewed and repeatedly re-evaluated over the years (Bayne et al., 1976; Charles et al., 1999; Cranford and Hill, 1999; Dral, 1967; Meyhöfer, 1985; Møhlenberg and Riisgård, 1978; Møhlenberg and Riisgård, 1979; Nielsen et al., 1993; Owen and McCrae, 1976; Riisgard and Larsen, 1995; Riisgård, 1988; Riisgård and Larsen, 2000; Riisgård et al., 1996; Riisgård and Møhlenberg, 1979; Riisgård et al., 2000; Silverman et al., 1996a; Silverman et al., 1996b; Smaal and Twisk, 1997; Ward, 1996; Ward et al., 1998; Ward et al., 1993; Winter, 1978). The volume of water filtered or cleared of particles per unit of time is defined as the clearance rate (CR). CR is sometimes referred to as the pumping rate when all particles (within the extractable size range) entering the mantle cavity are completely retained by the gills (Rajesh et al., 2001). When water is transported through the gills, suspension-feeding

bivalves obtain their food from the surrounding water by retaining suspended organic particles (Saraiva et al., 2011; Winter, 1978).

The process of particle filtration is initiated when water passes through the gills. The gills comprise parallel oriented filaments which possess ciliary tracts. There are three types of ciliary tracts; namely, lateral ciliary tracts, particle-transporting frontal ciliary tracts, and laterofrontal ciliary tracts. The laterofrontal cirri are composed of two types of ciliary structure: laterofrontal cirri and prolaterofrontal cilia (Riisgård, 1988) and are thought to act as filters to retain particles (Silvester and Sleigh, 1984). Studies combining flow cytometry and video endoscopy demonstrate that pre-ingestive selection may occur on the gills of oysters (*Crassostrea virginica, C. gigas*) and mussels (*Mytilus trossulus*) (Baker et al., 1998; Baker et al., 2000; Levinton et al., 2002; Ward et al., 1998) or is confined to selection by the palps of *Mytilus* sp. (Ward et al., 1998). According to an earlier estimate, one kilogram of zebra mussels (whole animal of multiple sizes) can filter 180 litres of water per hour (Lei, 1993). In another study, mussels (*Perna* v*iridis*) had a clearance rate of between 4.5 and 6.5 l/g/h and that clams (*Ruditapes philippinarum*) had a clearance rate of between 5 and 7 l/g/h on a dry weight basis (Li and Wang, 2001).

Bivalves obtain their food from the surrounding water by retaining food particles in the gills when water is transported through its system. Food intake is influenced by both plankton and suspended organic matter besides other environmental factors (Newell et al., 2005; Newell et al., 1989; Saraiva et al., 2011; Velasco and Navarro, 2003). Feeding activity and metabolism of suspension-feeding bivalves are considered key elements of benthopelagic coupling in coastal ecosystems. Their mode of feeding and dense masses of filter-feeders are responsible for the flux of matter from the water column to the bottom sediment. This process influences the nutrient cycling that plays a fundamental role at the ecosystem level (Sgro et al., 2005).

The non-assimilated (or undigested) material is expelled as pseudofaeces (material rejected before gut passage) and faeces (material rejected before absorption) and deposited in sediment (Saraiva et al., 2011). The bivalves can

reject excess particles in order to regulate ingestion when the particle concentration is above a threshold level (Arapov et al., 2010; Riisgård et al., 2011). Thus, the filter-feeding ability of bivalves can affect both the water column from which they extract suspended particles and the sediment on to which pseudofaeces and faeces are deposited, thereby exerting an impact on the benthic and pelagic compartments of the ecosystem. Filtration of particles can exert top-down control of phytoplankton and bacterial communities, as well as changes in nutrient cycling at the ecosystem scale (Dolmer, 2000; Zemlys et al., 2003). Bivalves can also increase deposition of pseudofaeces in sediment (Dame, 1993), enriching its nutrient content that stimulates microbial growth and may thus provide an important resource for surrounding benthos (Norkko et al., 2001; Saraiva et al., 2011). For example, Norkko et al. (2001) conducted a study on beds of the horse mussel *Atrina zelandica*, at three sites (4-7 m depth) on the East Coast of the North Island, New Zealand. Higher sedimentation rates and inputs of organic carbon and nitrogen were found close to individual *A. zelandica* (≤ 10 cm) compared to further away (≥30 cm) in bare areas. Further, sediments that were close to *A. zelandica* were enriched in carbon and nitrogen and had greater faunal abundance and diversity.

Bivalve filtering activity depends on organism size, phytoplankton concentration, and the size and quality of the food/suspended particles (Khalil, 1996). Abiotic factors such as temperature, salinity, and water flow can also affect filtration rates (Rajesh et al., 2001; Sgro et al., 2005). However, adaptation to temperature and food availability is also reported as Riisgård (1988) measured filtration rate of six species of suspension-feeding Northeast American bivalves at 27-28 °C which was close to the filtration rates measured at 10-13 °C in 13 species of Northwest European bivalves (Møhlenberg and Riisgård, 1979). The functional responses of filter-feeding bivalves – that is their ability to filter suspended particles – is related to the variations in total amount and nature of particles present in the water (Foster-Smith, 1976; Navarro and Iglesias, 1993; Newell et al., 1989; Riisgård, 2001a, b; Shumway et al., 1985) but contradictions and uncertainties still exist. Mostly experimental studies have been used for calculating the relationships between filtration of particles, pseudofaeces

formation and assimilation without a clear understanding of the mechanistic process of rejection of non-edible particles, non-assimilated material, or of the most appropriate quantity of food intake (Saraiva et al., 2011).

In this chapter, the effect of engineered nanoparticles on the CR of two species of bivalves *Amiantis umbonella* (clam) and *Asaphis violascens* (cockle), collected from coastal areas of Kuwait was studied using an indirect method of measurement. These species were chosen because of their economic importance, abundance in the coastal areas of Kuwait and for being understudied in the scientific literature. Using them to generate data on the toxic effects of engineered nanoparticles will be a novel contribution to the scientific literature and will help develop the scientific debate surrounding the broader aspects of bivalve filter feeding. CR is a dynamic index that reflects the physiological feeding state of suspension feeding animals. This endpoint has been used in many studies, about pollutant exposure (Al-Subiai et al., 2012; Al-Subiai et al., 2011; Buffet et al., 2013a; Buffet et al., 2013b; Canty et al., 2009; Widdows et al., 1995). The indirect method of measurement was selected to reduce the level of disturbance of the organisms during the experiment. In this method, the measurement of particle concentration (algal cell count) was done at different time intervals to determine CR. In ecotoxicology, this methodology can be used as an indicator of the functional activity of bivalves in response to the toxic action of nanoparticles.

The toxic effect of nanoparticle depends on its physical state, solubility, and bioavailability to the organism. It has been shown that nanoparticle in contact with aqueous medium undergo a variety of modifications like agglomeration, state of surface oxidation, dissolution and each state of the particle may have different toxicological reactions. It requires excessive efforts by using the expensive methodology to understand the phenomenon of each state of modification (Montaño et al., 2014). The characterisation of nanoparticles in the exposure medium was determined by using transmission electron microscopy (TEM) and dynamic light scattering (DLS). The phenomenon of agglomeration and settling of nanoparticles in the aqueous medium is well documented (Fabrega et al., 2011; Shang et al., 2014). However, a gentle stream of air passed through the medium in

the exposure vessel helps in avoiding settling and keeping the particles in suspension to make it bioavailable to the exposed organism.

It has been suggested that alternative to measuring the test substance itself in and on the organism is to determine its presence indirectly from biological responses of the whole organism, or preferably key target organs/cellular compartments (Selck et al., 2016). Considering the behaviour of nanoparticle in seawater where nanoparticles make a stable complex with chloride present, their agglomeration and aggregation, deposition on shells, it is more relevant to get an idea of dosimetry by estimating total metals accumulation in target organs on exposure to nanoparticles. Therefore, in this chapter, along with the measurement of the functional activity of exposed organism total metal concentration in gills and digestive gland of bivalve was determined using inductively coupled plasma optical emission spectrometry (ICP-OES) method. Two main nanoparticles, nano-titanium dioxide, and nano-silver, were selected for this study because of their extensive use and global production. However, in Kuwait, authentic information about the production and usage of selected nanoparticles is not available. In order to make a comparison between the nano and bulk material the oxides of nano-, and bulk-, zinc and copper were tested using *A. violascens*.

2.2 Materials and Methods

2.2.1 Nanoparticles

Inorganic nanoparticles of nano-titanium dioxide (n-TiO_2), nano-silver (n-Ag), nano-zinc oxide (n-ZnO), and nano-copper oxide (n-CuO) were purchased from Sigma-Aldrich, USA; supplied by Med Arabia, Kuwait. Their purity, particle size, and surface area are given in Table 2.1. The n-TiO_2 and n-Ag were the main particles of interest with n-CuO and n-ZnO used in some experiments for comparison.

Table 2.1. Properties such as purity, particle size, surface area and product code of the metallic nanoparticles purchased from Sigma-Aldrich.

	Metal oxides	Purity, %	Particle size, nm	Surface area, m^2/g	Product No.
1.	Nano-titanium dioxide	99.7	<25	200-220	637254
2.	Nano-silver	99.5	<100	5.0	576832
3.	Nano-copper oxide	--	<50	29	544868
4.	Nano-zinc oxide	--	<100	15-25	544906

*Information provided by the manufacturer

2.2.2 Preparation of nanoparticle suspensions

Stock suspensions of nanoparticles were freshly prepared in distilled water (DW) at a concentration of 1 mg/ml, and sonicated for 30 minutes (Bransonic, USA; 70W and 42- kHz frequency) and kept in constant shaking to aid mixing and breaking down of particle aggregates before adding it to the exposure vessel as recommended (Canesi et al., 2010a; Canesi et al., 2010b; Gomes et al., 2014a; Gomes et al., 2014b). Aliquots from the stock suspension were taken to prepare 0.05, 0.2, 1, and 2 mg/l nominal concentration of nanoparticle for exposure in the experiments.

2.2.3 Characterization of nanoparticles

Transmission Electron Microscopy (TEM)

For TEM imaging, a diluted suspension of nanoparticles was prepared after sonication for 15 min, and then 10 µl of this suspension was deposited on a carbon grid (copper carbon filmed grid). The drop was allowed to settle for 30-60 seconds on the carbon grid and dried at room temperature for several minutes before examination. Experiments were carried out on a Philips CM 100 compustage (FEI) Transmission Electron Microscopy at voltage 100 kV, and digital images were collected using an AMT CCD camera (Deben), Electron Microscopy Research Services, Newcastle University. It has a resolution of 0.19

nm, an electron probe size down to 0.5 nm and a maximum specimen tilt of ±10° along both axes.

Dynamic light scattering (DLS)

Nanoparticles were measured to calculate the average particle size using a laser diffraction particle size analyser (Malvern Zetasizer nanorange, Malvern Instruments Ltd, UK) using Hydro EV Flexible volume wet dispersion. Water was used as a dispersing medium for all the powders. The nanoparticles were dispersed using a small amount of dispersant medium (distilled water) and sonicated for 10 minutes before analysis. The dispersion was poured into the Hydro 2000 dispersion unit (Malvern, UK) until the obscuration was in range. The average particle size was calculated automatically using the Zetasizer nanorange software. The analysis was performed in triplicate.

2.2.4 Collection of bivalves

Two prevalent species of bivalves, *Amiantis umbonella* (Lamarck, 1818) and *Asaphis violascens* (Forskal, 1775) were collected from the coastal area of Kuwait and used as the test organism to generate data on the potentially toxic effects that ENPs may have on the Gulf ecosystems. The selection of these species was based on the premise that they are prevalent and well adapted to the Gulf conditions and no study is conducted on these species. The data generated on the toxicity of nanoparticles against these indigenous species may be suitably used in other areas of the Arabian Gulf. The predominant species found at Doha was *A. umbonella* (Lamarck, 1818) whereas the predominant species at Nuwaiseeb was *A. violascens* (Forskal, 1775). At both the sampling areas the nearest industrial activity was power generation coupled with a desalination plant. The Doha sampling site was 5 km away from Doha water, and power plant and Nuwaiseeb site were 10 km away from the Al-Zour water and power plant. The sampling was done in the non-spawning period November 2011 and 2012 from the Doha area at GPS location N 29^0 22.34; E 47^0 45.28 (Figure 2.1 A), and November 2013 and 2014 from the Nuwaiseeb at GPS location N 26^0 32.17; E 48^0 25.32 (Figure 2.1 B). It has been suggested that reproduction in *A. umbonella* is controlled by

temperature changes, during the very hot summer when temperatures are high, sexual development is restrained and resumes with the decline of temperature (Al-Mohanna et al., 2003). The major spawning event is observed in January that correlates with lower water temperature (Saeedi et al., 2010). Reproductive information for *A. violascens* is not available, and it is assumed that it may also have a winter spawning period.

At Doha, the animals were buried at 10- to 15 cm depth and at Nuwaiseeb the animals were buried in the sand near rocks under 30 cm of water. The animals were scooped gently with the help of a spade and were collected from an area around 200 m of the recorded position. In the laboratory, each animal was weighed, its shell length and width measured with digital Vernier Callipers. The animals were maintained in aquaria and were acclimatised for seven days before use in experiments. The natural seawater used for the study was obtained by pumping from the nearshore wells, which provide sand filtration, and was filtered in the laboratory through 0.45 µm before adding to the aquaria. Filtration was done using a Millipore filtration assembly by connecting to a vacuum line. The seawater in the aquaria was changed daily, and continuous aeration was provided. The temperature was 20±2 ºC, 39 ppt salinity with a photoperiod of 12 h light and 12 h dark. The animals were fed *ad libitum* with a diet of the microalga *Chlorella vulgaris* (always > 4.0 x 10^6 cells/ ml) that was routinely cultured in the Marine Fisheries Department (MFD). Seawater quality parameters were measured using a WTW 350 water quality probe, Germany, and the data is reported in Table 2.2. The water quality probe was calibrated on a regular basis.

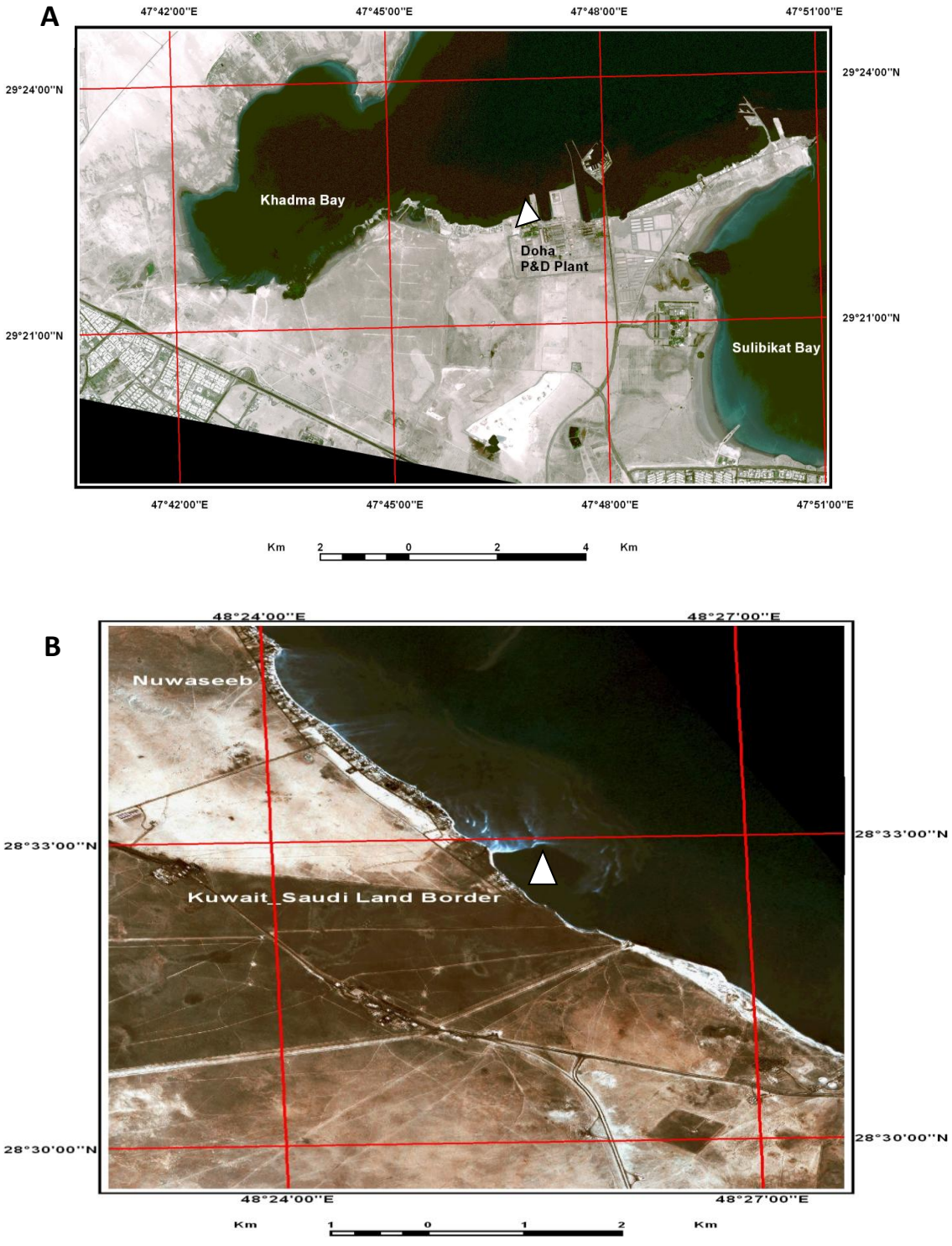

Figure 2.1. (A) Doha sampling station (Δ) N29^0 22.34/E47^{0}45.28 for *A. umbonella*, (B) Nuwaiseeb sampling station (Δ) N26^0 32.17/E48^0 25.32) for *A. violascens*.

Table 2.2. Water quality parameters for seawater supplied in the laboratory and the sampling area.

Parameters	Sea Water*	Doha	Nuwaiseeb
Temperature, °C	20.0	20.5	19.4
pH, units	8.0	7.8	7.7
Salinity, ppt	39.0	39.1	39.3
Dissolved Oxygen, mg/l	6.0	7.3	7.8
Total Suspended Solid, mg/l	--	15.9	15.8

*Filtered through 0.45 µm

2.2.5 Measurement of health condition index (CI)

Gross assessment of animal health was made by calculating health condition index (CI) for each animal using equation 1 (Cataldo et al., 2001). First, whole animal wet weight was recorded, then the soft tissue was removed from the shell and blotted on a paper towel to remove excess water; wet tissue and air-dried shells were then weighed. Initially, the CI was calculated using the fresh weight of the animals, but due to the water retention phenomenon in bivalves, it was observed that different amount of water was retained in different animals (water holding capacity was different in each). To avoid this discrepancy, dry weight of the animals was used considering the following equation, where dry weight and shell length were used for the calculation of CI.

CI = (Dry tissue weight / shell length of the animal) x 100 …… (1)

2.2.6 Nanoparticle exposure experiments

For the exposure study, animals of a similar length (*A. umbonella* 40-46 mm; *A. violascens* 52-57 mm) were selected for each experiment. A set of control animals (without nanoparticles exposure) was used for every exposure experiment. Preliminary experiments were performed to define the concentration of nanoparticles to be used for the exposure experiments. No effects in CR or oxidative enzymes were observed at 0.01 mg/l, and the treated animals were as

good as controls. The data for 0.01 mg/l exposure concentration is reported in Appendix I. At higher dose 5 mg/l, no filtration activity was observed as the animals did not open the shell. Therefore, the actual experiments with various nanoparticles were conducted at 0.0, 0.05, 0.2, 1, and 2 mg/l (Canesi et al., 2010a; Canesi et al., 2010b). The design of acute and chronic exposure that was used for all the nanoparticles tested in this study is given in Figure 2.2 (A-D).

2.2.7 Exposure to n-TiO_2 and n-Ag

Acute exposure. The two species of bivalves were exposed separately for 48 hours with n-TiO_2 and n-Ag at 0.0, 0.05, 0.2, 1, and 2 mg/l concentrations. Thus five concentrations of each nanoparticle including controls were used for exposure. The exposure regime required 100 animals for concentration-response toxicity determination of each nanoparticles testing. All the exposures were run in large (5 L) glass beakers. Five beakers were used for each exposure concentration and in each beaker 4L seawater was taken, and the desired concentration (0.05-2 mg/l) was made by using a stock solution of nanoparticles. In each beaker, four animals were gently placed at a stocking density of one animal per liter of seawater. The beakers were continuously aerated and maintained at 20±2 ºC using 12-hour light and 12-hour dark photoperiod. The process of aeration was expected to keep the particles in suspension as no deposition on the bottom was detected during exposure. The seawater was changed after 24 hours, and nanoparticles concentration was renewed. Animals were not fed in acute exposure (Figure 2.2 A & B).

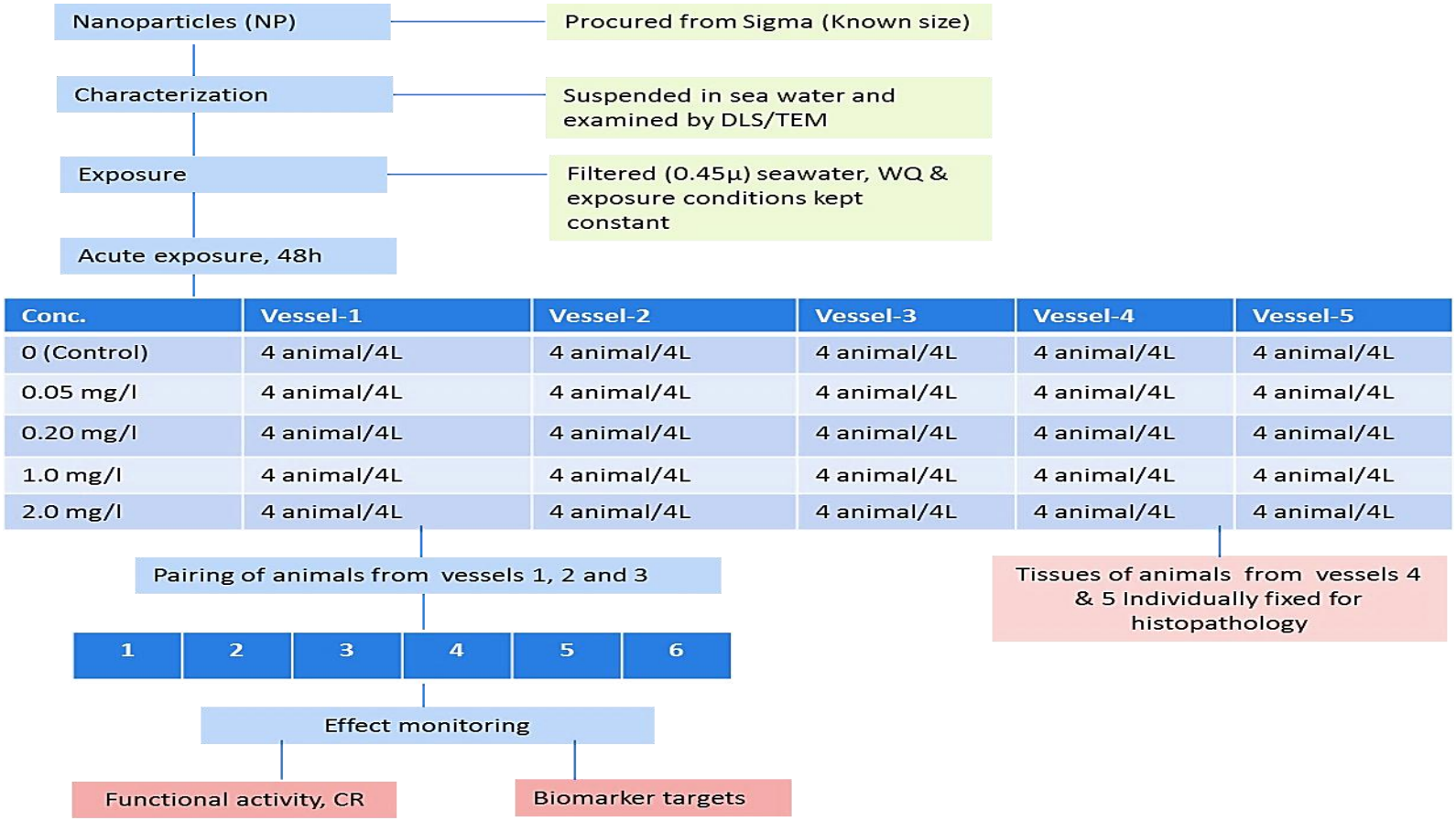

Figure 2.2A. Diagram showing the design and steps for the acute exposure of n-Ag and n-TiO_2 to *A. umbonella*.

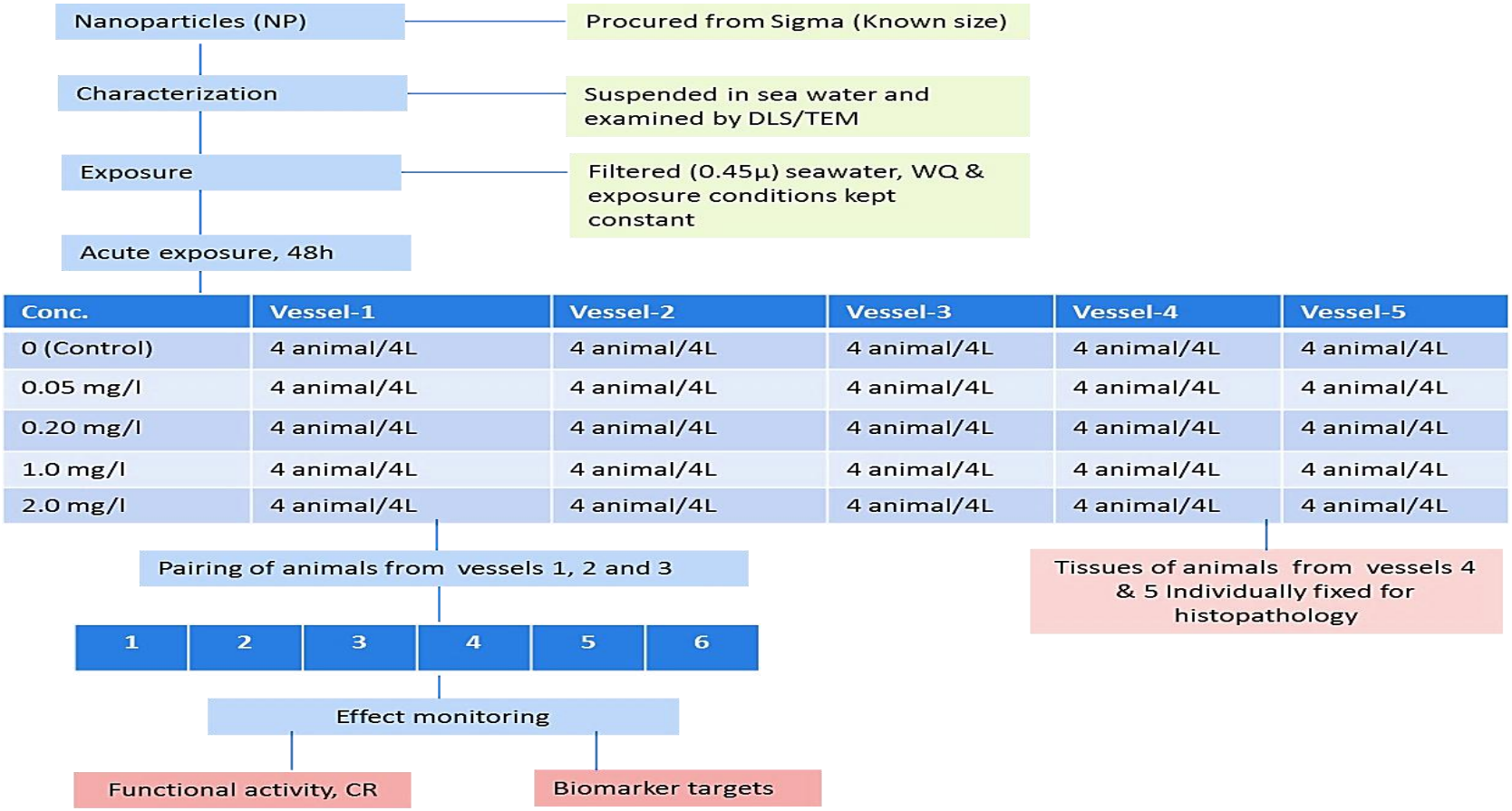

Figure 2.2B. Diagram showing the design and steps for the acute exposure of n-Ag and n-TiO_2 to *A. violascens*.

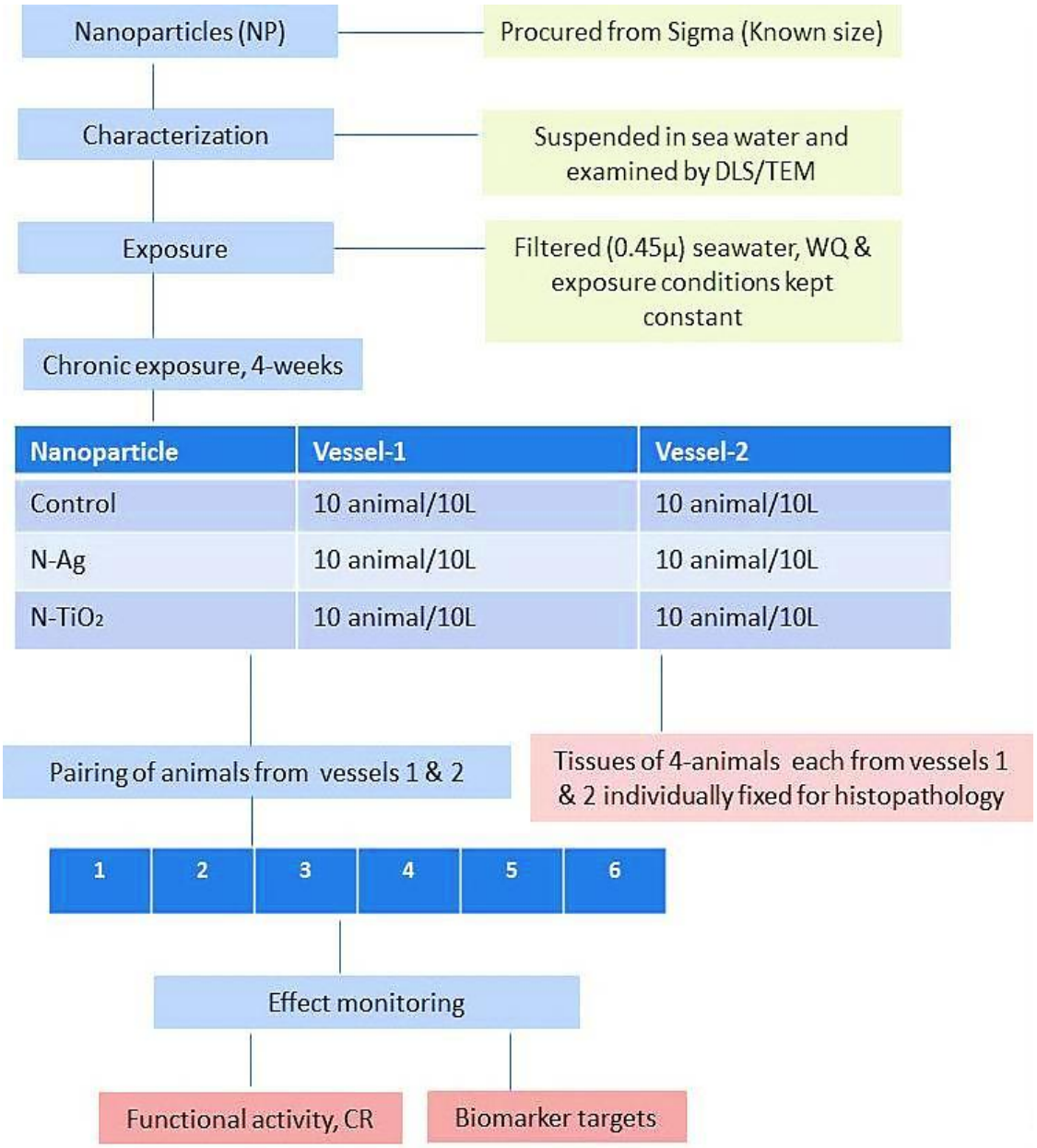

Figure 2.2C. Diagram showing the design and steps for the chronic exposure of n-Ag and n-TiO_2 to *A. umbonella*.

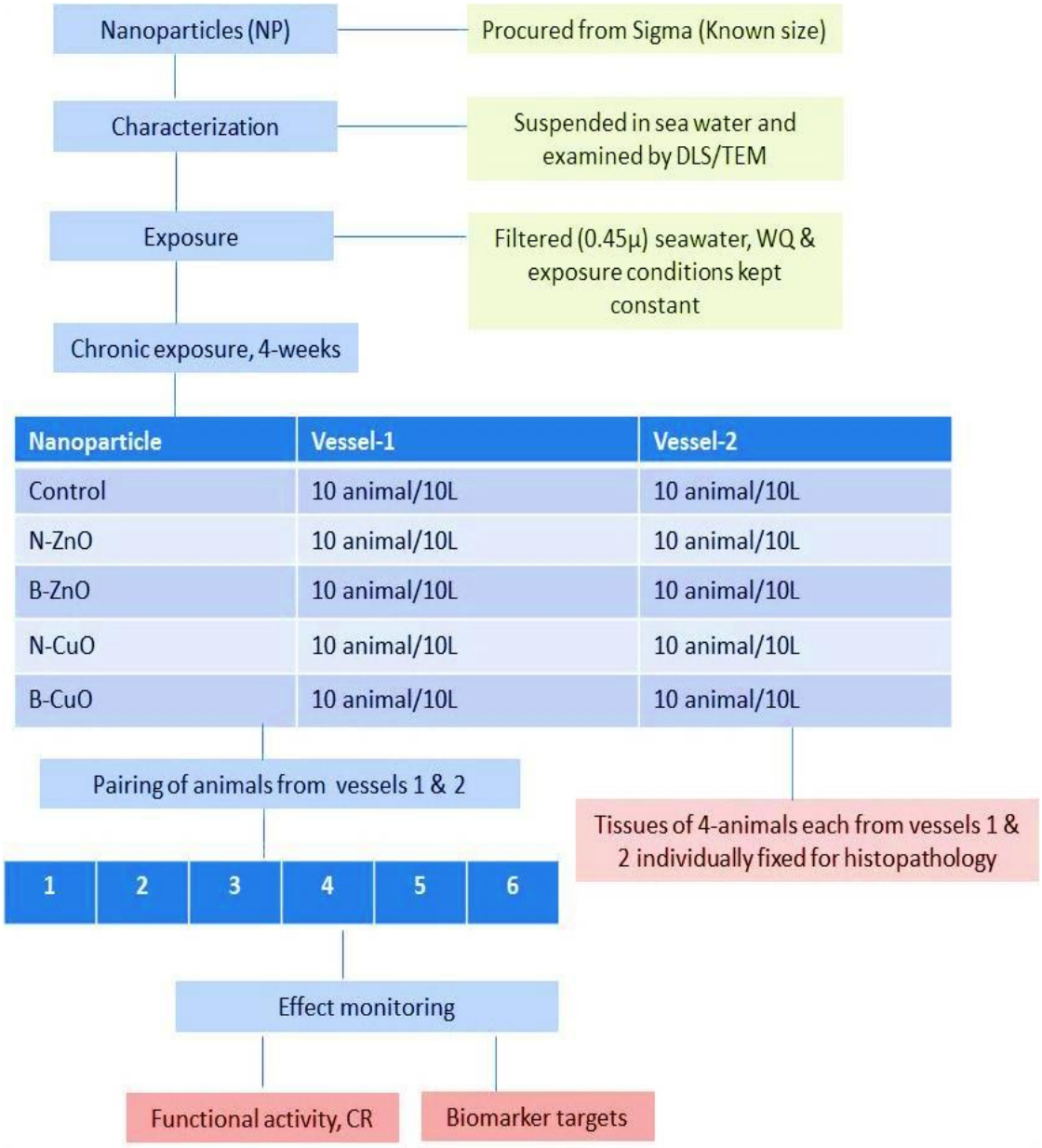

Figure 2.2D. Diagram showing the design and steps for the chronic exposure of nano- and bulk- ZnO and CuO to *A. violascens*.

Chronic exposure. The chronic exposure was done for four weeks at the lowest concentration (0.05 mg/l) of n-TiO_2 and n-Ag using *A. umbonella* (Figure 2.2 C). Two aquariums were used for each nanoparticle with a nominal concentration of (0.05 mg/l) of n-TiO_2 and n-Ag that was made by mixing with stock solution respectively. Controls were run simultaneously in a separate aquarium. In each aquarium, 10 animals were placed. The animals were immersed slowly with minimal disturbance or stress. The aquarium was aerated and maintained at 20±2 ºC using 12-hour light and 12-hour dark photoperiod. The seawater containing the same nanoparticle concentration (0.05 mg/l) was replaced each day. The animals were fed by adding *C. vulgaris* at 4.0 x 10^6 cells/ml concentration in replacement seawater.

2.2.8 Exposure to nano and bulk CuO and ZnO

Exposure to n-ZnO and b-ZnO, n-CuO, and b-CuO was done with an objective to determine the difference between nano and bulk state of the test material. A chronic exposure regime for four weeks at 0.05 mg/l concentration was used by using *A. violascens* (Figure 2.2 D). The method was the same as adopted for chronic exposure to n-TiO_2 and n-Ag. During exposure similar conditions were used and precautions were taken for minimal disturbance or stress on the animals during replacement of water. After the exposure period was over, the animals were immediately transferred to clean seawater and left for 24 hours to determine the clearance rate (CR).

Processing of animals. The controls and exposed animals were processed further in the following manner. After the exposure period was over, out of 20 animals per concentration 12 animals were used for clearance rate (CR) determination, and eight were individually fixed for histopathology. For CR determination six replicates were used by keeping 2 animals in 2L seawater in separate beakers. The pairing of animals was done with vessels 1, 2 and 3 (Figure 2.2 A). The procedure for CR determination is described in detail in subsequent section (2.2.9).

After CR determination the animals were sacrificed by cutting the adductor muscles. Gills and digestive gland from the two animals were excised and pooled to constitute one sample for biochemical analysis. Thus 6-samples of gills and 6-samples of digestive gland were individually analysed in triplicate for biochemical analysis that were obtained from 12 control and 12 exposed animals for each concentration of nanoparticle. Various steps of exposure and processing are shown in Figure (2.2 A-D).

2.2.9 Measurement of clearance rate

Clearance rate – a measure of the efficiency of the gills to retain particles – was estimated as the volume of water cleared of particles per unit time by measuring the reduction of particle density in a known volume of water. A well-documented indirect clearance method based on Famme et al. (1986) and Riisgård (1988) was used. This method agrees well with other methods used for measuring filtration rates.

In this study, *C. vulgaris* was used as feed, the size of which ranges from 2-10 µm. The concentration of algae used was determined by trial experiments with the endpoints being: 1) the animals do not produce pseudofaeces during acute exposure; and, 2) around 90% of particles are trapped during 24-hour exposure. During exposure, continuous aeration was maintained that kept the particles in suspension. No deposition of particles was detected on the bottom of the beakers. After the nanoparticle exposure period was over, the animals were immediately transferred to clean sea water containing 4.0×10^6 cells/ml algal concentration. The algal concentration used was pre-determined by exposing bivalves to varying algal concentrations, and at this concentration, food was not limiting, and pseudofaeces was not formed. Throughout the exposure experiments, control animals (untreated) were simultaneously examined with the exposed animals. For CR determination, two animals per two litres of seawater were kept thus using twelve animals in six beakers for each exposure concentration. The initial algal cell count (t=0 hours) was made before introducing the animals to the beaker, and then post-exposure samples were

collected after 24 hours to determine the algal concentrations. The water samples for algal cell counts were taken with a pipette in triplicate without disturbing the animals. The number of algal cells per millilitre of seawater was counted using a modified Neubauer haemocytometer and Olympus microscope with 100 x magnifications (B071, Japan). The disappearance of algae from the medium both in the controls and exposed animals is an indication of the feeding and clearance rate. The clearance rate was calculated using the following equation (2) (Nilin et al., 2012):

Clearance rate = V (InCi - InCf) / t …… (2)

Where V is the volume of the medium, Ci is the initial concentration of algal cells, and Cf is the final concentration after time t (hour). The clearance rate, ml/h/animal was determined and equated to dry weight of the soft tissue of an animal and expressed as ml/h/g dry wt. as explained below.

Since the clearance rate is determined in live animals, the convenient method of expression of CR is on the basis of per animal. However, in literature CR has been expressed by dry weight of soft tissue. Therefore, in this study dry weight was separately determined by taking 30 animals of each species. The animals were dissected, and the soft tissues were oven dried at 90ºC for 1 hour and then at 60 ºC until the tissues were completely dried. The percentage dry weight determined was used for the conversion of the clearance rate determined on a per animal basis. The CR values on soft tissue weight of each animal were converted to dry weight using a factor of the per cent dry weight of soft tissue.

For g dry wt. / animal = (% soft tissue dry wt. x soft tissue wet wt.) / 100…….. (3)

For CR/ g dry wt. of animal = (CR/ animal x 1)/ g dry wt. of the animal …… (4)

2.2.10 Bioavailability of nanoparticles

The bioavailability of nanoparticles was determined as a total metal concentration in the gills and digestive gland of *A. umbonella* after acute and

chronic exposures to nano silver and nano titanium dioxide. The exposure methodology was the same as used for the clearance rate determination described above. Three concentrations of n-Ag and n-TiO_2 were used, 0.05, 1.0, and 2.0 mg/l for acute exposure along with controls without nanoparticles for 48 h. For each exposure concentration, four animals were used, and the analysis was done in triplicate. Gills and digestive gland of four animals were pooled to constitute one sample. Thus, three samples (n = 3) were analysed to determine the metal content in the gills and digestive gland. Chronic exposure was done similarly as above at 0.05 mg/l nanoparticle concentration along with controls but continued for 4-weeks with the replacement of water and redosing every 24 h. After exposure, the animals were taken out, dissected and the gills and digestive glands were separated out for digestion to determine the total metal concentration in these tissues. However, pre and post-exposure to organism the concentration in the medium was not determined due to the non-availability of relevant equipment in the laboratory.

Trace metals in Tissues

The digestion procedure for the determination of total metal content in the bivalve tissues was done according to EPA Method 200.3. The excised gills and digestive gland were digested with a nitric acid solution, and heated with hydrogen peroxide to obtain a clear solution that was then analysed. The analysis was done using ICP-OES (High range Thermo Scientific iCAP 6000 series ICP Emission Spectrometer). Total metal concentration was determined by titanium and silver calibration against standards provided by Inorganic Ventures. The values were expressed as µg/g dry weight of the gills and digestive gland.

2.2.11 Statistical analysis

Statistical analysis was carried out using Minitab 17 statistical software. All data are presented as the mean ± standard deviation (S.D.) of at least six samples in triplicate. Student T-test was done to determine the difference in the morphological parameters of animals (whole weight, shell weight, shell length, shell width, tissue weight) and health condition index between the two samplings

of both species. The difference in physiological function (Clearance rate) of clams between control groups with exposed groups at various nanoparticle concentrations were detected using one-way analysis of variance (ANOVA) after the data had been checked for assumptions of normality and homogeneity (Leven's test). Fisher's multiple comparison test was used to differentiate between the groups of data and only $P<0.05$ was accepted as significant. Wherever the assumptions for normality were not met, the data were log ten transformed to meet the normality requirements. The literature referred for statistics supported and suggested the efficiency of log ten transformations and confined to normal distribution after log transformations. The Kruskal-Wallis test was done followed by Dunn's multiple comparison tests for multiple treatments. For every exposure experiment with various nanoparticles, ANOVA was done independently for each species.

2.3 Results

2.3.1 Characterization of nanoparticles by DLS and TEM

All the nanoparticles used in the study were characterized using dynamic light scattering (DLS) and transmission electron microscopy TEM. The average particle sizes are shown in Figure 2.3-2.6. The particle size determined by TEM was close to the size reported by the manufacturer (Table 2.1). Figure 2.3 (B and C) demonstrates the spherical shape and particle size distribution of n-Ag. In DLS analysis n-Ag displayed a unimodal pattern with an average size of 84.07 nm (Figure 2.3 A). The TEM images of n-TiO_2, n-ZnO and n-CuO shows the agglomeration behaviour of nanoparticles in suspension (Figure 2.4 B, Figure 2.5 B and Figure 2.6 B). However, the particle size was also measured for these nanoparticles given in their respective figures. The analysis of b-ZnO and b-CuO shows they were much bigger in size than the nanoparticles and these particles were not spherical in shape. The diameter for b-ZnO ranged from 103-123 nm for the measured particles, and they were rectangular in shape (Figure 2.5 C). For b-CuO, the diameter was comparable to that of n-CuO, but they were cylindrical in shape having a length of 278 nm for the measured particle (Figure 2.6 D). DLS analysis for n-TiO_2 showed a bimodal pattern with the first peak at 38.85 nm and the second peak at 6.2 nm (Figure 2.4 A). n-ZnO displayed a unimodal pattern showing an average size of 13.15 nm (Figure 2.5 A). n-CuO also displayed a bimodal pattern with the first peak at 7.628 nm and second at 0.81 nm (Figure 2.6 A).

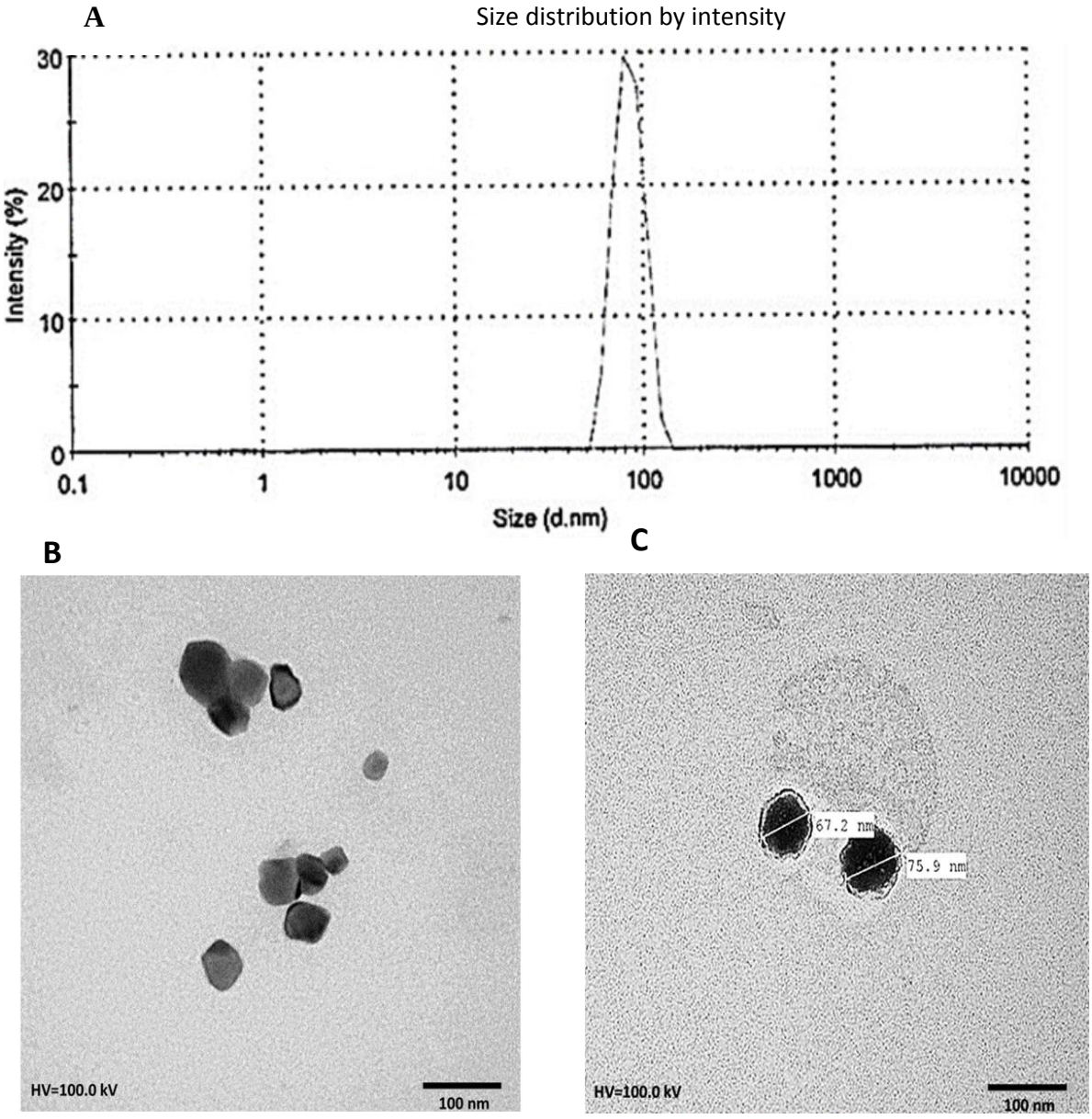

Figure 2.3. Characterization of n-Ag using (A), dynamic light scattering (DLS); (B and C), transmission electron microscopy (TEM).

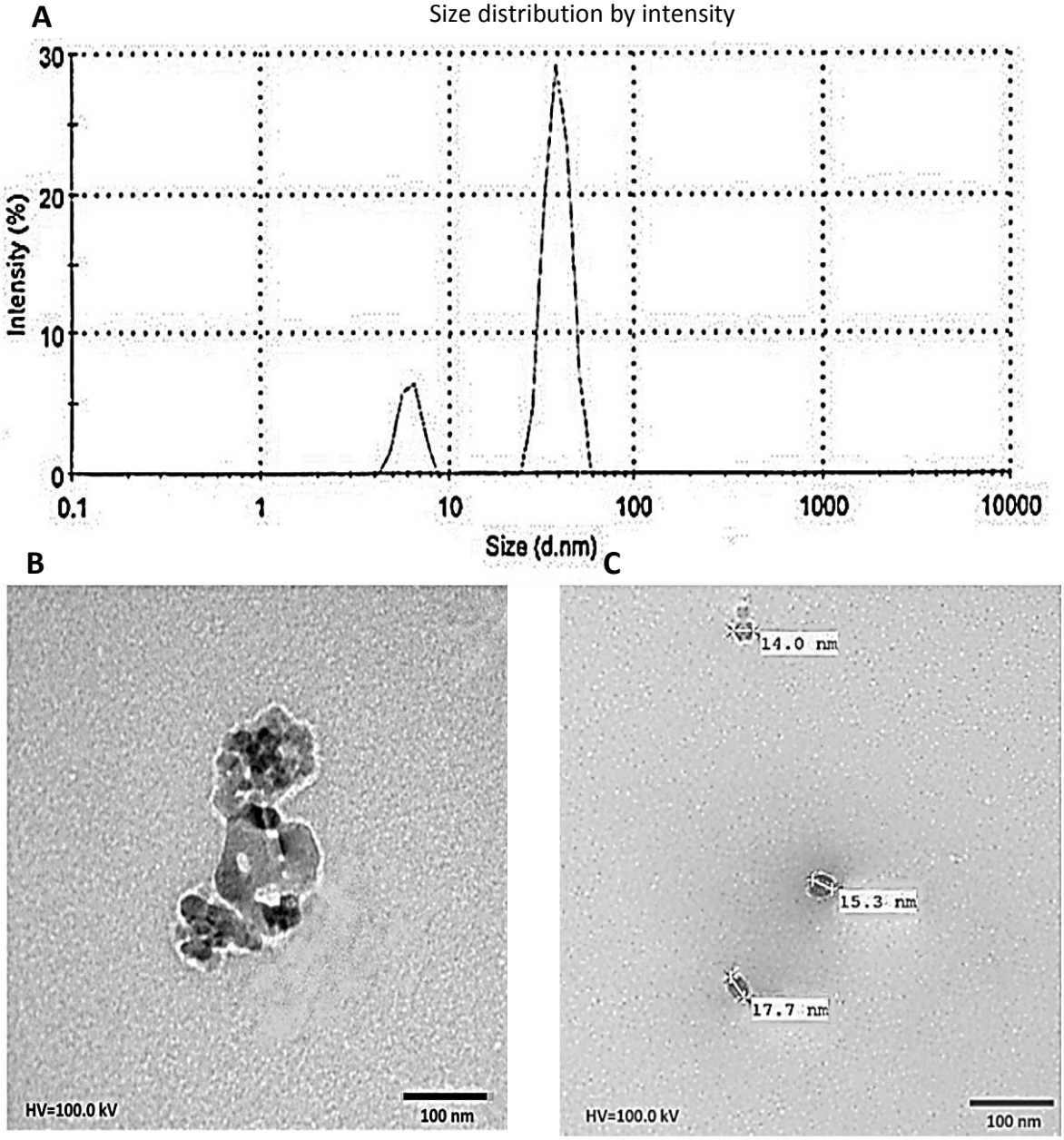

Figure 2.4. Characterization of n-TiO_2 using (A), dynamic light scattering (DLS); (B and C), transmission electron microscopy (TEM).

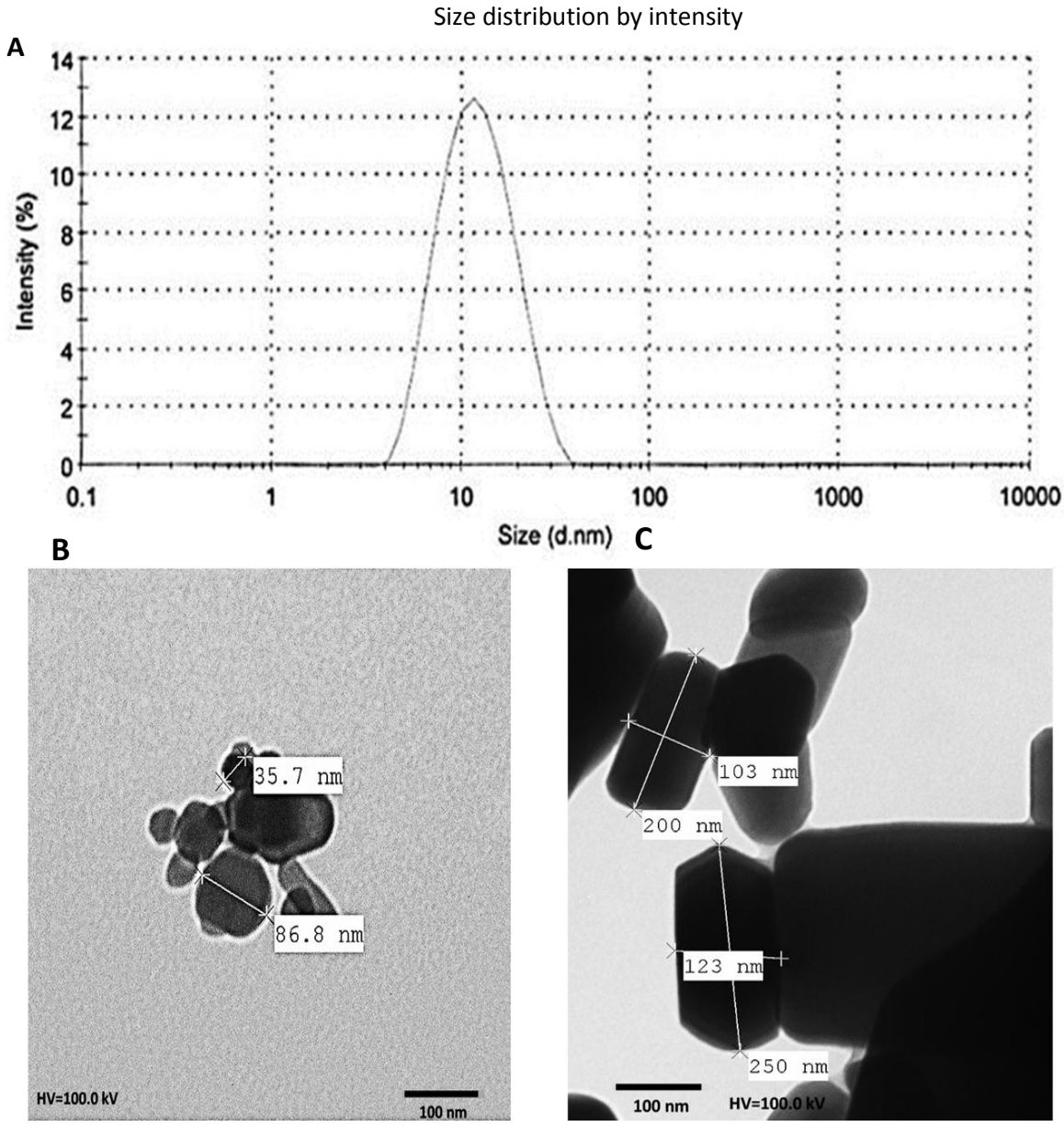

Figure 2.5. Characterization of n-ZnO using (A), dynamic light scattering (DLS); (B), transmission electron microscopy (TEM); (C), TEM analysis of b-ZnO.

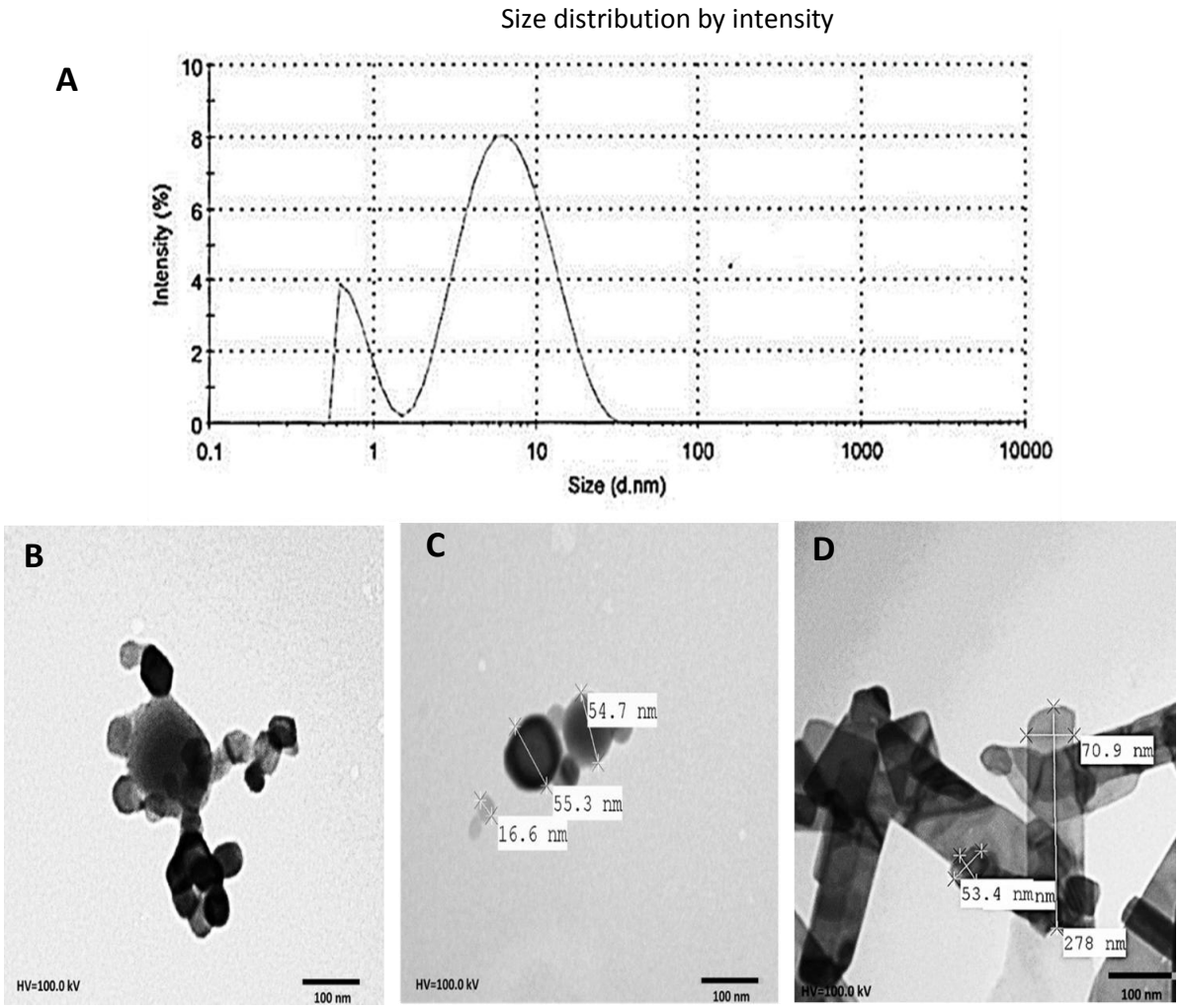

Figure 2.6. Characterization of n-CuO using (A), dynamic light scattering (DLS); (B & C), transmission electron microscopy (TEM); (D), TEM analysis of b-CuO.

2.3.2 Morphometrics, soft tissue measurements, and CI

The morphometric data for *A. umbonella* and *A. violascens* are reported in Figure 2.7. *A. violascens* had larger shell length, the higher whole weight of the animal and wet weight of the soft tissues. However, the shell width and shell weight in the two bivalve species were comparable. The soft tissue of *A. violescens* contained more water as percent dry weight yield was 18.5% compared to 22.9% in *A. umbonella*. These parameters are important for the expression of data for clearance rate as discussed in the following section.

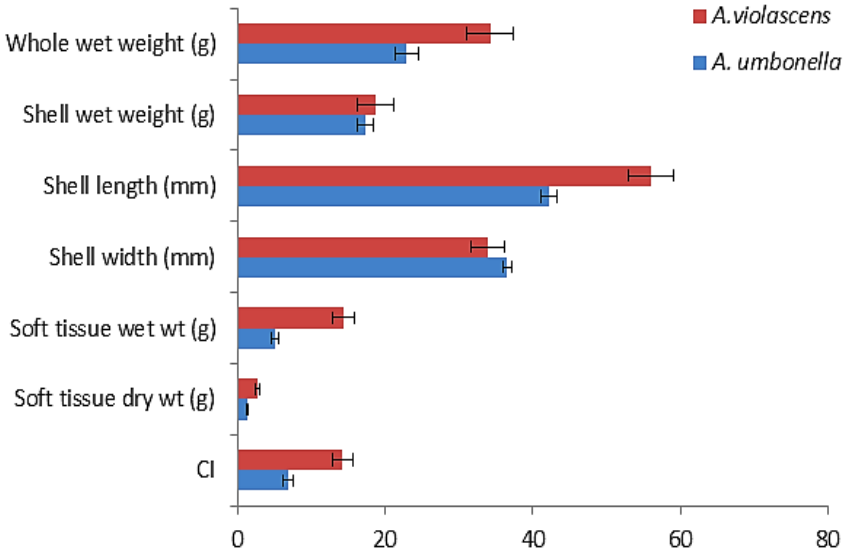

Figure 2.7. Morphometric measurements and health condition index (CI) of *A. umbonella* and *A. violascens* (Mean ± SD).

In Table 2.3, morphometric data for all the animals used in the acute and chronic exposures are tabulated. The data showed that exposure had not changed the morphometric parameters, and soft tissue weights, or the condition index (Table 2.3). The weight of the *A. umbonella* specimens collected for acute exposure was less than the animals collected in subsequent sampling for chronic exposures ($P < 0.01$). The difference in the whole weight

Table 2.3. Morphometric parameters, soft tissue measurements and health condition index of the two species used for various experiments.

Species and exposure	NPs type & conc.	Whole weight (g)	Shell weight (g)	Shell length (mm)	Shell width (mm)	Soft tissue wt. (g)	Dry tissue wt. (g)	Shell area (shell length x shell width)	Shell index (shell wt./ shell area)	CI (wet tissue wt. / whole wt.) x 100
A.umbonella-Acute exposure	Control	22.9±1.6	17.2±1.1	42.2±1.1	36.5±0.6	5.1±0.5	1.2±0.1	1540 ± 63.4	1.1 ± 0.1	6.9 ± 0.6
	n-Ag									
	0.05	22.7±1.7	16.7±1.4	42.3±1.3	37.2±1.4	5.6±0.7	1.2±0.1	1573 ± 90.4	1.1 ± 0.1	7.1 ± 0.3
	0.2	22.4±1.5	16.5±1.3	42.2±1.2	37.3±1.3	5.4±0.8	1.2±0.1	1574 ± 87.7	1.0 ± 0.1	7.2 ± 0.3
	1	22.8±1.9	16.9±1.4	42.2±1.2	36.5±0.6	5.3±0.5	1.2±0.1	1540 ± 63.4	1.1 ± 0.1	7.1 ± 0.6
	2	22.6±1.7	16.7±1.3	42.2±1.0	36.3±0.6	5.7±0.9	1.2±0.1	1532 ± 56.7	1.1 ± 0.1	7.2 ± 0.6
	n-TiO$_2$									
	0.05	23.0±3.0	17.0±2.2	42.4±1.7	37.4±1.0	5.8±0.8	1.3±0.2	1586 ± 102.5	1.1 ± 0.1	7.6 ± 0.5
	0.2	22.6±2.4	16.8±1.9	41.9±1.4	37.3±1.6	5.5±0.6	1.2±0.1	1563 ± 120.2	1.1 ± 0.1	7.1 ± 0.3
	1	22.6±2.6	17.0±2.2	42.1±1.4	36.9±1.3	5.2±0.6	1.2±0.1	1554 ± 107.6	1.1 ± 0.1	7.0 ± 0.5
	2	22.2±1.9	16.7±1.8	41.1±2.8	36.8±3.2	5.1±0.4	1.2±0.1	1513 ± 211.9	1.1 ± 0.4	7.2 ± 0.5
A.umbonella-Chronic exposure (0.05 mg/l)	Control	33.4±1.7	28.7±1.4	45.3±0.9	38.9±0.5	5.7±0.6	1.3±0.1	1762 ± 43.5	1.6 ± 0.1	4.5 ± 0.4
	n-Ag	33.8±2.6	29.4±0.6	44.8±1.3	38.3±1.0	5.5±0.8	1.3±0.1	1715 ± 92.2	1.7 ± 0.1	4.4 ± 0.3
	n-TiO$_2$	33.5±2.2	28.9±1.8	44.7±1.2	37.2±1.9	5.6±0.4	1.3±0.1	1663 ± 114.6	1.8 ± 0.1	4.5 ± 0.3
A.violascens-Acute exposure	Control	34.2±3.1	18.7±2.5	56.0±3.1	33.9±2.3	14.3±1.5	2.6±0.3	1898 ± 213.6	1.0 ± 0.1	13.9 ± 1.4
	n-Ag									
	0.05	34.8±3.8	20.2±3.1	56.3±2.8	33.20±2.0	14.6±1.6	2.7±0.3	1869 ± 193.6	1.0 ± 0.1	13.3 ± 1.0
	0.2	36.4±2.4	20.8±1.6	57.0±1.9	34.0±1.7	15.3±0.9	2.8±0.2	1938 ± 143.0	1.1 ± 0.1	13.5 ± 0.9
	1	35.8±2.8	20.6±2.0	57.1±1.8	33.9±1.6	15.0±0.9	2.8±0.2	1936 ± 127.3	1.0 ± 0.1	13.6 ± 1.6
	2	36.6±2.8	20.1±3.3	57.4±2.8	35.6±3.1	15.4±1.3	2.8±0.2	2043 ± 256.8	1.0 ± 0.1	13.9 ± 2.2
	n-TiO$_2$									
	0.05	35.5±3.1	20.0±2.1	56.4±2.1	34.4±3.0	14.6±0.9	2.7±0.2	1940 ± 236.4	1.0 ± 0.1	13.5 ± 1.6
	0.2	36.1±3.0	20.5±2.0	56.3±2.1	33.5±1.4	14.7±1.3	2.7±0.2	1886 ± 143.1	1.1 ± 0.1	13.2 ± 0.8
	1	35.6±2.0	18.5±2.4	56.5±1.4	33.6±1.1	14.8±0.6	2.7±0.1	1898 ± 98.3	1.0 ± 0.1	14.6 ± 2.1
	2	34.2±4.0	18.8±2.7	55.0±2.3	34.1±2.3	14.3±1.6	2.6±0.3	1875 ± 232.8	1.0 ± 0.1	13.8 ± 1.6
A.violascens Chronic Exposure (0.05 mg/l)	Control	23.6±2.5	12.7±1.6	52.9±2.3	32.7±1.9	9.1±1.2	1.7±0.2	1730 ± 174.2	0.7 ± 0.1	13.4 ± 1.9
	n-ZnO	23.8±2.4	13±2.5	53.1±1.7	32.2±1.3	9.2±0.8	1.7±0.3	1709 ± 122.0	0.8 ± 0.1	13.1± 2.2
	b-ZnO	23.9±2.4	12.7±2	53.0±1.7	32.5±1.5	9.5±0.7	1.8±0.1	1723 ± 131.8	0.7 ± 0.1	14.2 ± 1.8
	n-CuO	23.3±1.8	12.4±1.2	52.5±1.9	32.4±1.8	9.0±0.7	1.7±1.1	1701 ± 154.0	0.7 ± 0.1	13.7 ± 1.7
	b-CuO	23.6±1.7	12.9±1.2	52.9±2	32.9±1.9	9.2±0.9	1.7±0.2	1740 ± 163.3	0.7 ± 0.1	13.2 ± 1.8

of the animals was due to the increased shell weight in later sampling without any significant difference ($P > 0.05$) in soft tissue weight. However, increased shell weight reduced the CI in this dataset. Since CR of the animal depends on the functional activity of live tissue, it is not expected that the difference in CI due to increased shell weight influenced CR results.

2.3.3 Clearance rate

The CR was influenced by the concentration of algae in the medium. The CR trend line shows an increase in CR as the algal concentration in the exposure medium increased (Figure 2.8). A cell concentration 4.0 x 10^6 cells/ml was subsequently chosen for nanoparticle exposure because at this concentration more than 80% of the cells were consumed, food was not limiting in the medium and pseudofaeces were not evident.

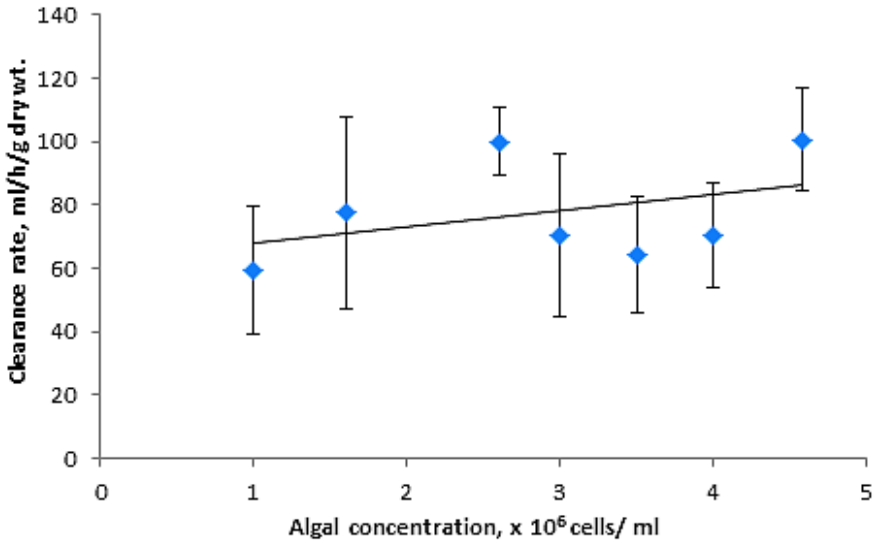

Figure 2.8. Effect of algal concentration on the clearance rate of *A. umbonella* (Mean ± SD).

The CR data for control *A. umbonella* and *A. violascens* are given in Figure 2.9. The data are expressed as conventionally reported in the literature by per animal, and on the basis of the wet and dry weight of the soft tissues. The data for CR were significantly different when expressed on the basis of per animal, wet and dry weight of soft tissue in *A. umbonella* (H = 9.22, DF = 2, P = 0.01) and in *A. violascnes* (H = 15.16, DF = 2, P = 0.001). As can be seen, the data for CR is an important consideration when comparing the efficiency of different species. *A. violascens* appeared more effective at clearing the particulate matter from the surrounding water as CR was higher per hour per animal. However, when expressed by unit wet weight and dry weight of soft tissue the CR looked higher in *A. umbonella*. This necessitated consideration of the expression of the data when comparing the efficiency of different species.

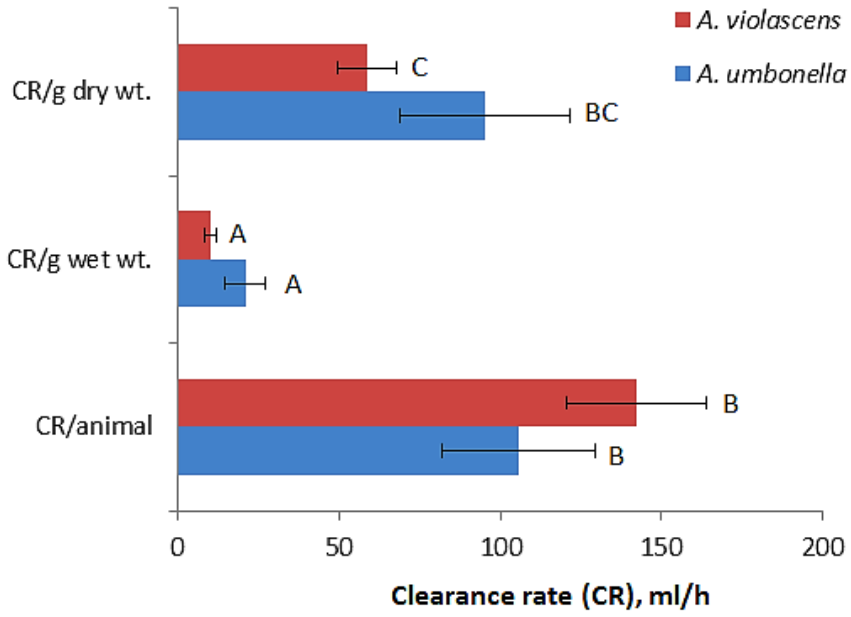

Figure 2.9. Clearance rate in control *A. umbonella* and *A. violascens* expressed on the basis of per animal and the wet and dry weight of soft tissue (Mean ± SD).

2.3.4 Clearance rate after acute exposure

The CR of *A. umbonella* exposed to n-Ag remained within the range of controls at exposure concentrations of 0.05 and 0.2 mg/l. Increasing the exposure concentration to ≥1 mg/l significantly decreased CR ($H = 24.76$, $DF = 4$, $P = < 0.001$) (Figure 2.10 A). In the case of n-TiO_2 exposure at up to 0.2 mg/l caused no change in the CR, and exposure to ≥1 mg/l decreased CR significantly ($H = 19.82$, $DF = 4$, $P = < 0.001$) with respect to the control (Figure 2.10 B).

The CR in n-Ag exposed *A. violascens* fluctuated within the range of controls up to 0.2 mg/l concentration (Figure 2.11 A). Further increase in exposure concentration (≥1 mg/l) resulted in a sharp decline in CR ($H = 23.43$, $DF = 4$, $P = < 0.001$). Exposure to n-TiO_2 up to 0.2 mg/l caused no change in the CR of *A. violascens* compared to controls. At 1 and 2 mg/l the CR was significantly decreased compared to controls ($H = 16.07$, $DF = 4$, $P = 0.003$) but the difference in comparison to 0.2 mg/l exposure was not significant (Figure 2.11 B).

2.3.5 Clearance rate after chronic exposure

Chronic exposure of *A. umbonella* at 0.05 mg/l nanoparticles induced significant decreases in CR. Both n-Ag and n-TiO_2 caused significant declines in the CR of chronically exposed animals compared to control animals. The decrease in CR during n-Ag exposure was more severe compared to n-TiO_2 exposure ($H = 15.17$, $DF = 2$, $P = 0.001$) (Figure 2.12). The CR of *A. violascens* chronically exposed to n-ZnO and b-ZnO was not affected and remained within the CR range observed with control animals. However, exposure to n-CuO and b-CuO caused significant declines in CR compared to control animals and also animals exposed to n-ZnO ($H = 23.43$, $DF = 4$, $P = < 0.001$). It was interesting to note that exposure to b-CuO produced more of an impact on the CR than animals exposed to n-CuO (Figure 2.13).

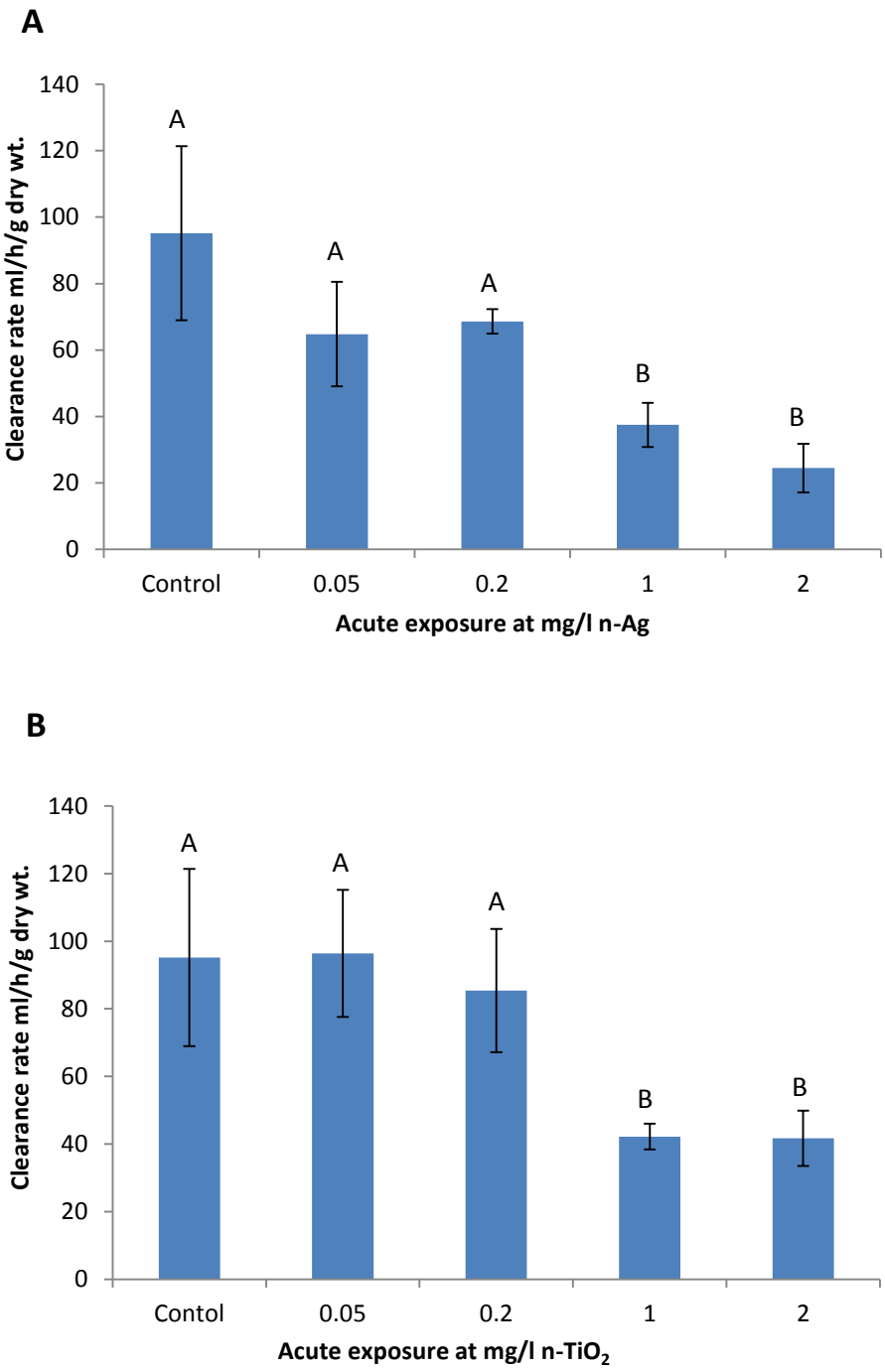

Figure 2.10. The clearance rate of *A. umbonella* after acute exposure to (A), n-Ag (B) n-TiO_2. Means that do not share a letter are significantly different (n = 6).

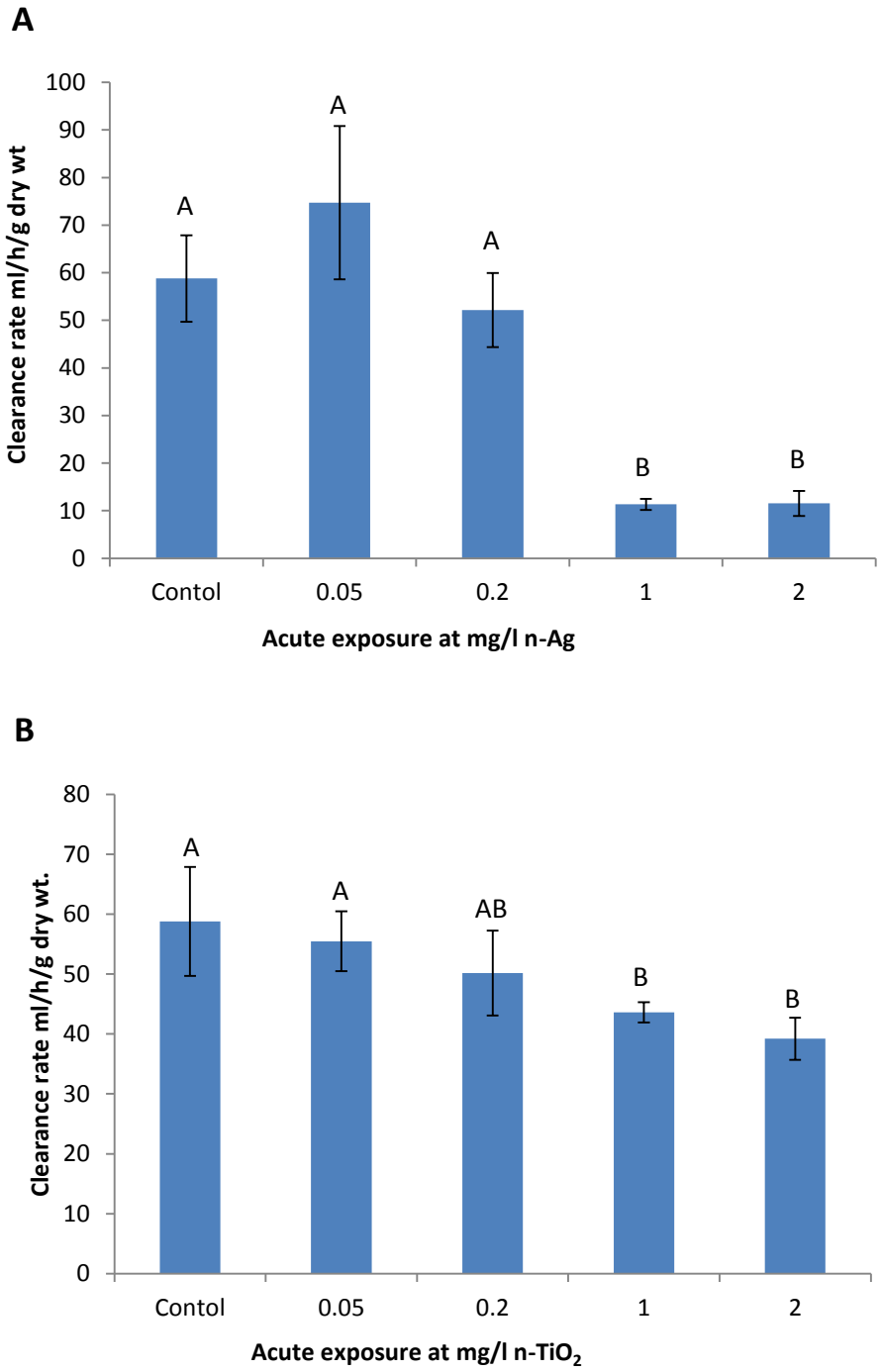

Figure 2.11. The clearance rate of *A. violascens* after acute exposure to (A), n-Ag (B) n-TiO_2. Means that do not share a letter are significantly different (n = 6).

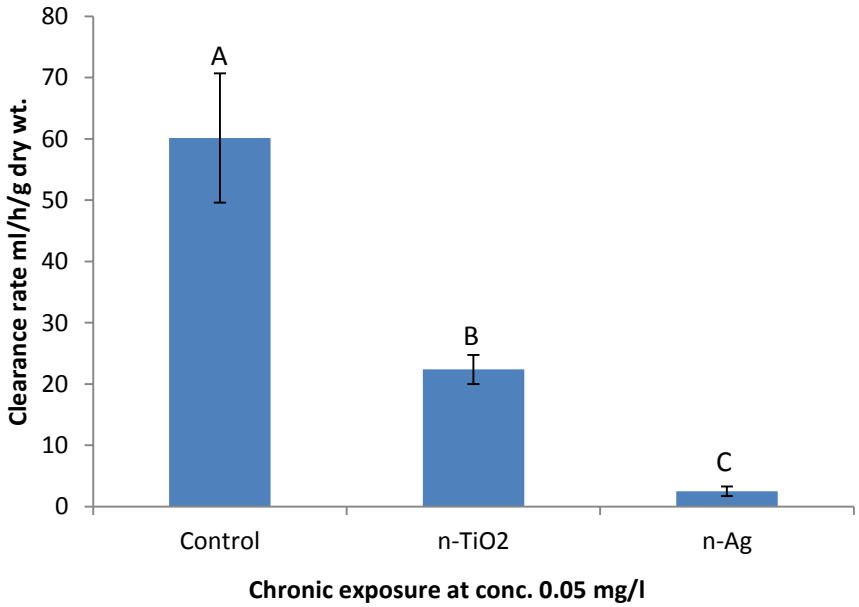

Figure 2.12. Clearance rate of *A. umbonella* after chronic exposure to n-Ag and n-TiO_2 at an exposure concentration of 0.05 mg/l. Means that do not share a letter are significantly different (n = 6).

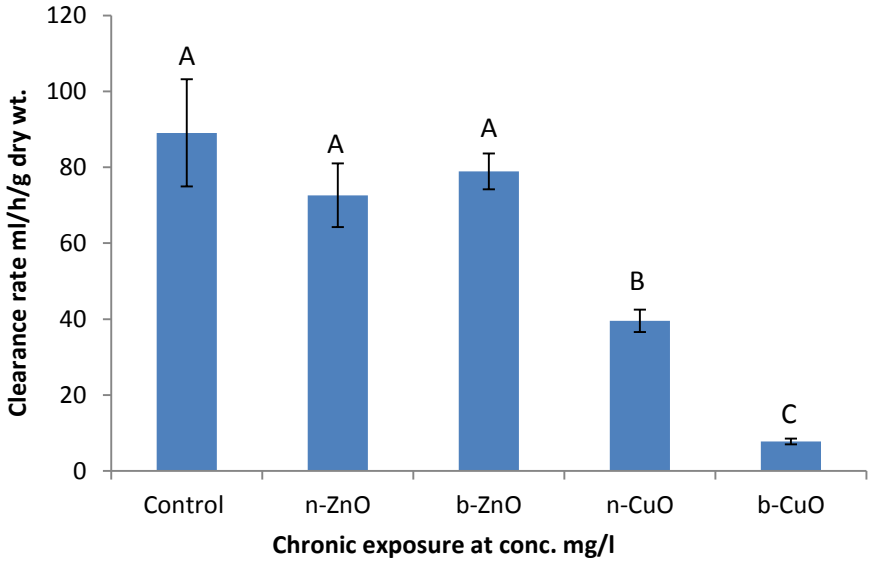

Figure 2.13. The clearance rate of *A. violascens* after chronic exposure to nano and bulk oxides of copper and zinc at an exposure concentration of 0.05 mg/l. Means that do not share a letter are significantly different (n = 6).

2.3.6 Bioavailability of nanoparticles after acute exposure

Total silver and titanium content in the gills and digestive gland of bivalves after acute exposure to n-Ag and n-TiO_2 are reported in Figure 2.14 (A & B). In controls the levels of n-Ag were 1.05±0.156 and 2.40±0.160 µg/g dry wt. in gills and digestive gland respectively. Exposure to 0.05 mg/l n-Ag increased silver concentration, 9-fold in gills and 26-fold in digestive gland. Further increase in exposure concentration to 1.0 mg/l caused a 116-fold and 211-fold increase in silver concentration in gills and digestive gland respectively. However, at 2 mg/l exposure concentration the concentration in gills was increased further to 173-fold compared to that in control gills, but in digestive gland, the concentration was 57-fold higher than controls but far less than that observed at 1 mg/l exposure. The accumulation pattern of titanium in gills and digestive gland after acute exposure to n-TiO_2 was similar to that obtained with n-Ag, but the concentration of titanium accumulated in the tissues is far less than silver to varying concentrations of n-TiO_2. In controls, titanium concentration was 0.239±0.020 and 0.454±0.052 µg/g dry wt. On exposure to 0.05, 1.0, and 2.0 mg/l n-TiO_2 exposure concentration the increase in tissue concentration of total titanium in gills was 11-, 15-, and 19-fold and in digestive gland was 10-, 13-, and 16-fold.

2.3.7 Bioavailability of nanoparticles after chronic exposure

The results of total silver and titanium content in gills and digestive gland of chronically exposed bivalves to 0.05 mg/l n-Ag and n-TiO_2 concentrations are reported in Figure 2.15 (A & B). The accumulation of silver in gills after chronic exposure to n-Ag was 4.51±0.61 and in digestive gland was 11.03±1.52 µg/g dry wt. The increase in the accumulation of total silver after chronic exposure to 0.05 mg/l n-Ag was 8- and 6-fold in gills and digestive gland as compared to control. However, the total silver in tissues after chronic

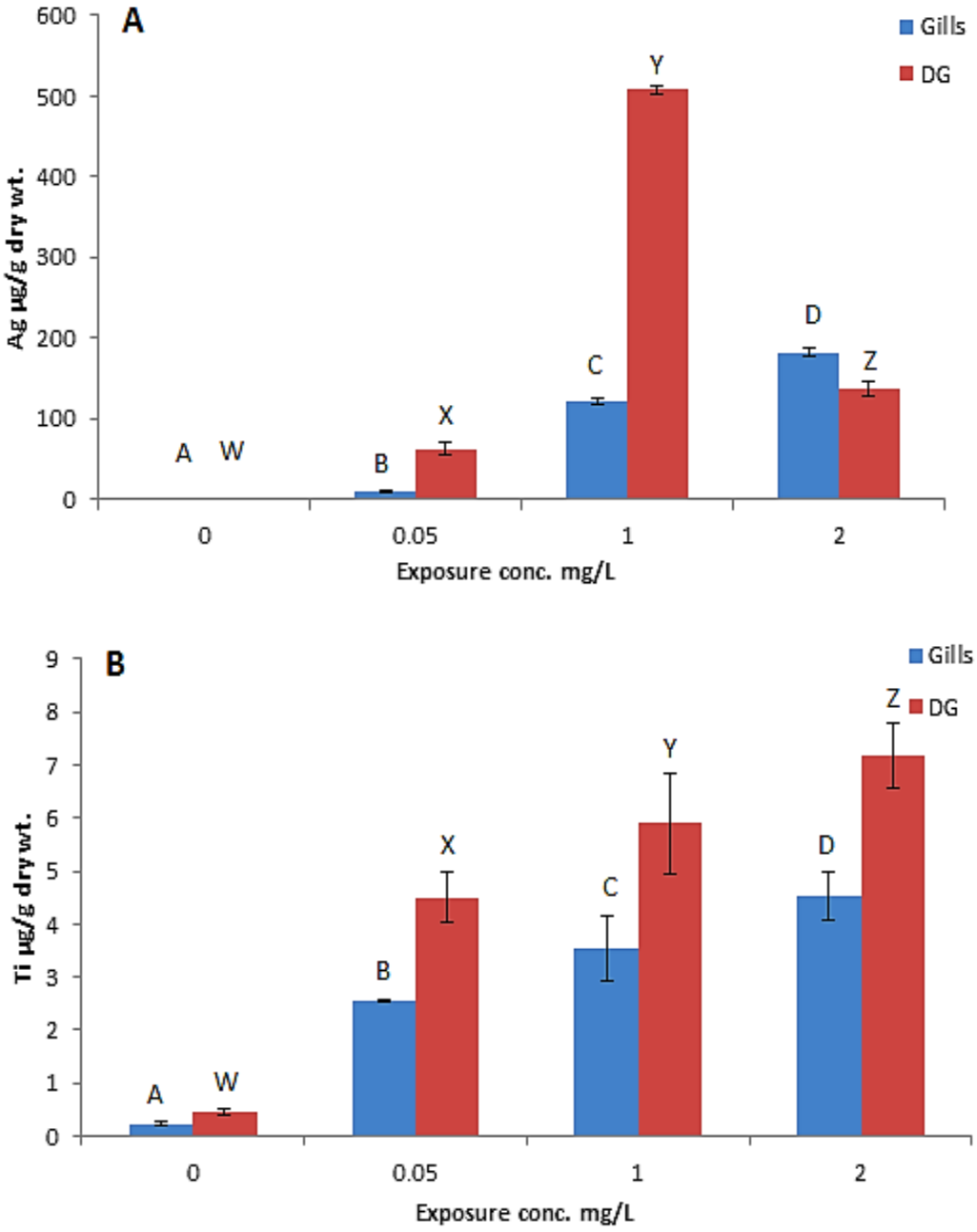

Figure 2.14. The concentration of A) silver and B) titanium (µg/g dry wt.) in gills and digestive gland of *A. umbonella* after acute exposure to silver and titanium dioxide nanoparticles at various concentrations (n = 3)

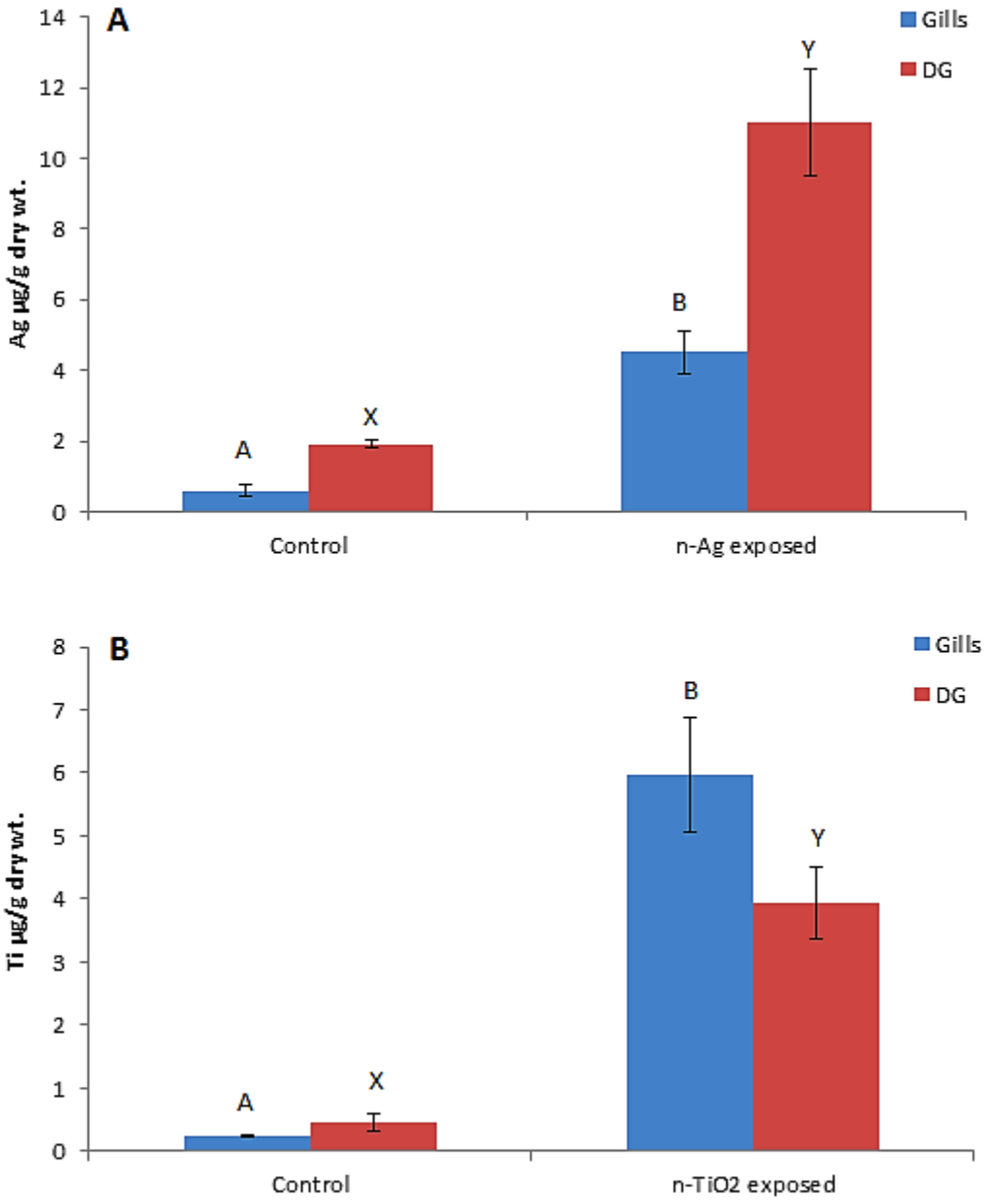

Figure 2.15 Concentration of A) silver and B) titanium (µg/g dry wt.) in gills and digestive gland of *A. umbonella* after chronic exposure to silver and titanium dioxide nanoparticles at 0.05 mg/l concentration (n = 3).

exposure was marginally higher than in the exposed tissues of acutely exposed animals. Chronic exposure to n-TiO_2 also resulted in the low accumulation of titanium in the tissue. In the exposed gills, 6.0±0.92 and digestive gland 3.93±0.57 µg/g dry wt titanium concentration was found. The concentration of titanium was increased in chronically exposed organisms by 25-fold in gills and 9-fold in digestive gland compared to that in controls. The increase in titanium after acute exposure to 0.05 mg/l n-TiO_2 concentration was 11.0-fold in gills and 13.2-fold in digestive gland.

2.4 Discussion

Two techniques, dynamic light scattering (DLS) and transmission electron microscopy (TEM), were used for nanoparticle size characterisation and the state of agglomeration in an aqueous medium. It was observed that nanoparticles suspended in the aqueous medium had some degree of agglomeration; however, a large number of particles were also freely suspended. In DLS, n-Ag revealed a unimodal pattern and the average particle size in suspension was close to the size reported by the manufacturer, whereas n-TiO_2 showed a bimodal pattern showing a small peak of very fine particles and the second peak of particles averaging larger than reported by the manufacturer. This shows the agglomeration of particles in n-TiO_2 suspension along with the presence of smaller size particles. A bimodal pattern was found in the n-CuO suspension, also with a small peak of very fine particles of <1nm and the other major peak of 7.6 nm which were far less than the size reported by the manufacture. However, using TEM analysis particles of larger size were observed. In the n-ZnO suspension, a unimodal pattern in DLS was obtained, and the particle size of 13.1 nm was detected which was lower than <50 nm reported by the manufacturer but using TEM, larger particles were detected, and some particles were big enough to show agglomeration. In general, agglomeration in n-TiO_2, n-CuO and n-ZnO was higher than n-Ag as revealed in TEM images. The particle size of n-Ag being greater than the other nanoparticles may be one of the reasons that agglomeration was less in its

suspension. Bulk ZnO and CuO were of a larger size, and the particles were not spherical in shape. The study revealed that the two methods of measurement do not provide the same measure of particle size and the same has been reported in earlier studies (Mahl et al., 2011). It has been reported that nanoparticle aggregation during sample preparation is a limiting factor in size determination by TEM (Shang et al., 2014) and that may be the reason for the discrepancy in the two methods observed in the present study. However, the two methods showed particles used that were in the nano-size range, and there was a difference in the nano and bulk material in size and shape of the particles and wherever agglomeration was found it was detected. It has been shown that a change in agglomeration state occurs due to the presence of biological fluids, protein and polysaccharide in the medium (Fabrega et al., 2011). The effect of the presence of bivalve in the exposure medium on the agglomeration pattern was not determined in the present study. Such information could be generated if a facility for a continuous flow system for real-time monitoring of the particle agglomeration in the exposure system was available. The possibility of agglomeration cannot be eliminated, though nanoparticle preparation was sonicated before mixing with the exposure medium and a continuous stream of air was bubbled to keep particles in a dispersed state.

The CR responses of both the bivalves were similar to nanoparticle exposure, i.e. no change at lower concentrations and then a decline at concentrations of ≥1mg/l. The data suggest that *Asaphis violascens* were more sensitive to n-Ag by exhibiting a significant decrease at a lower concentration and a sharp decline at concentrations of ≥1mg/l.

Bivalve gills function as water transporting and particle trapping organs. The gill structure contains a lateral ciliary tract for water pumping and a frontal ciliary tract for particle transportation. The retention efficiency depends on particle size since bivalves can reject unwanted particles as pseudofaeces. The volume of water cleared of particles per unit time is estimated by measuring the reduction of particles in a known volume of water. It is documented for mussels (Famme et al., 1986;

Riisgård, 1988) that the indirect clearance method agrees well with other both indirect and direct methods used for measuring filtration rates. In the filtration process, the particles are trapped on the gills and consumed as a source of food. CR is used as a means to measure the efficiency of particle removal by the animals.

The data for morphometric parameters showed no change due to exposure to engineered nanoparticles as the soft tissue weights and condition index in the exposed animals remained similar to that found in controls (Table 2.3). The weight of the whole animals used for chronic exposures was more than the animals used for acute exposure, which was mainly due to the higher shell weight whereas the soft tissue weight of the animals in the two set of exposure was not different. Since the CR is the function of the soft tissue of the animal the higher shell weight is not expected to influence the results of the CR reported in this study. However, the increased shell weight resulted in the reduction of the calculated CI values in the dataset with no change in soft tissue weight. Similar observations were also reported by Gimin et al. (2004).

A preliminary experiment designed to find the most appropriate microalgae concentration to be used for CR determination revealed that CR increased by increasing the algal concentration from $2.6x10^6$ cells/ml to $4.6x10^6$ cells/ml. However, the increase was not proportional to the algal concentration in the medium. In some of the reported studies, a reduction in CR at high algal concentrations was observed which was attributed to the closure of the valves due to the phenomenon of food saturation reduction (Clausen and Riisgård, 1996; Dolmer, 2000; Riisgård, 2001a, b). However, the phenomenon of saturation reduction was not observed with the concentration of algae used in the present study. A concentration of 4.0×10^6 cells/ ml was chosen for exposure because at this concentration more than 80% of cells were removed, food in the medium was not limiting, and no pseudofaeces was observed.

Expression of data for comparing the CR in different species is an important consideration because of the differences in size of the animal, fresh and dry weight of the soft tissue. In literature, CR is mainly expressed by per animal and related to its size (Reeders and De Vaate, 1990); however, some other reports expressed the data by dry weight of the soft tissue (Li and Wang, 2001; Riisgård, 1988). It was observed in the present study that among the two species the CR, when expressed on per animal basis, was higher for *A. violascens* (140 ml/h/animal) compared to *A. umbonella* (90 ml/h/animal) this may be related to the former being of larger size. Zebra mussels filter water at about 50 ml/h/animal (Reeders and De Vaate, 1990). According to Zebra Mussel Research (1993), the mussels do not filter continuously; however, it has been estimated that an individual zebra mussel of shell length 20 mm is likely to filter one litre of water per day and about 42 ml/h/animal (Lei, 1993). The same was observed in this study where *A. umbonella* with a shell length of 40 mm filtered around 90 ml of water per hour which is around two litres of water filtered per day, and for *A. violascens* with the shell length of 60 mm, three litres was filtered per day. So, it can be said that filtration rate in bivalves is directly related to the size of the animal; however, this is not always true, since some other estimates showed very high filtration rates of mussel, i.e., 300 ml/h/mussel (Kryger and Riisgård, 1988).

It appears that discrepancies in CR reported in mussel may be due to different sizes of animals. Therefore, it is better to express the data by the unit weight of the soft tissue. It was observed that the soft tissue weight of *A. umbonella* was 5.2 g/animal compared to 14.3 g/animal in *A. violascens*, more so the per cent dry weight was 22.9% and 18.5% respectively showing that the soft tissue of *A. violascens* contained more water compared to *A. umbonella*. When the CR data were expressed on the basis of dry weight of soft tissue, the filtration rate was found to be 58 ml/h/g dry weight in *A. violascens* compared to 95 ml/h/g dry weight in *A. umbonella*, showing that *A. umbonella* is more efficient in clearing the surrounding water. This subject has been debated in the literature and the filtration rate is correlated with the

size of animal, length of the shell, soft tissue mass and dry weight of the soft tissue of the animals, whereas other studies showed no correlation of CR with these parameters (Rajesh et al., 2001; Riisgård, 1988, 2001a, b). However, these studies were mainly conducted to judge the efficiency of bivalves present in a given area on the filtration of water and utilisation of bacterio-plankton and phytoplankton. It has been calculated that zebra mussels process from 39-96% of the entire water column daily in various regions (Bunt et al., 1993). The measurement procedures also differed; both direct and indirect methods for the measurement of CR have been used, and no method was allocated superiority over the others. In direct methods, exhaled water was separated from the surrounding water and measured (Famme et al., 1986). A variety of direct methods using flow-through chamber systems and suction methods for the collection of reliable data on CR of bivalves have been described by Riisgård (2001b). Each method has pros and cons, and the variation in the CR data was partly because of differences in experimental conditions. In the indirect method, the volume of water cleared of suspended particles per unit of time is measured. Coughlan (1969) studied indirect methods and suggested that indirect methods are based on the rate of removal of particles from a known volume of suspension. It requires that a suspension feeder be allowed to feed for a given time in a volume of suspension.

The indirect clearance method is frequently used by counting the number of unicellular algae at different time intervals in the surrounding medium. The time interval chosen by many authors ranged from 15 minutes to several hours, and the results were expressed on a per

hour basis (Li and Wang, 2001; Nilin et al., 2012; Rajesh et al., 2001). A longer period for the measurement of CR was used because the nature of bivalves used in the study was very sensitive, they take 15-30 minutes to open the shell and sometimes with little agitation they quickly closed their shell, and short-term measurement have not produced reproducible data. In one experiment the CR measured after 2 hours was 2.5-times higher than measured at 24 hours showing that the CR decreased with time, but 24h data provided an average value which was consistent and reproducible.

Another consideration was the behaviour of the treated animals that took a longer time than control animals in opening the shells when transferred from the exposure medium to clean water. The objective of this study was to investigate the effect of nanoparticle exposure on the functional activity of bivalves and for that collection of data at the stabilized condition of control and treated animals was essential. The time period used for CR measurement produced uniform data in multiple controls.

Exposures to varying concentrations of nanoparticles produced an uneven response, and CR varied with nanoparticle concentration. Exposure to n-Ag caused a concentration-dependent decrease in CR in *A. umbonella*, and the change was significant at ≥1mg/l concentration, whereas, n-TiO_2 insignificantly enhanced CR at the lowest exposure concentration but decreased significantly at higher exposure concentrations. The effect of n-Ag and n-TiO_2 on *A. violascens* was similar, both increased CR at the lowest exposure concentration but decreased it at higher exposure concentrations. The decrease in CR was statistically significant on exposure to n-Ag at all the tested concentrations, but exposure to n-TiO_2 caused an insignificant increase in comparison to controls at lower concentration but a significant decrease in CR at higher concentrations.

At higher concentration, the inhibition in CR persisted in n-Ag treatment, and n-TiO_2 also caused significant lowering at higher concentration, but the response was less compared to n-Ag. The difference in the effect on the functional activity may be attributed to the process of agglomeration of nanoparticles in seawater. It has been shown by dynamic light scattering studies that n-Ag in seawater quickly agglomerate and the size increases from 20 to 40 to greater than 400 nm (Buffet et al., 2013a).

It was interesting to note that exposure to a low concentration of nanoparticles (50µg/l) did not cause a significant effect on *A. umbonella* in short-term exposure, but the same concentration in chronic exposure trials of 4-weeks significantly decreased the CR. The inhibitory effect on the CR of n-Ag was severe in comparison to the n-TiO_2. In one recent study, Buffet et al. (2013a) observed no effect on CR of bivalves

after up to 8 days of exposure whereas after 10 days of exposure a significant effect was observed in comparison to controls. The effect of n-Ag on the functional activity of bivalves observed in our study was similar to that reported by Buffet et al. (2013a) and differences in the degree of effect are attributed to the concentrations used for exposure and also the differences in test species. In the present study, the two species of bivalves showed broadly similar responses to exposure to nanoparticles.

In order to test the effect of other nanoparticles and also to examine the difference in response by nano and bulk ZnO and CuO, *A. violascens* were chronically exposed for 4-weeks. Treatment with n-CuO and b-CuO caused a significant reduction in CR; the effect of b-CuO was more severe. Copper was found to significantly inhibit the CR of bivalves at concentrations comparable to that used in this study (Al-Subiai et al., 2011). One recent study on mussels exposed to n-CuO reported a decrease in CR at >1mg/l exposure concentration and n-CuO were much less toxic than Cu ions to marine mussels (Hanna et al., 2014). The difference in bulk and nano CuO observed in the present study may be due to their solubility since n-CuO does not readily dissolve in aqueous media (Baek and An, 2011; Mortimer et al., 2010). The toxicity caused by n-CuO is mainly due to the particle and not the ion, suggesting a nano-specific toxic effect (Hanna et al., 2013; Midander et al., 2009). Contrary to n-CuO, exposure to n-ZnO caused a significant decrease in CR, b-ZnO also reduced CR, but the degree of effect was lower than n-ZnO. It is reported that n-ZnO dissolve rapidly in seawater (Miller et al., 2010), the effect observed in this chapter may probably be due to Zn^{2+}. Not many reports are available on n-ZnO effects on bivalves. Recently, Hanna et al. (2013) reported that n-ZnO increased respiration rate in mussels after chronic exposure and their survival decreased.

The nanoparticles selected for the present study are the most widely used nanoparticles, and their release in marine ecosystems is predicted/documented in several studies (Kägi et al., 2008; Mueller and Nowack, 2008; Ward and Kach, 2009; Zou et al., 2013). Therefore, the hypothesis that the nanoparticles present in the marine water will influence the functional activity of bivalves was tested. Metal

analysis using ICP-OES showed that exposure to nanoparticles results in bioaccumulation of metals in gills and digestive glands of exposed animals. Estimation of total metals by ICP-OES method does not provide an idea whether the metals were accumulated in ionic form or in nano form, but it ascertains that by exposure to metallic nanoparticles, total metal content in tissues is increased depending on the concentration in exposure medium.

In the case of n-Ag, it has been reported in earlier studies that mussels quickly accumulated Ag in both gills and digestive glands, regardless of the Ag form used (Gomes et al., 2014b). The nano metal content in digestive gland was several folds higher than in the gills. It has been documented that silver is the choicest metal that is accumulated in bivalves in the highest concentration and gills play an important role in metal accumulation (Rainbow, 1990). There is a quick translocation of n-Ag or silver ions from gills that are in direct contact with the exposure media, towards the digestive glands, and consequently the accumulation is always higher in digestive gland (Al-Sid-Cheikh et al., 2013; Canesi et al., 2012; McCarthy et al., 2013; Ward and Kach, 2009).

Contrary to silver the concentration of total titanium accumulated in gills and digestive gland of animals exposed to n-TiO_2 was extremely low. However, between the gills and digestive gland, a much larger amount was found in the digestive gland. The reason may be attributed to the solubility of n-TiO_2 in the exposure medium. Canesi et al. (2014) reported that a very low concentration of titanium was accumulated in the digestive gland after exposure to 100 ug/l nominal exposure concentration of n-TiO_2. The author considered the reason for low accumulation to the availability of n-TiO_2 in seawater suspension, as they observed the actual concentration of dispersed n-TiO_2 was found to be far lower than the nominal concentration, with both agglomeration and sedimentation mainly depending on n-TiO_2 initial concentration and, to a minor extent, on salt content, ionic strength, and dissolved organic material. Determination of nanoparticle concentration in exposure medium is still a challenge because of interaction with salt in seawater and other

confounding factors like organism's secretion of mucous and faecal pellets and adsorption on its shell. It has been calculated that only a minor fraction (about 10%) of the nominally added n-TiO_2 is available to the organism in the suspended form (Balbi et al., 2014). However, if compared to control the total titanium concentration in the gills and digestive glands was manifold higher and the effect observed in biochemical response was the result of the presence of titanium in the tissue. Earlier electron microscopic study demonstrated the presence of small n-TiO_2 agglomerates within the lysosomes of digestive gland cells under the same exposure conditions (Barmo et al., 2013).

The present study also revealed that in chronic exposure for 4-weeks the total metal concentration in gills and digestive glands was not distinctly increased compared to acute exposure for 48h. This observation indicated that on the chronic exposure the organism prepared itself to deal with the thrust of contaminants in surrounding water by enhancing metabolic disposition. Gomes et al. (2014b) also suggested that on the prolonged exposure the metal concentration in the tissue of exposed organism decreases possibly due to activation of detoxification mechanism. However, the exposed organism suffers due to metabolic adjustment as observed in the significant decrease in CR after chronic exposure in the present study.

Bivalves are important for maintaining coastal marine ecosystems and help maintain water quality through filter-feeding. The study suggested that acute exposure to nanoparticles causes concentration-dependent changes in the functional activity of bivalves and chronic exposure reduces functional activity as CR decreased drastically, the degree of change depends on the nature of nanoparticles and their concentration in the exposure medium, length of exposure and species of bivalve. How far this change in functional activity is reflected in the tissue damage (mainly gills and digestive glands) and the enzymes of oxidative stress is examined in subsequent chapters.

Chapter 3. Effects of Nanoparticles on Biomarkers of Oxidative Stress in Marine Bivalve Molluscs

3.1 Introduction

The toxicity of metal-based nanoparticles can arise either due to the release of dissolved metal ions into the environment or due to their surface physico-chemical characteristics (Buffet et al., 2014b). The released metal ions cause damage at various levels within cells due to their long retention time. Nanoparticles may be taken up and can exert toxic effects in different ways in an organism; however, the exact modes of toxic action are not yet completely known (Bhatt and Tripathi, 2011). Chemical mechanisms mainly include the production of reactive oxygen species (ROS), dissolution and release of toxic ions, disturbance of the electron/ion cell membrane transport activity, and oxidative damage through catalysis and lipid peroxidation (Elsaesser and Howard, 2012). If an imbalance (i.e., oxidants > antioxidants) occurs between oxidants and antioxidants, oxidative stress arises that can damage tissue, molecules and cellular function (Baker et al., 2014)

Bivalves can detoxify ROS (Bigot et al., 2009; Solé et al., 2010). Oxidative stress is recognized as one of the most common effects of nanotoxicity (Klaine et al., 2008), therefore the antioxidant defence mechanism assumes considerable significance for the survival of the organism. The antioxidant defence mechanism can be subdivided into: i) enzymatic antioxidants such as catalase, superoxide dismutase (SOD), glutathione S-transferase (GST), glutathione peroxidase (GPx), and glutathione reductase (GR); and ii) non-enzymatic antioxidants such as glutathione, vitamin E, ascorbate, β-carotene, and urate (Valavanidis et al., 2006). The level of thiobarbituric acid reactive substances (TBARS) - a gross biomarker of oxidative injury - indicates if this protective mechanism is compromised (Bergayou et al., 2009; Parvez et al., 2006; Regoli et al., 2011). In addition, metallothioneins, which are

involved in both antioxidant defence and metal detoxification, have also been recognized as useful biomarkers to reveal the presence of bioavailable metals (Amiard et al., 2006; Buffet et al., 2013a).

Antioxidant enzymes act jointly to destroy ROS within cells. Catalase is one of the primary enzymes that degrade hydrogen peroxide produced within the cell. The other enzyme that catalyzes organic and inorganic peroxides is selenium dependent glutathione peroxidase (Se-GPx), the other isoform of which (selenium independent GPx) reduces only organic peroxides (Bigot et al., 2009). Glutathione reductase is another antioxidant enzyme that maintains the balance of reduced glutathione (GSH) and oxidized glutathione (GSSG) in favour of its reduced form GSH, which acts as soluble antioxidant by itself and also plays a role as a cofactor in other enzymatic activities (Solé et al., 2010). Glutathione S-transferase represents a major group of detoxification iso-enzymes whose 'natural' substrates range from molecules of foreign origin to by-products of cellular metabolism. It plays an important role in catalysing the conjugation of reduced glutathione to various electrophilic compounds but can also act as binding proteins sequestering hydrophobic molecules (Manduzio et al., 2005; Prohaska, 1980). Metallothioneins are also known to play important roles in quenching the effects of ROS. The assessment of these antioxidant enzymes has been shown to be a suitable tool for monitoring environmental pollutants in mussels (Box et al., 2007; Cheung et al., 2004; Lionetto et al., 2003; Regoli and Principato, 1995), reflecting the levels of exposure to environmental toxicants.

In this chapter, the impacts of acute (48 hour) and chronic (four weeks) exposure to metallic nanoparticles on oxidative stress in the gills and digestive glands of two bivalve species, *Amiantis umbonella* and *Asaphis violascens* were investigated. Catalase, GST, GPx, and GR activities were evaluated as biomarkers of oxidative stress, TBARS as a measure of membrane lipid peroxidation, and metallothioneins as metal scavenging proteins.

3.2 Materials and Methods

The methods for animal collection and maintenance, preparation of nanoparticle suspensions and the nanoparticle exposure experiments are described in Chapter 2. The gills and digestive glands of twelve exposed animals were excised and for enzyme assays the tissues from two animals were pooled to constitute one sample of gill and one of digestive glands. Thus six samples were analysed for protein estimation, enzyme assays and metallothionein determination. All the reagents used below were prepared using distilled water (DW).

3.2.1 Tissue homogenisation

The excised gills and digestive glands were weighed separately and stored on ice before homogenization. The weighed samples were homogenized in chilled Tris buffer containing 150 mM KCl, 1 mM dithiothreitol (DTT), and 0.1 mM phenylmethylsulfonyl fluoride (PMSF), with a pH of 7.4 using a Potter-Elvehjem homogenizer to prepare 10% homogenates. The homogenate was centrifuged at 15, 000 x g for 20 minutes at 4 °C in a Beckman refrigerated centrifuge to obtain the post-mitochondrial supernatant (PMS). The supernatant obtained was separated using Pasteur pipettes and stored at -80 °C until analysis. Enzymatic assays of the PMS were conducted in accordance with Pandey et al. (2003), with some modification.

3.2.2 Total protein estimation

Total protein content of the PMS was determined by the Lowry method (Ulmer VerlagLowry et al., 1951) from a 10 µl aliquot of PMS made up to 1 ml in distlled water. The concentration of protein in the sample was determined against a standard curve prepared with 0.05 % stock solution of bovine serum albumin (BSA) in distilled water. A series of dilutions (0-250 µg) were made in replicates of four with a final volume of 1 ml. Samples were diluted such that they fell within the BSA

standard range. After standards and samples were diluted, 5 ml of copper reagent was added to each test tube and mixed thoroughly. Copper reagent was prepared by mixing 0.5 ml of 1 % cupric sulfate with 0.5 ml of 2 % sodium potassium tartrate, followed by the addition of 50 ml of 2 % sodium carbonate in 0.1 N NaOH. The mixture was then allowed to incubate at room temperature for 10-15 minutes prior to the addition of 500 µl of 1 N Folin-Ciocalteu reagent. Samples were vortexed immediately and the colour was allowed to develop for 30 minutes at room temperature. A reagent blank was also prepared along with the standard and samples. The absorbance was measured at 600 nm with a quartz cuvette using a UV-visible spectrophotometer (UV-1601, Shimadzu, Japan).

3.2.3 Oxidative enzymes

Catalase assay

Catalase activity was determined using the method of Claiborne (1985). The reaction mixture consisted of 2.38 ml of 0.1 M phosphate buffer (pH 7.4), 600 µl of 0.5 M hydrogen peroxide (H_2O_2), and 20 µl of PMS, for a final volume of 3 ml. Changes in absorbance were recorded at 240 nm using a UV-visible spectrophotometer at room temperature (20±2 ºC) for three minutes and enzyme activity was calculated using an extinction coefficient of 0.04 mM^{-1} cm^{-1}.

Glutathione peroxidase (GPx) assay

GPx activity was assayed using the method of Mohandas et al. (1984). The reaction mixture consisted of 2.3 ml phosphate buffer 0.1 M phosphate buffer (pH 7.4), 100 µl 30 mM ethylene diamine tetraacetate EDTA, 100 µl of 30 mM sodium azide, 100 µl of IU/ml glutathione reductase, 100 µl of 30 mM reduced glutathione GSH, 150 µl of 4 mM nicotinamide adenine dinucleotide phosphate reduced NADPH, 100 µl of 7.5mM hydrogen peroxide and 50 µl of PMS in a total volume of 3 ml. Changes in absorbance were recorded at 340 nm using a UV-visible

spectrophotometer at room temperature (20±2 ºC) for three minutes using an extinction coefficient of 6.22 mM^{-1} cm^{-1}.

Glutathione reductase (GR) assay

GR activity was assayed using the method of Carlberg and Mannervik (1975), as modified by Mohandas et al. (1984). The reaction mixture consisted of 2.7 ml of 0.1 M phosphate buffer (pH 7.4), 100 µl of 10 mM EDTA, 100 µl of 20 mM oxidized glutathione (GSSG), 50 µl of 4 mM NADPH and 50 µl of PMS, for a total volume of 3 ml. GR enzyme activity was recorded at 340 nm at room using a UV-visible spectrophotometer for three minutes at room temperature (20±2 ºC) using an extinction coefficient of 6.22 mM^{-1} cm^{-1}.

Glutathione S-transferase (GST) Assay

GST activity was determined using the method of Habig et al. (1974), with some modifications. The reaction mixture consisted of 2.69 ml of 0.1 M phosphate buffer (pH 7.4), 100 µl of 30 mM EDTA, 100 µl of 30 mM reduced glutathione (GSH), 10 µl of PMS and 100 µl of 30 mM 1-chloro-2,4-dinitrobenzene, for a total volume of 3 ml. Changes in absorbance were recorded at 340 nm using a UV-visible spectrophotometer for three minutes at room temperature (20±2 ºC) using an extinction coefficient of 9.6 mM^{-1} cm^{-1}.

3.2.4 Lipid peroxidation

Lipid peroxidation was assayed by measuring thiobarbituric acid reactive substances (TBARS) in PMS by the method of Camejo et al. (1998) as modified for spectrophotometric analysis to suit bivalves preparations. To a 400 µl aliquot of PMS 100 µl of 1 nmol l^{-1} butylatedhydroxytoluene (2,6-Di-O-*tert*-butyl-4-methylphenol or BHT) was added to stop further oxidation of the sample. To this was added 1.4 ml of 0.1 M phosphate buffer at pH 7.5 to bring the volume up to 1.9 ml followed by the addition of 50 µl of 50 % (w/v) trichloroacetic acid (TCA) to each tube and 750 µl of

1.3 % (w/v) thiobarbituric acid (TBA) dissolved in 0.3 % sodium hydroxide (NaOH) to each tube. The tubes were sealed and incubated for 60 minutes at 60 °C, centrifuged for 20 minutes at 20, 000 x g and the colour change was measured at 586 nm against a blank. The activity was expressed as pmol of malondialdehyde/min/mg protein using the extinction coefficient 1.56 $x10^5$ M^{-1} cm^{-1}.

3.2.5 Metallothionein determination

The method of Podrug and Raspor (2009) was chosen to adopt the protocols for metallothionein determination by Differential Pulse Voltametry (DPV). A different homogenization process was used to prepare the PMS for metallothionein determination. The digestive gland and gills of animals were excised as per standard protocols and homogenized in buffer containing Tris 100 mM; dithiotheritol (DTT) 1 mM; potassium chloride (KCl) 150 mM (pH 7.5 keeping the tissue: buffer ratio as 1:5). The homogenate was centrifuged at 30,000 g at 4 °C to collect the supernatant, and the protein was determined by the Lowry method. The supernatant obtained was diluted ten times with 0.9 % saline solution and heated at 85 °C for 10 minutes. After heating, the solution was kept in an ice bath for 30 minutes and centrifuged at 4 °C at 20,000 g for 15 minutes. The supernatant was stored at -80 °C until analysis. For the detection of metallothionein by DPV, 5 ml of 2 M ammonia buffer (NH_4Cl/NH_4OH) of pH 9.6 and 5 ml of Brdicka supporting electrolyte, and 1 mM hexamine cobalt (III) chloride [$Co(NH_3)_6Cl_3$] solution were taken in the cell of 797-VA Computrance Metrohm Voltameter (3-electrode system) with a hanging mercury drop electrode (HMDE) of surface area of 0.4 mm^2, counter or supporting electrode-platinum tip and a reference electrode Ag/AgCl/saturated KCl. It was noted that in HMDE mode the linearity at high concentrations was erratic giving problems of reproducibility. In static mercury drop electrode (SMDE) mode higher concentrations of metallothionein could be determined with linearity up to 60 µl addition of stock solution. Therefore SMDE mode was used in preference to HMDE.

The equipment setup parameters were; scan from = -0.9 V to -1.65 V; scan rate, 0.005 V/second; voltage pulse amplitude, 0.025 V; duration of the pulse application, 0.057 seconds; and clock time, 0.5 seconds. Rabbit liver zinc-metallothionein-1 was obtained from Alexis Biochemicals, Switzerland. The preparation was supplied in 25 mM Tris-HCl buffer of pH 8.0 containing 50 mM NaCl at 1380 µg/ml concentration. A working standard (50 µg/ml) was prepared by diluting in 0.25 M NaCl and was used to prepare the standard curve (Figure 3.1). The concentration of metallothionein in the samples was determined from the calibration line of the standard. In several gill preparations the concentration of metallothionein was very low, therefore a standard addition method was adopted to determine the concentration in the tissue samples. The concentration of metallothionein was expressed as µg/g fresh tissue.

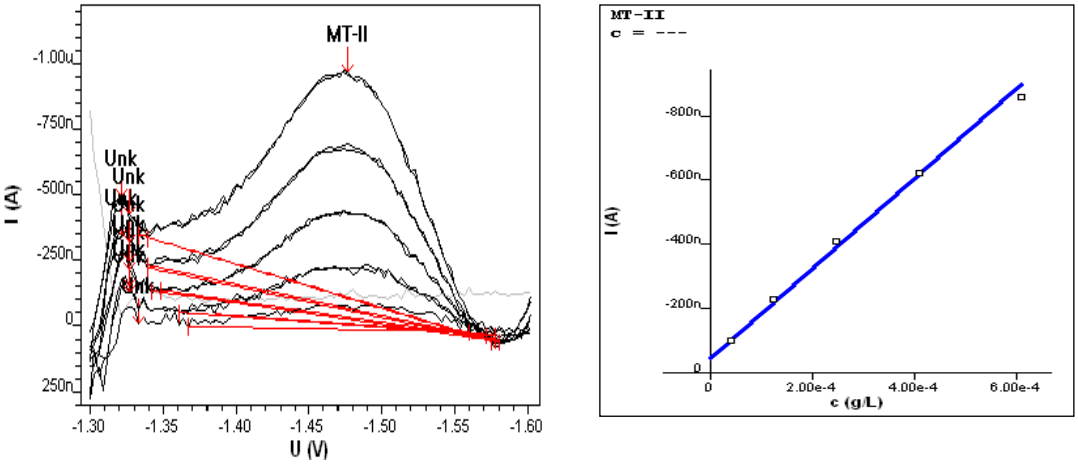

Figure 3.1. Calibration curve of rabbit liver metallothionein using Metrohm 797 VA Computrace (Version 1.3.2.65) Voltameter, SMDE mode A) Peak height of standard metallothiein at 0.5, 1.0, 1.5, 2.0, 2.5 µg concentration B) Standard curve of metallothionein concentration versus current (I = current; A = ampere; U = voltage; V = volts; C = concentration).

3.2.6 Calculation method for enzyme activities

The enzyme assays were conducted in 3 ml assay volume in a cuvette of 1 cm light path, wherein 0.02 ml aliquot of post mitochondrial supernatant of 10% homogenate was used for the assay. The change in optical density (OD) of substrate was followed for 3 min and averaged as change per min. This change in OD/min by 0.02 ml of enzyme was equated to 10 ml (equivalent to 1 g tissue weight) and value obtained was expressed OD change/gram tissue weight. This value was divided by the mg protein/g tissue weight to obtain specific activity of enzyme. Thus, the specific activity of enzyme obtained was OD change/min/mg protein. The OD value was converted to molar values by using molar extinction coefficient of substrate and the data are finally expressed as, milli mole, micro mole, nano mole, or pico mole of substrate changed/min/mg protein. The extinction coefficient of substrate used is given under the assay method of each enzyme described above.

3.2.7 Statistical analysis

Statistical analysis was carried out using Minitab 17 statistical software. All data are presented as mean ± standard deviation (S.D.) of at least six samples in triplicate. Significant differences in the biochemical parameters (CAT, GPx, GR, GST, LPO and metallothionein) between the control groups and the exposed groups at various nanoparticle concentrations were detected using one-way analysis of variance (ANOVA) after the data had been checked for assumptions of normality and homogeneity (Leven's test). Fisher's multiple comparison test was used to differentiate between the groups of data and only $P<0.05$ was accepted as significant. Wherever the assumptions for normality were not met, the data were log ten transformed to meet the normality requirements. The literature referred for statistics supported and suggested the efficiency of log ten transformations and confined to normal distribution after log transformations. The Kruskal-Wallis test was done followed by Dunn's multiple comparison tests for multiple treatments. For every

exposure experiment with various nanoparticles, ANOVA was done independently for each species.

3.3 Results

3.3.1 Oxidative stress in *Amiantis umbonella* in response to acute exposure to nanoparticles

The levels of antioxidant enzymes were analyzed in the gills and digestive gland of *A. umbonella* after acute exposure to n-TiO_2 and n-Ag. The data are presented below.

Protein

The protein content in the digestive gland was 4-fold higher than in gill tissue (Figure 3.2 A and B). Exposure to nanoparticles did not cause any significant change in the mean gill or digestive gland protein concentration in both n-TiO_2 (gills: DF = 4, F = 1.88, P = 0.145; digestive gland: DF = 4, F = 2.25, P = 0.092) and n-Ag exposure (gills: DF = 4, F = 0.79, P = 0.546; digestive gland: H = 5.49, DF = 4, P = 0.241) (Figure 3.2 A and B).

Catalase

There was no consistent pattern in catalase activity between the two nanoparticles. Exposure to n-TiO_2 caused a significant increase at 0.05 mg/l in both gills and digestive gland compared to controls. However, exposure to higher n-TiO_2 concentrations caused a decrease in catalase activity which was significant in gills at 1 mg/l; in digestive gland the decrease was significant at 0.2 and 1 mg/l but not at 2 mg/l (gills: H = 24.57, F = 4, P = < 0.001; digestive gland: DF = 4, F = 30.29, P = < 0.001) (Figure 3.2 C). Exposure to n-Ag resulted in an increase in catalase activity at all exposure concentrations but this increase was only significant for gills at 0.2 and 1 mg/l (DF = 4, F = 6.95, P = 0.01) and in the digestive gland despite the activity in all

of the exposed groups of animals being higher than controls but the change was not significant (DF = 4, F = 1.86, P = 0.149) (Figure 3.2 D).

Glutathione peroxidase (GPx)

Nano-titanium dioxide exerted an opposite response on GPx in gills and digestive gland at 0.05mg/l (Figure 3.2 E). In gills, the activity decreased (DF = 4, F = 4.54, P = 0.007) whereas in the digestive gland GPx activity increased significantly (DF = 4, F = 31.03, P = < 0.001). However, at higher exposure concentrations GPx was also reduced in the digestive gland. Treatment with n-Ag caused a peculiar response. GPx activity increased significantly at the lowest (0.05 mg/l) and highest (2 mg/l) concentrations compared to controls both in the gills and digestive gland. The other two concentrations (0.2 and 1.0 mg/l) registered a decrease in GPx activity in both tissues (gills: DF = 4, F = 28.23, P = < 0.001; digestive gland: DF = 4, F = 10.58, P = < 0.001) (Figure 3.2 F).

Glutathione reductase (GR)

Acute exposure to n-TiO_2 increased GR activity in gills and digestive gland at the 0.05 mg/l but the change was not significant (Figure 3.3 A). At 0.2 and 1 mg/l the decrease in GR activity was significant in both gills and digestive gland. At 2mg/l n-TiO_2 exposure GR reduced significantly in the digestive gland with no change in gill tissue (gills: DF = 4, F = 6.23, P = 0.001; digestive gland: DF = 4, F = 9.08, P = < 0.001). Exposure to n-Ag caused a significant increase in GR activity in gills and digestive gland at 0.05 mg/l and 2 mg/l. At other exposure concentrations the increase was not significant except in digestive gland at 1 mg/l (gills: DF = 4, F = 5.63, P = 0.002; digestive gland: DF = 4, F = 6.70, P = 0.001) (Figure 3.3 B).

Glutathione S-transferase (GST)

Exposure to n-TiO_2 caused no change in GST activity of gills and the average activity fluctuated in the exposed group within the same range found in controls (DF = 4; F = 1.05; P = 0.402). However, in digestive gland GST significantly increased at

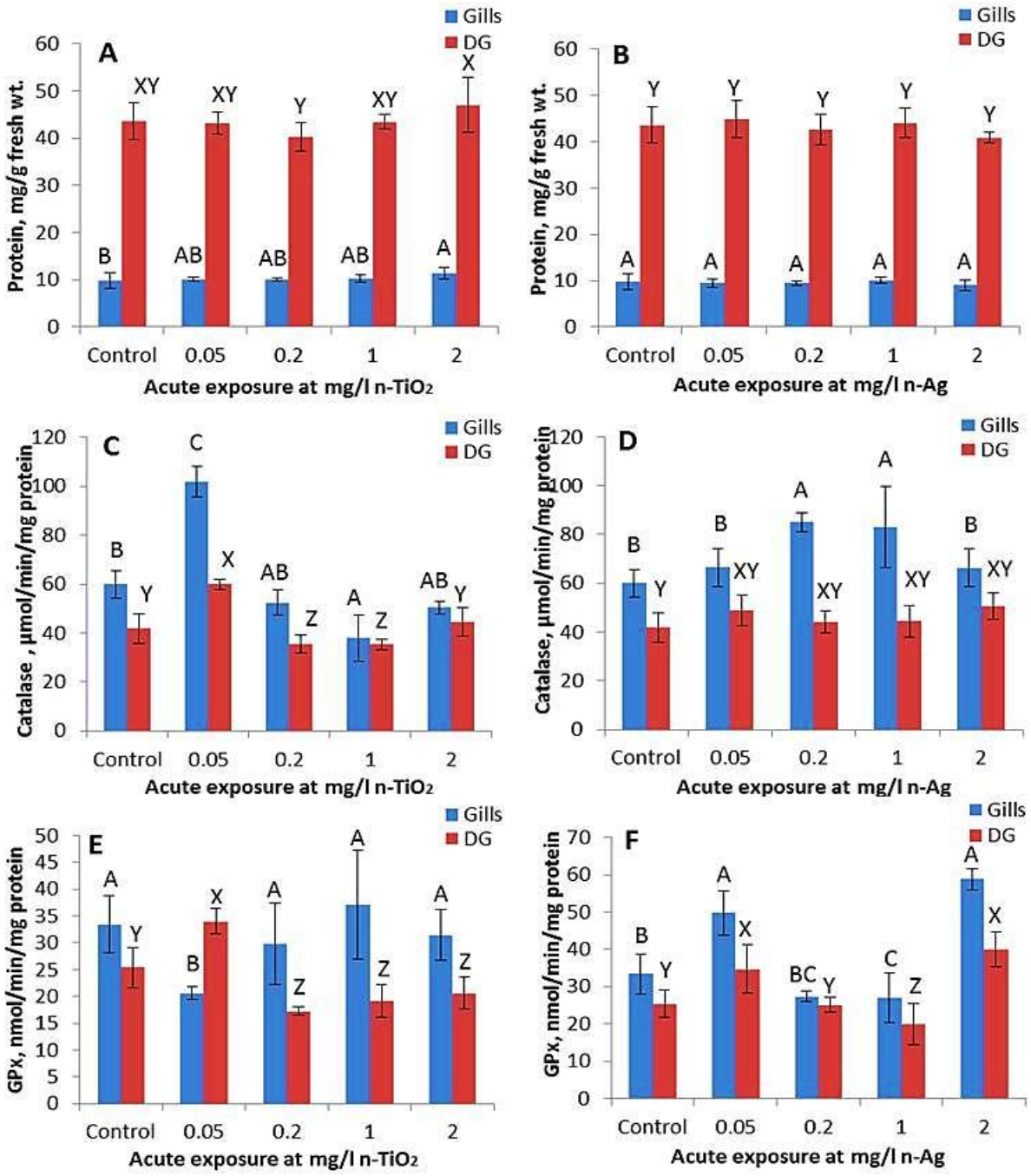

Figure 3.2. Antioxidant enzymes in *A. umbonella* gills and digestive glands from animals that were acutely exposed to engineered nanoparticles (TiO_2 = A, C, E; Ag = B, D, F) at various concentrations for 48 hours. A, B = Total protein content; C, D = catalase activity; E, F= glutathione peroxidase (GPx) activity. Means that do not share a letter are significantly different (n = 6).

0.05 mg/l and decreased at 0.2 mg/l with no change at 1 and 2mg/l (DF = 4, F = 15.80, P = < 0.001) (Figure 3.3 C). Exposure to n-Ag caused a gradual increase in GST in gills with increasing exposure concentrations which was significant at 2 mg/l concentration compared to controls (H = 17.83, DF = 4, P = 0.001). In digestive gland of n-Ag exposed group GST was not changed at 0.05 mg/l concentration but increased significantly with further increases in exposure concentration compared to controls (DF = 4, F = 37.82, P = < 0.001) (Figure 3.3D).

Lipid peroxidation levels

Exposure to n-TiO_2 caused a consistent increase in lipid peroxidation levels in gills with maximum enhancement at the lowest exposure concentration (H = 24.40; DF = 4; P = < 0.001).

In the digestive gland, lipid peroxidation was increased at all exposure concentrations except 0.2 mg/l where a decrease was registered (H = 25.49, DF = 4, P = < 0.001). Animals exposed to n-Ag showed a significant increase in lipid peroxidation at 0.05 mg/l and 1 mg/l in gills and at other exposure concentrations the lipid peroxidation in gills fluctuated within the range of controls (H = 19.20, DF = 4, P = 0.001). In the digestive gland of n-Ag exposed animals a significant increase in lipid peroxidation was observed at all the exposure concentrations with the highest response at 2 mg/l n-Ag exposure concentration (DF = 4, F = 32.76, P = < 0.001) (Figure 3.3 E and F).

Metallothionein

Exposure to n-TiO_2 caused a concentration dependent increase in metallothionein levels in the gills and digestive gland and the change was significant in all the exposed groups (gills: DF = 4, F = 9.38, P = < 0.001; digestive gland: DF = 4, F = 18.02, P = < 0.001) (Figure 3.3 G). In n-Ag exposed animals metallothionein in gills was similar to that in controls at 0.05 and 0.2 mg/l and at 1 mg/l and 2 mg/l

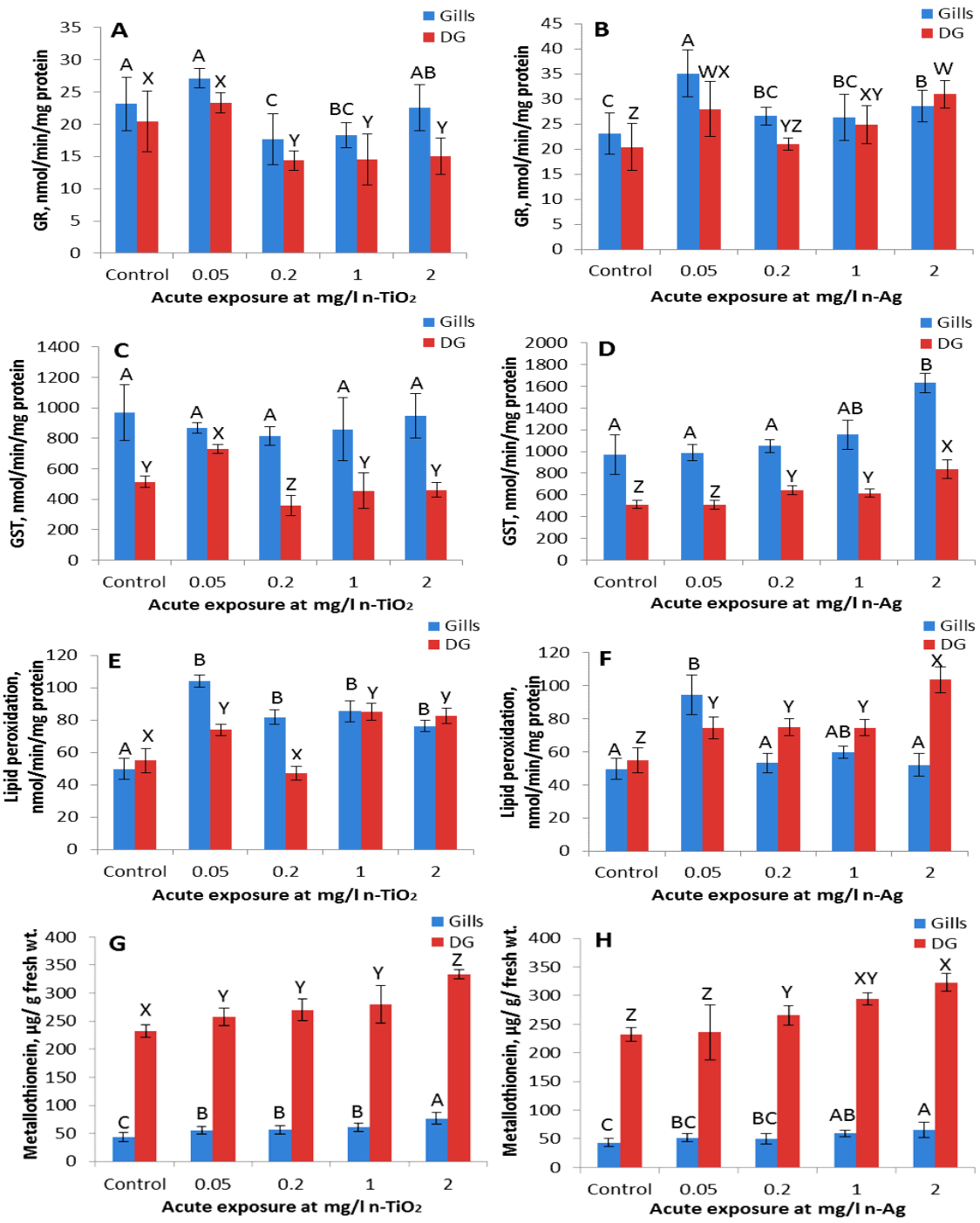

Figure 3.3. Antioxidant enzymes in *A. umbonella* gills and digestive glands from animals that were acutely exposed to engineered nanoparticles (TiO_2 = A, C, E, G; Ag = B, D, F, H) at various concentrations for 48 hours. A, B = glutathione reductase (GR) activity; C, D = glutathione-S-transferase activity; E, F = lipid peroxidation levels; G, H = metallothionein levels. Means that do not share a letter are significantly different (n = 6).

exposure concentrations a significant increase was observed compared to controls (DF = 4, F = 4.34, P = 0.008). In the digestive gland of n-Ag treated animals a significant increase in metallothionein was observed at 1 and 2 mg/l with no change in 0.05 and 0.2 mg/l exposed animals compared to controls (DF = 4, F = 11.13, P = < 0.001) (Figure 3.3 H).

3.3.2 Oxidative stress in *Asaphis violascens* in response to acute exposure to nanoparticles

This species of bivalves which is widely present along the southern coast of Kuwait was examined for its oxidative response on exposure to n-TiO_2 and n-Ag at the same concentrations as *A. umbonella*. The purpose was to determine whether different species respond to nanoparticles in a similar fashion.

Protein

The protein levels in the digestive glands were always higher in comparison to the gills. Exposure to n-TiO_2 caused an increase in the gill protein levels but the increase was significant only at 0.2 mg/l (DF = 4, F = 3.50, P = 0.021) (Figure 3.4 A). The digestive gland suffered a significant decrease in protein level in exposed animals at 2 mg/l n-TiO_2 but at other exposure concentrations no changes were observed compared to controls (DF = 4, F = 2.16, P = 0.103). In n-Ag exposure, compared to controls, the protein was higher in exposed gills, but the change was significant at 0.2 and 2 mg/l (DF = 4, F = 2.87, P = 0.044). In the digestive gland no change was found in protein levels on exposure to n-Ag up to 1 mg/l. At the highest exposure concentration of n-Ag (2 mg/l) a decrease was found in the protein content of the digestive gland of exposed animals compared to controls but it was not significant (DF = 4, F = 1.71, P = 0.179) (Figure 3.4 B).

Catalase

Gill catalase activity was significantly inhibited at the lowest (0.05 mg/l) n-TiO_2 exposure concentration (Figure 3.4 C). An increase in exposure concentration of n-TiO_2 to 0.2 mg/l caused a significant increase in catalase activity compared to controls, which was not further increased by the increase in n-TiO_2 concentration in the exposure medium but remained higher than controls (DF = 4, F = 20.48, P = < 0.01). In the digestive gland catalase activity fluctuated within the range of the controls (DF = 4, F = 1.87, P = 0.148). Exposure to n-Ag reduced (19.8 %) catalase activity in gills at 0.05 mg/l but a significant increase was observed in the digestive gland. In the gills, a further increase in n-Ag exposure concentration caused a significant increase in catalase activity over controls (H = 19.66, DF = 4, P = 0.001). In the digestive gland the increase observed at 0.05 mg/l was not detected at higher exposure concentrations as the activity was within the range of controls (DF = 4, F = 8.83, P = < 0.001) (Figure 3.4 D).

Glutathione Peroxidase (GPx)

In acute n-TiO_2 exposure, GPx activity significantly increased in gills at 0.05, 1 and 2 mg/l exposure compared to controls but at 0.2 mg/l n-TiO_2 exposure, GPx in gills was found within the range of controls (DF = 4, F = 5.83, P = 0.002) (Figure 3.4 E). In the digestive gland of n-TiO_2 exposed animals GPx activity was higher than controls only at 0.05 mg/l but it was not significant statistically (DF = 4, F = 1.99, P = 0.126). In acute n-Ag exposure GPx was significantly higher in gills of exposed animals compared to controls except in gills of animals exposed to 0.2 mg/l concentration (DF = 4, F = 10.78, P = < 0.001). In the digestive gland no significant effect was observed in GPx at any exposure concentration of n-Ag (DF = 4, F = 1.14, P = 0.362) (Figure 3.4 F).

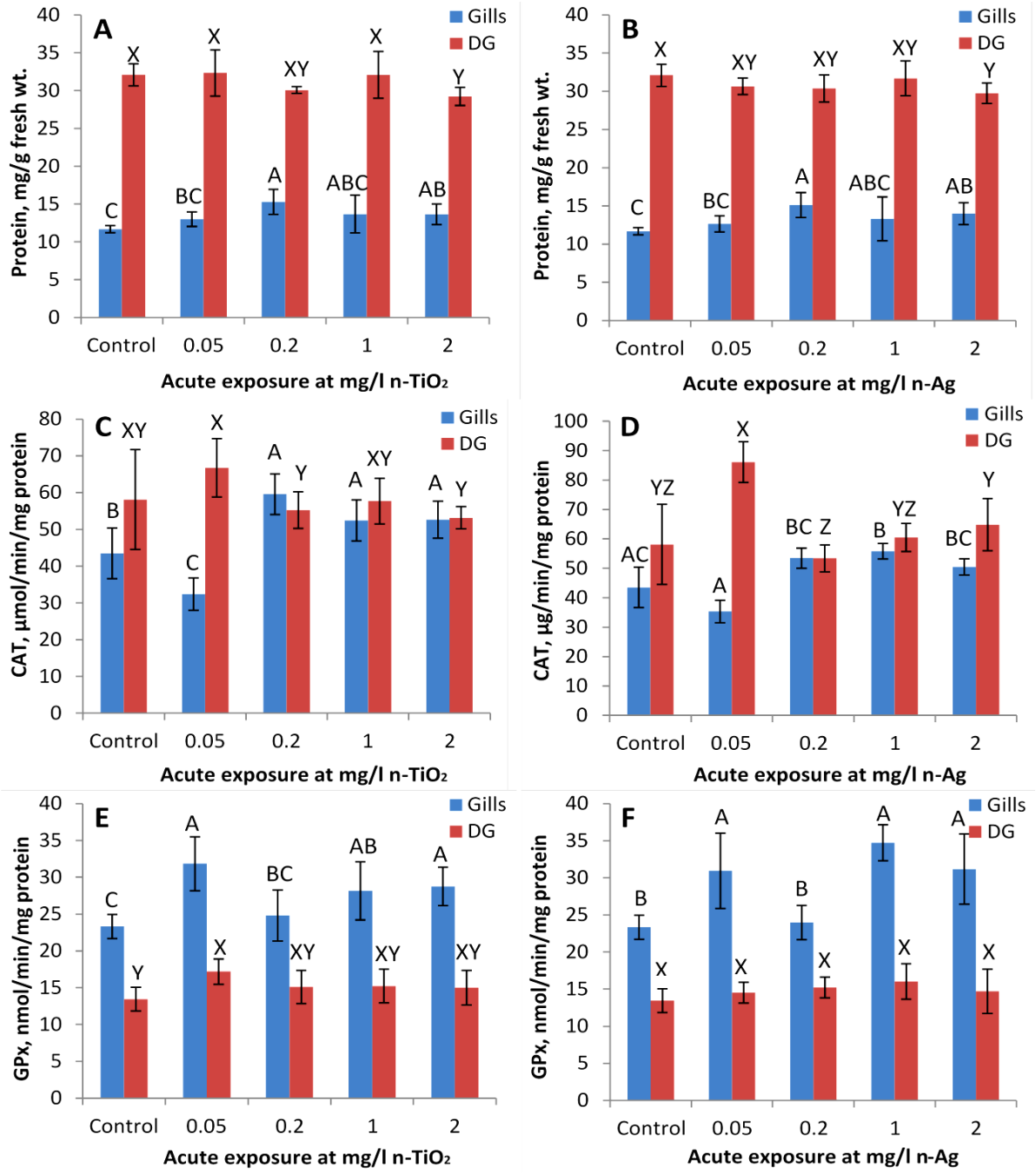

Figure 3.4. Antioxidant enzymes in *A. violascens* gills and digestive glands from animals that were acutely exposed to engineered nanoparticles (TiO_2 = A, C, E; Ag = B, D, F) at various concentrations for 48 hours. A, B = Total protein content; C, D = catalase activity; E, F = glutathione peroxidase (GPx) activity. Means that do not share a letter are significantly different (n=6).

Glutathione Reductase (GR)

The activity of GR in gills was unchanged at all the n-TiO_2 exposure concentrations (DF = 4, F = 1.51, P 0.230) (Figure 3.5 A). In acute n-Ag exposure there was no change in GR of gills observed (DF = 4, F = 0.23, P = 0.918). An increase was observed in the digestive gland of the animals exposed to n-TiO_2 compared to controls (DF = 4, F = 3.93, P = 0.013). In the digestive gland of n-Ag exposed animals a significant increase in GR was found at 0.05 mg/l whereas at other exposure concentrations the change was not significant compared to that in controls (DF = 4, F = 4.79, P = 0.005) (Figure 3.5 B).

Glutathione S-transferase (GST)

Acute exposure to n-TiO_2 caused no change in GST activity of gills in exposed animals (DF = 4, F = 0.35, P = 0.843), whereas in the digestive gland the GST activity was significantly increased at 0.05 mg/l exposure concentration compared to controls (DF = 4, F = 4.19, P = 0.010) but at other exposure concentration the change was insignificant (Figure 3.5 C). In n-Ag treatments, similar to that observed in n-TiO_2 treatment GST in the gills was not affected by the exposure to n-Ag (DF = 4, F = 0.38, P = 0.822). In the digestive gland, n-Ag treatment also resulted in a significant increase in GST at 0.05 mg/l and the increase in GST activity persisted at higher exposure concentrations (DF = 4, F = 5.44, P = 0.003) (Figure 3.5 D).

Lipid Peroxidation (LPO)

Exposure to n-TiO_2 did not cause any significant changes in LPO in gills and digestive gland abd remained within the range of controls (gills: DF = 4, F = 1.88, P = 0.145; digestive gland: H = 8.04, DF = 4, P = 0.090) (Figure 3.5 E). In the n-Ag treatment LPO was increased in gills at 0.05 mg/l, however the change was not significant at any of the other exposure concentrations (DF = 4, F = 2.70, P = 0.054). In digestive gland LPO on n-Ag exposure remained within the range of control in

exposed animals at all the exposure concentrations (DF = 4, F = 1.68, P = 0.185) (Figure 3.5 F).

Metallothionein

Exposure to n-TiO_2 caused a steady increase in metallothionein levels in both gill and digestive gland tissues with increasing concentrations (Figure 3.5 G). The increase was significant at and above 0.2 mg/l exposure concentrations (gills: DF = 4, F = 11.77, P = < 0.001; digestive gland: DF = 4, F = 16.61, P = < 0.001). However, animals exposed to n-Ag showed significant increase in metallothionein levels in gills and digestive gland up to 1 mg/l exposure concentration, and then a decrease in the activity was observed at 2 mg/l exposure concentration (gills: DF = 4, F = 6.24, P = 0.001; digestive gland: DF = 4, F = 4.59, P = 0.006) (Figure 3.5 H).

3.3.3 Oxidative stress in response to chronic exposure to nanoparticles

Amiantis umbonella were chronically exposed to n-TiO_2 and n-Ag at a concentration 0.05 mg/l for four weeks. Chronic exposure caused no change in the protein content of gills or digestive gland in animals exposed to n-TiO_2 or n-Ag (gills: DF = 2, F = 0.47, P = 0.635; digestive gland: DF = 2, F = 1.01, P = 0.388) (Figure 3.6 A). Exposure to both types of nanoparticles caused a significant decrease in catalase activity in gills (DF = 2, F = 42.25, P = < 0.001). In the digestive gland the activity significantly increased in n-Ag exposure with an insignificant increase observed with n-TiO_2 exposure (DF = 2, F = 21.60, P = < 0.001) (Figure 3.6 B). GPx activity in gills was higher than controls but the change was significant only for n-TiO_2 (DF = 2, F = 18.61, P = < 0.001). In the digestive gland, the activity of GPx in clams exposed n-TiO_2 was significantly increased whereas in n-Ag exposure GPx fluctuated within the range of controls (DF = 2, F = 10.63, P = < 0.001) (Fogure 3.6 C). GR activity was significantly increased in the gills with no change in the digestive gland of clams exposed to n-TiO_2. Exposure to n-Ag caused a significant decrease in the GR activity in the digestive gland with no change in GR in the gills compared to controls (gills:

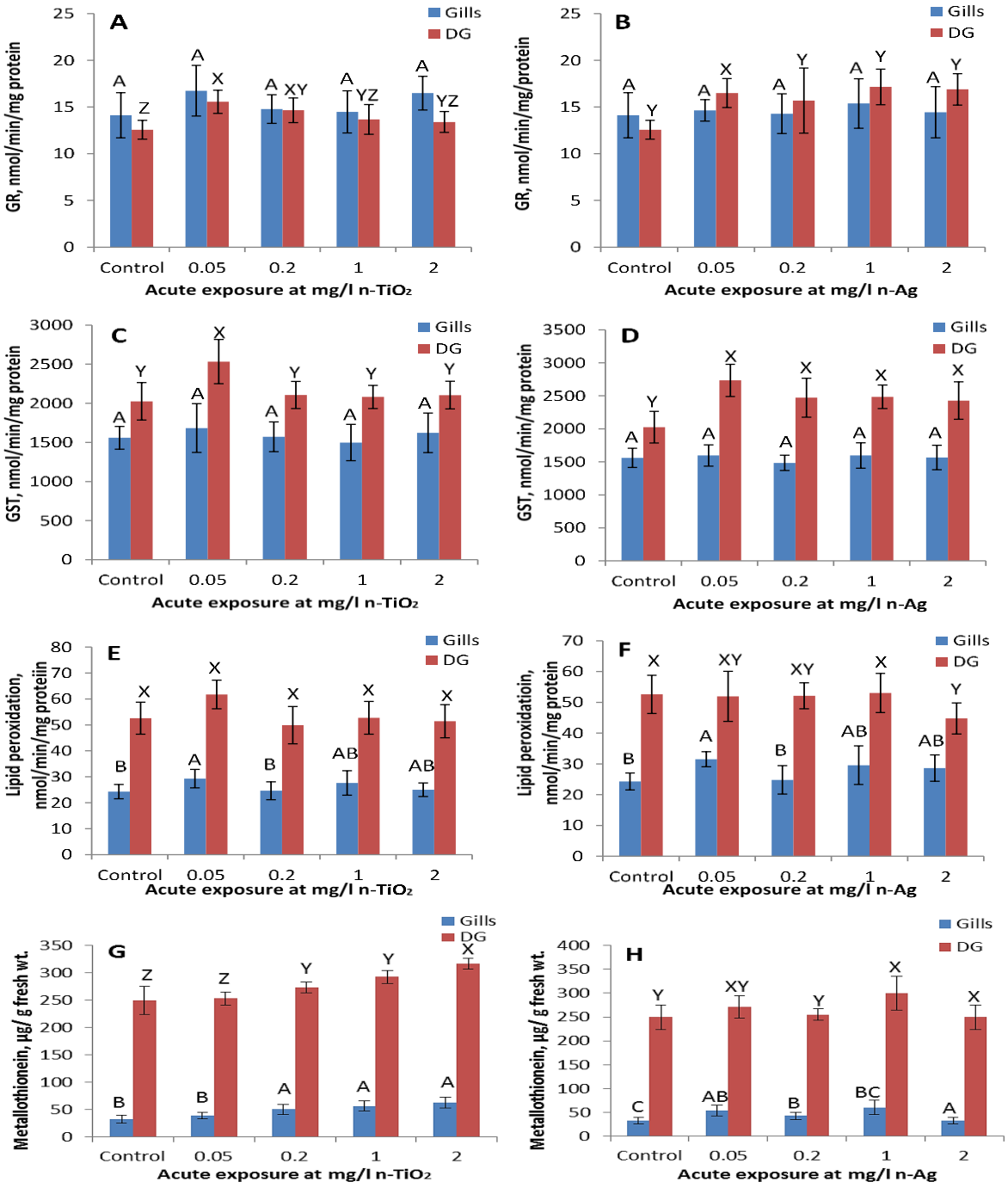

Figure 3.5. Antioxidant enzymes in *A. violascens* gills and digestive glands from animals that were acutely exposed to engineered nanoparticles (TiO_2 = A, C, E, G; Ag = B, D, F, H) at various concentrations for 48 hours. A, B = glutathione reductase (GR) activity; C, D = glutathione-S-transferase (GST) activity; E, F = lipid peroxidation levels; G, H = metallothionein levels. Means that do not share a letter are significantly different (n=6).

DF = 2, F = 33.62, P = < 0.001: digestive gland: H = 11.79, DF = 2, P = 0.003) (Figure 3.6 D). GST levels did not show any changes in the gills but significantly increased in the digestive gland of n-TiO_2 exposed animals. In the n-Ag treatment GST was not changed in the gills compared to the controls but in the digestive gland of chronically exposed clams GST increased significantly (gills: H = 0.57, DF = 2, P = 0.751: digestive gland: DF = 2, F = 34.87, P = < 0.001) (Figure 3.6 E). Lipid peroxidation levels were not affected in either the gills or digestive glands of either n-TiO_2 or n-Ag exposed animals compared to controls (gills: DF = 2, F = 0.78, P = 0.475; digestive gland: DF = 2, F = 4.16, P = 0.036) (Figure 3.6 F). Exposure to both types of nanoparticles caused a significant increase in metallothionein levels in gills and the digestive glands compared to controls (gills: DF = 2, F = 6.0, P = 0.012; digestive gland: DF = 2, F = 19.61, P = < 0.001) (Figure 3.6 G).

3.3.4 Oxidative stress on chronic exposure to nano and bulk metal oxides

There are reports in the literature that nano and bulk metal oxides produce different responses in exposed organisms; therefore, four week exposure experiments were conducted on *A. violascens* using n-CuO and n-ZnO along with their bulk oxides. The data are presented below.

The protein content in the gills and digestive glands of nano and bulk metal oxide exposed animals were not significantly different from control animals (gills: DF = 4, F = 0.99, P = 0.432; Digestive gland: H = 1.92, DF = 4, P = 0.75) (Figure 3.7 A).

Catalase activity in the gills was insignificantly increased in gills and decreased in the digestive gland on exposure to n-ZnO. Exposure to b-ZnO caused significant activation of catalase in the digestive gland and inhibition in the gills. Exposure to n-CuO and b-CuO caused a significant activation of catalase in the gills. In the digestive gland both n-CuO and b-CuO caused a significant increase in catalase activity. The magnitude of the increase was more in b-CuO treatments than n-CuO

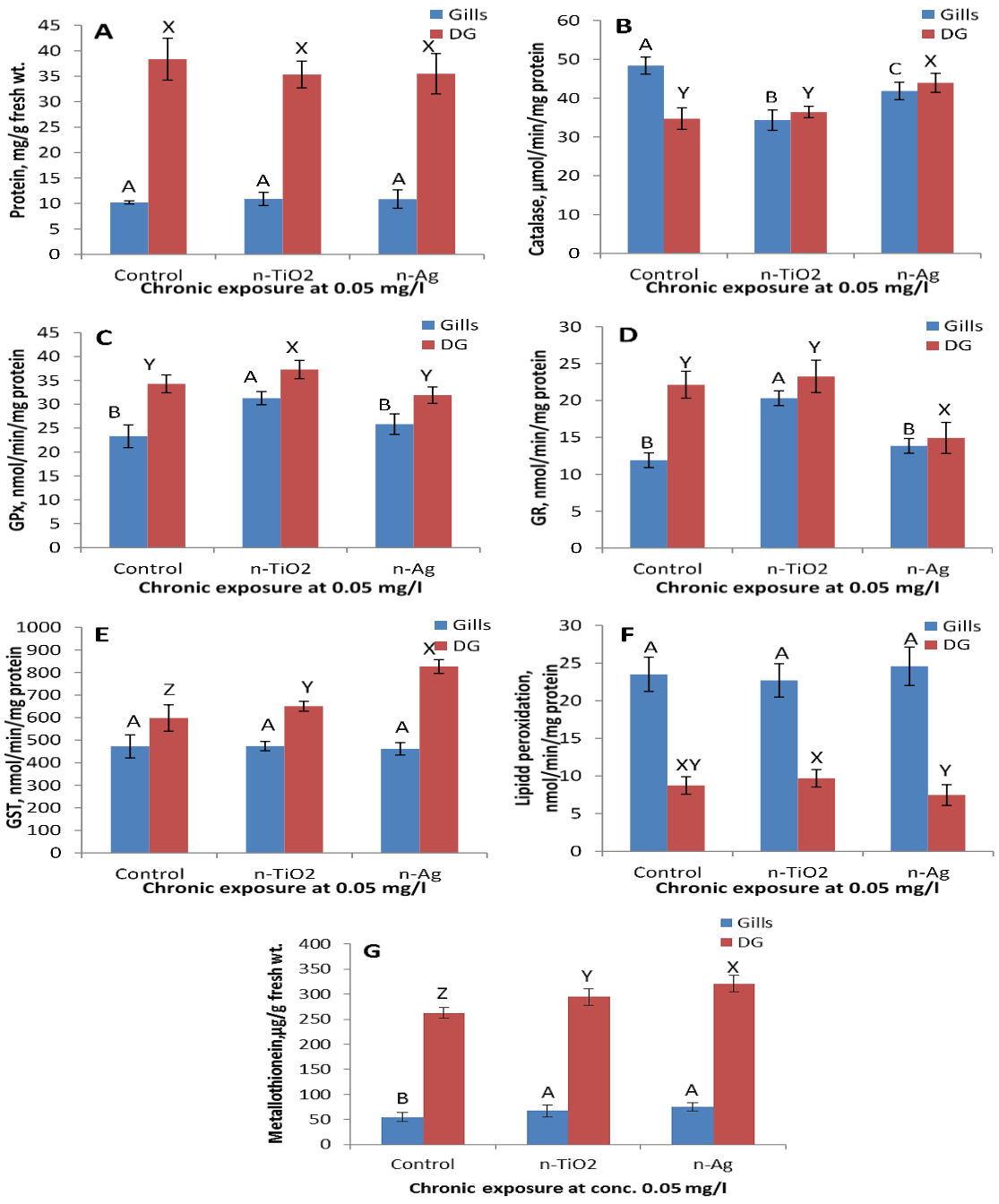

Figure 3.6. Antioxidant enzymes in *A. umbonella* gills and digestive glands from animals that were chronically exposed to engineered nanoparticles (0.05 mg/l TiO_2 and Ag) for four weeks. A, total protein content B, catalase activity C, glutathione peroxidase (GPx) activity D, glutathione reductase (GR) activity E, glutathione-S-transferase (GST) activity F, lipid peroxidation levels G, metallothionein levels. Means that do not share a letter are significantly different (n=6).

compared to controls (gills: H = 16.79, DF = 4, P = 0.002; digestive gland: DF = 4, F = 24.31, P = < 0.001) (Figure 3.7 B).The GPx activity in the gills and the digestive gland was significantly decreased by both n-ZnO and b-ZnO exposure in comparison to controls, whereas GPx activity responded differently in the gills and the digestive gland following n-CuO and b-CuO exposure. The activity of GPx increased in the gills of exposed animals whereas a significant decrease was observed in GPx of digestive glands with both nano and b-CuO treatment (gills: H = 23.73, DF = 4, P = < 0.001; digestive gland: H = 21.17, DF = 4, P = < 0.001) (Figure 3.7 C).

The activity of GR was significantly inhibited in the gills and increased in the digestive gland of n-ZnO exposed animals. Exposure to b-ZnO resulted in significant inhibition in GR in both the gills and the digestive gland tissues of exposed animals compared to controls. Exposure to n-CuO caused a significant inhibition in GR in the gill tissue whereas GR remained within therange of control activity in the gills after the treatment with b-CuO. In the digestive glands, GR was increased after exposure to n-CuO, but exposure to b-CuO caused an insignificant decrease in GR of the digestive gland (gills: DF = 4, F = 42.74, P = < 0.001; digestive gland: DF = 4, F = 21.22, P = < 0.001) (Figure 3.8 A).

The activity of GST was not affected in the gills and digestive gland by n-ZnO while a significant decrease in GST was found in the gills exposed to b-ZnO. Also, there was no change in GST of the gills and the digestive gland by n-CuO or b-CuO treatment except for a significant lowering in b-CuO exposed gills (gills: DF = 4, F = 3.50, P = 0.021; digestive gland: DF = 4, F = 0.35, P = 0.839) (Figure 3.8 B).

Lipid peroxidation was significantly increased in gills of n-ZnO treated animals but not affected in the digestive gland tissue exposed to n-ZnO. Exposure to b-ZnO caused a significant decrease in lipid peroxidation in both gills and digestive gland compared to controls. In the n-CuO treatment gill lipid peroxidation was not affected but in the digestive glands a significant decrease was observed. Exposure to b-CuO caused a significant decrease in lipid peroxidation in the gills with no change

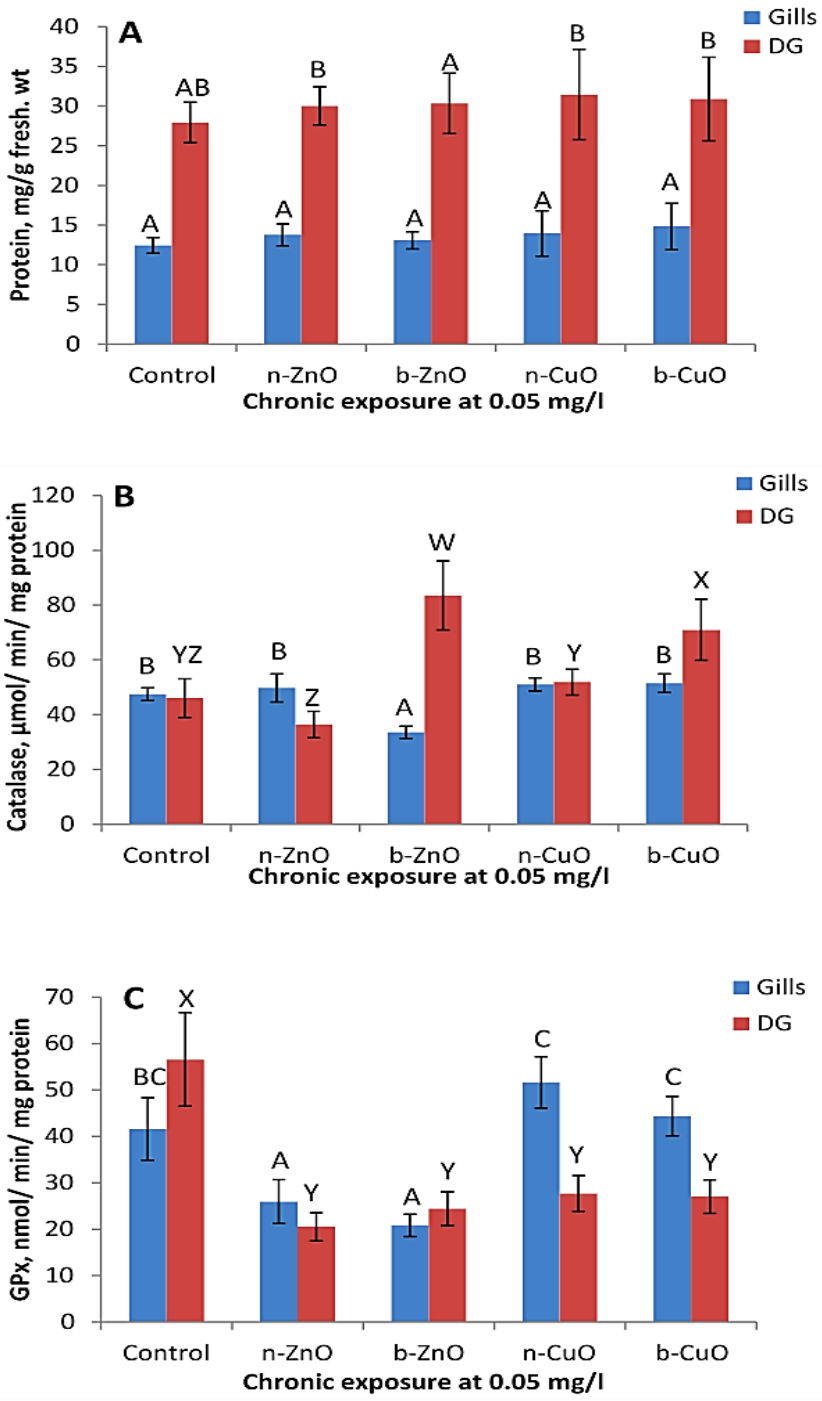

Figure 3.7. Antioxidant enzymes in *A. violascens* gills and digestive glands from animals that were chronically exposed to nano and bulk oxides of zinc and copper at 0.05 mg/l for four weeks. A, Total protein content B, catalase activity C, glutathione peroxidase (GPx) activity. Means that do not share a letter are significantly different (n=6).

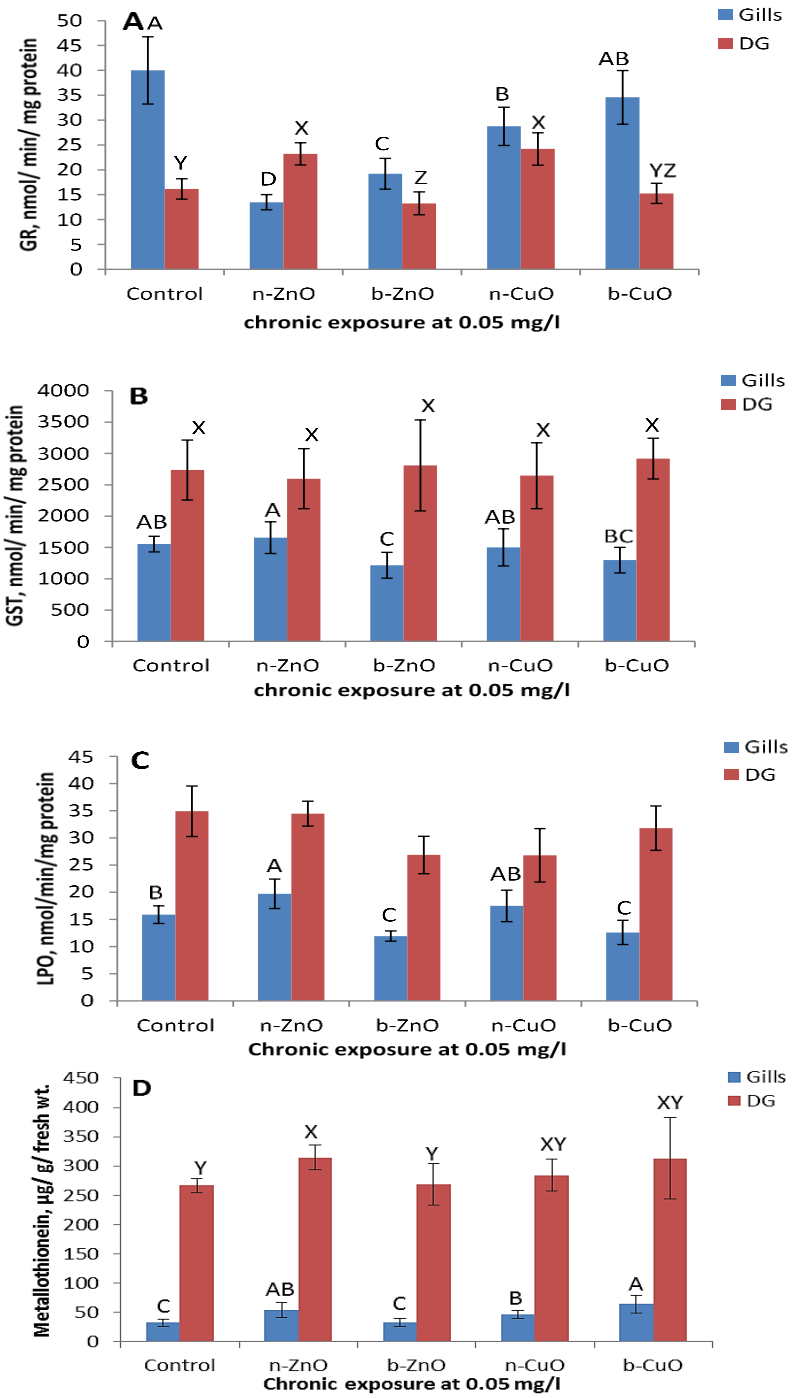

Figure 3.8. Antioxidant enzymes in *A. violascens* gills and digestive glands from animals that were chronically exposed to nano and bulk oxides of zinc and copper at 0.05 mg/l for four weeks. A, glutathione reductase (GR) activity B, glutathione-S-transferase (GST) activity C, lipid peroxidation levels D, metallothionein levels after exposure to nano and bulk oxides of zinc and copper. Means that do not share a letter are significantly different (n=6).

in the digestive gland compared to control (gills: DF = 4, F = 11.47, P = 0.021; digestive gland: H = 11.96, DF = 4, P = 0.018) (Figure 3.8 C).

There was a significant increase in the metallothionein levels in the gills by n-ZnO with no change in b-ZnO exposure compared to controls. In the digestive gland metallothionein was enhanced significantly by the n-ZnO treatment but not in the b-ZnO treatment. Exposure to n-CuO and b-CuO caused a significant increase in the metallothionein levels in the gill tissues compared to controls but in the digestive gland an insignificant increase was observed in both treatment (gills: DF = 4, F = 8.02, P = < 0.001; digestive gland: DF = 4, F = 2.03, P = 0.120) (Figure 3.8 D).

3.4 Discussion

Extensive studies are being conducted to determine the toxicity of nanoparticles to aquatic organisms (Al-Subiai et al., 2012; Al-Subiai et al., 2011; Buffet et al., 2012; Buffet et al., 2013a; Buffet et al., 2014a; Buffet et al., 2013b; Buffet et al., 2011; Buffet et al., 2014b; Canesi et al., 2015; Canesi et al., 2008; Canesi et al., 2012; Canesi et al., 2010a; Canesi et al., 2010b; Canesi et al., 2014; Gomes et al., 2013a; Gomes et al., 2014a; Gomes et al., 2013b; Gomes et al., 2012; Gomes et al., 2014b; Gomes et al., 2011); however, the mechanisms of action and target biomarkers are not yet fully defined (Rocha et al., 2015). There is increasing evidence showing that metal–containing nanoparticles exert cellular toxicity through a free radical mechanism. On this basis the choice of key biomarkers for this study was made. A battery of biomarkers monitored at the same time in the same organisms (Bodin et al., 2004; Porte et al., 1998; Roméo et al., 2003) helps in the evaluation of the effect of exposure and aids in establishing a relationship between injury and cellular defence. Since oxidative stress is tissue specific, biomarker levels in the gills and digestive glands were determined separately. It is interesting to observe that the levels of antioxidant enzymes were less in the digestive gland than in the gill tissue of *A. umbonella* suggesting a lower antioxidant defence in the digestive gland, an

observation similar to that found in the marine mussel, *Perna viridis* (Cheung et al., 2001). The present study indicated that the effect of exposure to nanoparticles was concentration dependent but the effect was not proportional to the exposure concentration. Secondly, the response of the two tissues, gills and digestive gland, was not always similar. Thirdly, n-TiO_2 and n-Ag did cause oxidative stress but the target biomarkers of antioxidant defence were not uniformly affected. However, tissue damage was commonly observed in both gills and digestive gland exposed to the two types of nanoparticles (chapter 4).

Exposure to n-TiO_2 at the lowest concentration (0.05 mg/l) caused the highest level of catalase activation, a primary scavenger of hydrogen peroxide, and the increase was found both in gills and digestive glands. In general, n-TiO_2 at the lowest exposure concentration resulted in increases in antioxidant enzymes of gills as well as digestive glands of *A. umbonella* but this activation was not sufficient to protect against membrane damage as an increase in lipid peroxidation was also observed. When antioxidant defences are unable to cope with the generation of oxy-radicals there is an imbalance between the production and removal of oxidants, a situation known as oxidative stress resulting in membrane lipid peroxidation (Barata et al., 2005; Halliwell and Gutteridge, 1986; Livingstone et al., 1990). By increasing exposure concentrations all the enzymes that were increased showed a depression in activities in comparison to controls but lipid peroxidation persisted at higher levels. A recent study using a freshwater bivalve (*Corbicula fluminea*) exposed to n-TiO_2 found an increase in catalase after three days but levels returned to normal by 15 days; however, lipid peroxidation increase persisted (Vale et al., 2014). This indicated that a change in enzyme activity may quickly occur whereas any resulting membrane damage takes longer to repair. Earlier, Canesi et al. (2010a) observed that mussels (*Mytilus galloprovincialis*) exposed to 0.05 to 5 mg/l n-TiO_2 concentrations showed no clear trend in catalase and GST in gills; however, in digestive gland a consistent increase was observed in GST at all the exposure concentrations but catalase was only increased at higher (1 and 5 mg/l) concentrations.

Nanoparticle toxicity is attributed to their small size and large surface area (Xiong et al., 2011). Cell membrane disruption may be the result of physical damage due to the size and surface properties of the nanoparticles, or due to ROS production and lipid peroxidation that can ultimately cause cell damage (Elsaesser and Howard, 2012). However, the response observed in the present study indicates complexity in the effect of nanoparticles on organisms with respect to the varying concentrations in the exposure medium. After activation of oxidative defence enzymes at 0.05 mg/l n-TiO_2, a decrease in enzyme activity was observed up to 1 mg/l exposure indicating a failure in oxidative defence with increased concentration. However, at 2 mg/l the enzyme levels were close to the controls or only marginally activated. It was observed that bivalves closed their shells at the highest exposure concentration thereby protecting themselves from the assault of toxicants present in the surrounding medium. The initial intake of particles was sufficient to signal the animal to close its shell. This is supported by the fact that clearance rates decreased at this exposure concentration (Chapter 2). It is interesting to consider another possibility to explain why oxidative stress is still observed at the higher exposure concentration when the animal closes its shell. When bivalves close their shells they retain a volume of water (albeit very small) within the mantle cavity and thereby create a condition of hypoxia. Logically, hypoxia may result in the decreased production of free radicals but it has been found that a decreased oxygen concentration may actually induce oxidative stress as demonstrated in goldfish exposed to anoxia where superoxide dismutase and catalase activities were enhanced (Lushchak, 2011). This phenomenon is termed "preparation for oxidative stress" (Hermes-Lima et al., 1998). Hypoxia-induced oxidative stress has also been found in medaka (*Oryzias latipes*) where GST was increased (Oehlers et al., 2007) and in the freshwater clam *Corbicula fluminea* where catalase and GPx activities increased (Vidal et al., 2002). Although it is worth pointing out that the mechanism of hypoxia-induced oxidative stress is not yet established.

Another interesting finding was the increase in metallothionein with exposure concentrations in the acute exposure and also in the chronic exposure at low concentrations where metallothionein increased with time. The metallothionein data suggested that the effect at higher exposure concentrations was not only because of shell closure but also due to metabolic adjustments. Metallothionein is involved in the metabolism of essential metals and also in sequestrating toxic metals, the process that reduces the toxicity of pollutant metals in the cell (Polizzi et al., 2014). It was observed that exposure to metal nanoparticles and metal oxides induced metallothionein concentration in a time-dependent manner; this was also reported in other bivalve molluscs including n-Ag and n-CuO exposure to *M. galloprovincialis* (Gomes et al., 2014a; Gomes et al., 2012; Gomes et al., 2014b; Gomes et al., 2011); n-ZnO exposure to *Elliptio complanata* (Gagne et al., 2013b); and Co^{2+} containing nano-polymeric complex exposure to *Anodonta cygnea* (Falfushynska et al., 2012). The role of metallothionein in detoxifying metal nanoparticles in bivalves has been studied and the detoxification mechanism is by controlling the intracellular availability of the metals released from nanoparticles by binding with it and detoxifying excessive metal concentrations (Bebianno et al., 2004; Regoli and Principato, 1995). Therefore, a protection of the system was expected because of increased binding of metals from nanoparticles with this soluble protein (Gomes et al., 2014a).

Exposure to n-Ag caused significant increases in catalase in gill tissues at 0.2 and 1 mg/l exposures. An increase in catalase in gills and digestive gland tissues was found in oysters (*Crassostrea virginica*) exposed to 0.2, 2, and 20 µg/l n-Ag (McCarthy et al., 2013). Contrary to n-TiO_2 exposure, GPx and GR were significantly elevated in n-Ag treatments at the lowest exposure concentration. In addition to catalase, GPx in cells plays an additional role in scavenging hydrogen peroxide; however, this enzyme is energy dependent and oxidizes reduced glutathione. To regulate the supply of GSH, GR was also activated in the n-Ag exposed animals to convert GSSG to its reduced form GSH. GSH acts as a substrate for peroxide scavenging enzymes and also as a direct scavenger of oxy radicals (Jena

et al., 2009). During n-TiO_2 exposure, GST was decreased in general whereas in n-Ag exposure there was an increase in GST in both the gills and digestive gland. Lipid peroxidation was persistently high in the digestive gland of n-Ag exposed animals, but in the gills lipid peroxidation was enhanced only at 0.05 mg/l. At other concentrations lipid peroxidation was unaffected compared to controls.

In comparing the two bivalve species, the data for enzymes of oxidative defence and lipid peroxidation after exposure to n-TiO_2 and n-Ag revealed that acute exposure to various concentrations induced a disturbance in their levels. However, the severity of response differed. The basal levels of catalase, GPx, GR and metallothionein were lower but GST was higher in untreated *A. violescens* compared to *A. umbonella*. This suggests that *A. violescens* was better equipped for handling conditions of oxidative stress. It was also evident that the most effective assay concentration was 0.05 mg/l. Tissue total protein levels in the gills and digestive glands were unaffected by any of the nanoparticles tested. The response of catalase in the digestive gland to n-TiO_2 exposure was similar in the two species of bivalves at higher exposure concentration. In the gills, catalase was activated in *A. umbonella* and inhibited in *A. violascens* at the lowest exposure concentration. Interestingly, it was also observed that GPx activity was enhanced when catalase was reduced. The response in gill GPx in the two species was opposite to that of catalase at the lowest exposure concentration. This shows that the two enzymes which deal with hydrogen peroxide in cells 'back up' for one another, i.e. if one decreases the other increases. Increases in GPx and catalase were observed in the digestive glands of both species. GR also increased in both tissues of both species at the lowest exposure concentration and an increase in concentration exerted irregular patterns of increases or decreases in the two tissues. GST increased in the digestive gland of both species. Lipid peroxidation was generally increased in the gills and digestive gland at lower exposure concentration and higher concentrations caused irregular changes in lipid peroxidation. Thus, n-TiO_2 exposure data revealed that the lowest assayed concentration was the most effective with subsequent concentration increases

producing an irregular response in oxidative defence and tissue damage. It is considered that ROS toxicity is reflected in antioxidant enzyme increases to ameliorate oxidative stress; however, excessive ROS production due to higher exposure concentrations can reduce the anti-oxidant defence due to the exhaustion of the detoxification mechanism (Jacobson and Reimschuessel, 1998; Valavanidis et al., 2006). This is supported by the observations in the present study as antioxidant enzymes increased in acute exposure and then were depleted with time in chronic exposure.

Treatment with n-Ag also indicated that the most effective exposure concentration was the lowest concentration used in both species. The response of gill catalase was different between the species whereas it was same in the digestive gland. GPx increased significantly in both tissues of *A. umbonella* whereas the increase in GPx was significant only in the gills of *A. violascens*. GR increased in the digestive gland of both species but only in the gills of *A. violascens*. GST was mostly unaffected with the exception of the digestive gland of *A. violascens* where a significant increase was observed. Lipid peroxidation increased in both tissues of *A. umbonella* but only in the gills of *A. violascens*.

Acute exposure experiments revealed that the moment a toxic compound enters the exposure medium the organism starts metabolic adjustment to deal with it. In the present study acute exposure to nano-metals at the lower concentration (0.05 mg/l) enhanced antioxidant defence enzymes to combat oxidative stress; however, the exposure also caused cellular damage as revealed by the increase in lipid peroxidation. When the animals were exposed to the same concentration for a longer period the response of animals was not the same as that found in acute exposure. Chronic exposure of *A. umbonella* to n-TiO_2 at 0.05 mg/l caused a significant inhibition of catalase in gills - contrary to that found in acute exposure. This means the persistent presence of n-TiO_2 in the surrounding water caused a failure in the primary defence enzyme that deals with hydrogen peroxide. Interestingly, in gills the secondary defence enzymes GPx and GR were significantly activated during chronic

exposure playing a ROS scavenging role and protecting membrane oxidation as lipid peroxidation was not increased in gills of chronically exposed animals. In digestive glands of clams chronically exposed to n-TiO_2, all of the oxidative defence enzymes were higher than the controls but the increase was significant only for GPx; however, this increase was not enough to protect against membrane damage as more lipid peroxidation was found in exposed clams than in controls.

Chronic exposure of *A. umbonella* to n-Ag caused inhibition in catalase in gills whereas other antioxidant enzymes and lipid peroxidation persisted within the range of the control. In the digestive gland, catalase and GST increased but GPx and GR were inhibited and an insignificant inhibition in lipid peroxidation was also observed in comparison to controls. A similar observation was found with *Scrobicularia plana* exposed to n-Ag for 14 days where catalase and GST in whole soft tissues increased with no significant change in lipid peroxidation as examined by measuring TBARS (Buffet et al., 2013a). In a recent study, n-Ag exposed *M. galloprovincialis* showed an increase in catalase, GPx and lipid peroxidation in gills, but in the digestive gland while catalase and GPx were increased, lipid peroxidation was inhibited after 15 days of exposure (Gomes et al., 2014b). A lack of lipid peroxidation production has also been seen in *S. plana* exposed to n-Ag with an efficient mechanism of defence (Buffet et al., 2013a). The lack of lipid peroxidation production was explained as an intensification of the antioxidant system of animals or the storage/detoxification of n-Ag as nontoxic insoluble silver sulphide precipitates (Berthet et al., 1992; Geffard et al., 2004; Gomes et al., 2014a; Gomes et al., 2014b).

Nanoparticles are generally considered more toxic in comparison to their bulk state due to their small size and greater surface area that provides enhanced surface activity and ability to penetrate cells (Xiong et al., 2011). However, in relation to oxidative stress, a comparison of n-CuO and n-ZnO with b-CuO and b-ZnO revealed a much more complex picture. The activity of catalase in exposed gills and digestive glands was more than controls but the increase was significant only in the digestive gland of animals treated with b-CuO, but lipid peroxidation was not significantly

changed in both nano- and bulk- CuO exposure. In Gomes et al. (2012), *M. galloprovincialis* exposed to n-CuO showed an increase in catalase in the digestive gland, as a first response within three days after exposure, whereas GPx was higher in exposed animals but the increase became significant in long-term exposure by that time catalase returned to the level of controls. In the present study, after a four week exposure, catalase was comparable to controls but GPx in gills was significantly increased, but decreased in the digestive gland. Lipid peroxidation was not observed in the present exposure to n-CuO and b-CuO. Buffet (2011) found an increase in catalase and GST in n-CuO exposure without any change in lipid peroxidation.

Significant changes were seen in GPx and GR indicating that the target of n-CuO toxicity was the glutathione cycle. Cysteine thiol groups present in proteins and low molecular mass thiols react fast with oxidizing species and thus contribute to antioxidant defence (Hansen et al., 2009). These reduced proteins (enzyme protein in the present case) may signal more widespread toxicity affecting overall protein synthesis or increased degradation rate of protein as reported for n-Ag by McCarthy et al. (2013). Some of these aspects may be understood by proteomic studies that enable broad comparison by simultaneously examining hundreds of proteins (Kultz et al., 2007).

With n-ZnO, the catalase activity was comparable to controls except for a slight inhibition in the digestive gland of exposed animals, whereas with b-ZnO, a significant increase was found in the digestive gland compared to controls. A decreased GPx and GR activity in the exposed animals was the main effect observed in this study; however, the response was tissue specific and opposite in the gills and digestive gland with both nano- and bulk- ZnO. Similar responses were also observed in the oyster *C. gigas* by Trevisan et al. (2014), who reported inhibition in GR activity at 4 mg/l both in gills and digestive gland with no change in the catalase compared to controls, although GPx was increased in gills after 48 hours of exposure but remained unchanged in the digestive gland. Inhibition of GR has also been detected in various organisms including mussels (Franco et al., 2006; Trevisan et al.,

2014), fish (Franco et al., 2008b) and rats (Franco et al., 2008a; Maris et al., 2010), suggesting a key role for this enzyme in the mechanism of zinc toxicity. Alterations in GR activity can cause a disturbance to the reduction of GSSG under oxidative conditions, thereby increasing the susceptibility of the organism to oxidative damage (Mitozo et al., 2011). GST activity did not show any changes compared to controls in the n-ZnO exposed animal which is similar to the findings of Buffet et al. (2012), where n-ZnO particles did not affect the activity of GST in the whole tissue of *S. plana*; however, b-ZnO showed a significant inhibition in the exposed gills. When the defence mechanism is not efficient, it may be revealed by TBARS, as gills exposed to n-ZnO in this study also showed a significant induction of lipid peroxidation, whereas the levels in the digestive gland were similar to controls; however, lipid peroxidation was significantly induced in the exposed tissues by b-ZnO.

The toxicity of zinc oxide nanoparticles can be related to the accumulation of nanoparticulated or soluble zinc ions, as solubility of nanoparticles strongly influences cytotoxicity (Brunner et al., 2006; Heinlaan et al., 2008). However, there is not an extensive literature on n-ZnO toxicity to bivalves despite soluble zinc toxicity being well documented in bivalves, showing impaired development (Fathallah et al., 2010), physiology (Hietanen et al., 1988), with accumulation in epithelial tissues (George and Pirie, 1980), immunologic disturbance (Taylor et al., 2013) and oxidative and cellular stress (Devos et al., 2012; Franco et al., 2006; Trevisan et al., 2014). Recently it has been indicated that mussels can efficiently filter suspended n-ZnO particles, and can further excrete them in pseudofaeces but still a high fraction accumulates in the soft tissue (Montes et al., 2012). Slowed shell growth rate, increased mortality, zinc accumulation and increased respiration rates were observed in mussels exposed to n-ZnO, which indicates a high energy demand to cope with excessive zinc uptake (Hanna et al., 2013). These data are further supported by behavioural disturbances and increased catalase activity in marine clams (Buffet et al., 2012) and an increase in lipid peroxidation and metallothionein levels in freshwater mussels (Gagne et al., 2013a).

In exposure situations where catalase or GPx are increased in the cell but lipid peroxidation is decreased, it is presumed that the activation of anti-oxidation defence enzymes mitigated oxidative stress; therefore, lipid peroxidation was not increased and damage to membrane was protected. However, a lowering in lipid peroxidation needs further explanation. Normally ROS damage membrane lipids and produce lipid hydroperoxides which trigger an adaptive response in cells and/or cause cell death (Girotti, 1998). Phospholipid hydroperoxidase can be detoxified directly by phospholipid hydroxyperoxides-GPx or classical-GPx or by phospholipase-A2 (Ursini et al., 1991), affecting ultimate formation of malonaldehyde that was detected as TBARS in the present study. A decrease in lipid peroxidation was observed in the digestive gland of CdTe-quantum dots treated freshwater mussels (*Elliption complanata*) that played a favourable role in increasing phagocytosis to assist in the elimination of nanoparticles and protecting the gut epithelium (Gagne et al., 2008).

The present study suggests oxidative injury occurs in both tissues i.e., gill and digestive gland of the two species exposed to various nanoparticles for different exposures; however, the target biomarkers of antioxidant defence were not the same. The levels of antioxidant defence enzymes were in general lower in the digestive gland compared to the gills. The effects were more evident at the lowest exposure concentration used. Acute exposure at the lower exposure concentration enhanced antioxidant defence enzymes to combat oxidative stress, yet the exposure caused cellular damage. Whereas in chronic exposure some of the effects observed in acute exposure were recovered by the organism but in the process metabolic defence is compromised.

Chapter 4. Effect of Nanoparticles on Histopathological Indices in Marine Bivalves

4.1 Introduction

Acute exposure to the test nanoparticles depressed the functional activity of *Amiantis umbonella* and *Asaphis violascens* (data reported in Chapter 2). To gain further insight into the cellular processes involved, a histological investigation of the gill and digestive gland tissues was conducted. Bivalves, being filter feeders, are considered sentinels of sediment quality at a specific site and of the quality of the overlying water column (Costa et al., 2013). These organisms have been used in many toxicological studies focusing on a variety of indicators (Bjørnstad et al., 2006; Muralidharan et al., 2012). The alterations caused by the presence of contaminants can be measured as exposure and effect biomarkers, as these contaminants can affect bivalve molluscs at biochemical and physiological levels (Ramos-Gómez et al., 2011). The Clean Seas Environment Monitoring Programme of the UK considers bivalves (mussels) as biomonitoring organisms (particularly given their sensitivity, availability and commercial relevance) (Lyons et al., 2010), and bivalve histopathology is considered a useful tool for biomonitoring coastal ecosystems.

Histopathology is used in biomonitoring programs as a means to document various biomarkers to effectively demonstrate the health status of an organism (Papo et al., 2014; Teh et al., 2000; Usheva et al., 2006; Van der Oost et al., 2003). In marine pollution monitoring analysis of histopathological alterations in resident (Aarab et al., 2008; Wedderburn et al., 2000) and transplanted bivalves (Morales-Caselles et al., 2008; Nasci et al., 1999) are being increasingly used. Histopathology is a proven method for the *in situ* assessment of toxic effects in both short- and long-term studies (Handy et al., 2002; Morales-Caselles et al., 2008; Watermann et al., 2008).

Histopathological changes in target tissues occur due to exposure to a wide range of contaminants (Au, 2004), and they indicate the status of target tissues, providing a general view of the damage received by the organism. In bivalves, the gills and the digestive gland are directly exposed to environmental contaminants, and play an important role in food collection, absorption and digestion. The gills filter large volumes of water in order to obtain nutrients, and are therefore in near continuous contact with pollutants present in the water column (and indeed resuspended from sediment pools). The digestive gland actively participates in the detoxification and elimination of xenobiotics and is the main site for metabolic processes, especially metal accumulation and deposition (Canesi et al., 2010b; Koehler et al., 2008). It is also involved in the mechanisms of immune defence and homeostatic regulation of the internal medium (Moore and Allen, 2002). Exposure to contaminants can cause cell damage, and epithelial cells may disappear in the gills, while haemocytic phagocytosis may occur in both organs that can lead to tissue dysfunction and can also have detrimental effects on the health status of the individual.

There are few reports available on the effects of engineered nanoparticles on the bivalve gill (being a primary target of waterborne contaminants and the site of uptake of substances present in water) and the digestive gland (as an organ of nanoparticle accumulation) (Al-Subiai et al., 2012; Barmo et al., 2013; Koehler et al., 2008). Further, there are no available detailed histological studies of bivalves in response to nanoparticle exposure; therefore, in this chapter information is generated about the histopathological effect of nanoparticles on the structural integrity of the gills and digestive glands of experimentally exposed bivalves. Histological investigations were conducted on the gill and digestive gland tissues of *A. umbonella* and *A. violascens* after acute (48 hours) and chronic (4 weeks) exposure to selected nanoparticles. The presence or absence of histological changes in target tissues of exposed animals was compared with tissues from control animals.

4.2 Materials and Methods

The methods for animal collection and maintenance, preparing nanoparticle suspensions and the nanoparticle exposure are described in Chapter 2. After cessation of exposure, tissues were processed for histological studies following the standard procedure detailed below (Roberts and Rodger, 2012). Nanoparticle type, concentration, and the exposure time used for both species is given in Table 4.1.

Table 4.1. Types of nanoparticles, concentrations and times used to study the effects of engineered nanoparticles on histopathological indices in marine bivalves.

Bivalve species	Nanoparticle	Concentration (mg/l)	Exposure time	No. of animals per treatment
Acute exposure				
A. umbonella	Nano silver	0.05	48 hours	8
	Nano titanium dioxide	0.05	48 hours	8
A. violascens	Nano silver	0.05	48 hours	8
	Nano titanium dioxide	0.05	48 hours	8
Chronic exposure				
A. umbonella	Nano silver	0.05	4 weeks	8
	Nano titanium dioxide	0.05	4 weeks	8
A. violascens	Nano zinc oxide	0.05	4 weeks	8
	Nano copper oxide	0.05	4 weeks	8

4.2.1 Tissue preparation for histopathology

The bivalves were opened by quickly cutting the adductor muscle(s) as close to the shell as possible. The animals were examined for any clinical signs that can be observed on the shell (blisters, boring sponges, brown rings, malformations, mud worm tunnels, pustules, scars) or on the soft parts (abscesses, abnormal pigmentation, gill erosion, pustules, watery condition). The soft tissues were gently removed from

the shell and placed on paper toweling prior to slicing. The gills and digestive gland were excised and preserved in 10% neutral buffered formalin (NBF). After 24 hours the solution was changed and the samples were transferred to fresh NBF, and then to 50% ethanol (EtOH) solution after 24 hours. An automatic processing unit (Shandon Citadel® Tissue Processor, UK) for tissue processing using a series of solvents, with a 12-bucket solvent and a processing system for the preserved samples was conducted by treating each sample with the following solutions in sequence for one hour each: 50% EtOH, 70% EtOH, 80% EtOH, 90% EtOH, 90% EtOH, 100% EtOH, and 100% EtOH. Tissue clearing was conducted for one hour using 100% xylene, and then it was repeated for another hour. After, paraffin was added for hot infiltration for one hour and repeated for another hour. The tissues were then placed in tissue processing cassettes (Figure 4.1).

The processed tissues in plastic moulds were transferred to steel mould trays and the embedding was done using a tissue wax embedder (Leica, USA). The wax-embedded tissue blocks were stored overnight in a refrigerator. Tissue sections were obtained using a microtome (Leica, USA) mounted on disposable steel blades. Uniform thin (5 to 6 µm) sections were obtained, that were then floated on a warm water bath, taken onto glass slides with the sections stabilized to hold onto the slide by holding the slides on a heated platform at 37 to 40^{o}C. The stabilized tissue sections were further fixed by keeping the slide tray for a further 24 hours in an incubator at 35^{o}C. This was followed by a regular haematoxylin and eosin staining method (Figure 4.1).

4.2.2 Haematoxylin-eosin staining procedure

Briefly, the slides were mounted onto slide baskets and were immersed in different solvents and stains as described below. Staining was done using the following steps: 1) 100% xylene: five minutes, 2) 100% xylene: two minutes (two changes to ensure removal of paraffin), 3) absolute alcohol: two minutes, 4) 95%

alcohol: one minute, 5) 70% alcohol: one minute, 6) 50% alcohol: one minute, 7) distilled water: one minute, 8) hematoxylin: five to seven minutes, 9) water: two to three minutes, 10) 50% propanol: one minute, 11) 50% eosin in 50% propanol: two to three dips, each 10 seconds, 12) 70% propanol: one minute, 13) 90% propanol: one minute, 14) 100% propanol: one minute, 15) 100% propanol: one minute, 16) absolute alcohol-xylene (1:1): two minutes, 17) xylene: two minutes, 18) xylene: two minutes. The samples were mounted using DPX (dibutyl phathalate xylene) as a mounting agent. A cover slip was added, and then the sample was dried and photographed for internal abnormalities.

The slides were examined for histopathological changes using a Leica, DM 2500 microscope. The glossary of terms used to describe the histopathological changes in the gills and digestive tissues is given in Table 4.2.

Figure 4.1. Slide preparation for histopathology.

Table 4.2. Glossary of the terms used for bivalve histopathology.

Terminology	Meaning	Reference
Epithelium	Layer of cells covering both ectodermal and endodermal derived tissues of a body's surface and canals. Bound together by various junctions and substances to provide strength and facilitate metabolite exchange.	Dorland (2006); Stedman (1995)
Haemocytes	Blood cells of bivalves and/or other invertebrates.	Canesi et al. (2010b)
Pigmentation (Pg)	Abnormal insoluble deposits, yellow, brown or black in the cell/tissue that typically results from disease.	Smith and Hafer (2010)
Necrosis (Ne)	Cell death characterized by irreversible damage, the earliest of which is mitochondrial. Changes visible by light microscopy are nucleus related and generally accompanied by cytoplasm shrinkage or fragmentation.	Stedman (1995)(http://medsci.indiana.edu/histo/docs/glossary.pdf)
Hypertrophy (Ht)	An increase in individual cell size, which in turn leads to an increase in the size of tissue mass/organ.	(https://www.schulich.uwo.ca/pathol/about_us/resources/glossary_of_medical_terms.html)
Hyperplasty (Hp)	An increase in the size of an organ due to an increase in the number of normal cells in normal arrangement in a tissue or organ.	Dorland (2006); Stedman (1995)
Degeneration	Retrogressive but sometimes reversible pathological change in cells or tissues. Results in impairment, deterioration, or destruction of functions.	Dorland (2006); Stedman (1995)
Tubule (T)	A minute canal/ small tube found in various structures or organs of the body.	(http://medical-dictionary.thefreedictionary.com/tubule)
Neoplasia (Np)	Pathological process that results in the formation and growth of a neoplasm. Neoplasm is an abnormal tissue that grows by cellular proliferation more rapidly than normal and continues to grow after the stimuli that initiated the new growth ceases. It is usually characterized by partial or complete lack of structural organization and functional coordination with the normal tissue, usually forming a distinct mass.	Stedman (1995)
Pyknosis (Pyk)	Reduction or condensation of the size of the cell or its nucleus.	Pharma (2006); Stedman (1995)
Deposit / deposition	A pathologic accumulation of inorganic material in a tissue / Extraneous inorganic matter collected in the tissues or in an organ of the body	(http://medical-dictionary.thefreedictionary.com/depositing)
Inflammation	Pathological process which destroys, dilutes, and walls off the injurious agent. Inflammation consists of a dynamic complex of cytological and chemical reactions that occur in affected tissues in response to an injury or abnormal stimulation. The injury can be caused by a physical, chemical, or biologic agent, including the local reactions and resulting morphological changes. The destruction or removal of injurious material, and the responses that lead to repair or healing.	Dorland (2006); Stedman (1995)

4.3 Results

The histopathology of the gill and digestive gland tissues of the exposed group of animals was compared with controls to derive information about the histopathological changes, if any, in nanoparticle exposed animals. The histopathological alterations observed after acute and chronic exposure to various nanoparticles at 0.05 mg/l are described below and summarized in Table 4.3.

4.3.1 *Amiantis umbonella*

The histopathological changes observed in the gills and digestive gland tissues of *A. umbonella* after acute and chronic exposure to n-Ag and n-TiO_2 are detailed below:

Gills

Representative images of haematoxylin and eosin–stained sections of the gills from control *A. umbonella* and those exposed to n-Ag and n-TiO_2 are shown in (Figure 4.2). Normally, the gills comprise two gill-plates at each side of the animal. The gill-plate is composed of a number of gill filaments. The wall of the gill filament is lined with ciliated columnar epithelial cells and between them there are a number of mucous secreting cells. The core of the gill-plates, as well as the gill filament, is made of loose connective tissue with interlamellar tissue. In the control group, the gills exhibited well-preserved histological structures; intact gill lamellae with rich lamellar foliation were observed and the gill filaments were covered with ciliated epithelia on their external surfaces (Figure 4.2 A and Figure 4.3 A).

In acute exposure, the gills from n-Ag and n-TiO_2 exposed clams exhibited slight histological alterations. Erosion (E) of the gill epithelium and necrosis (Ne) was observed in some of the regions of the gill filaments (Figure 4.2 B and C). The overall damage in the gills was less frequent and less severe in clams after acute exposure compared to chronic exposure. The gills after chronic exposure to n-Ag and

Table 4.3. Summary of histopathological evaluations of different organs (G = gills, DG = digestive gland) of *A. umbonella* and *A. violascens* following exposure to different nanoparticles.

Species and exposure	Nanoparticle	Organ	Percentage abnormality *	Observed effects
A. umbonella				
Acute	n-Ag	G	75	Erosion
		DG	75	Loss of lumen
	n-TiO_2	G	75	Erosion, Necrosis
		DG	87.5	Degeneration of tubule, sloughing
Chronic	n-Ag	G	87.5	Deposition, sloughing
		DG	87.5	Necrosis, haemocyte infiltration, disintegration of lumen, erosion
	n-TiO_2	G	100	Ghost cells, swollen eroded gill filaments, necrotic erosion, deposition
		DG	100	Degeneration of tubule, sloughing, pyknosis, vacuolation, loss of lumen, deposition, erosion, ghost cells
A. violascens				
Acute	n-Ag	G	62.5	Haemocyte infiltration
		DG	62.5	Degeneration of tubule, degeneration of epithelium, intertubular neoplasia
	n-TiO_2	G	75	Haemocyte infiltration
		DG	62.5	Loss of tubule structure, necrosis, pyknosis
Chronic	n-ZnO	G	100	Necrosis, haemocyte infiltration, exfoliated lamellae, epithelial erosion
		DG	100	Necrosis, deposition
	n-CuO	G	100	Degeneration, necrosis, pigmentation
		DG	100	Necrosis, deposition, pyknosis

*For each treatment eight individuals were analyzed

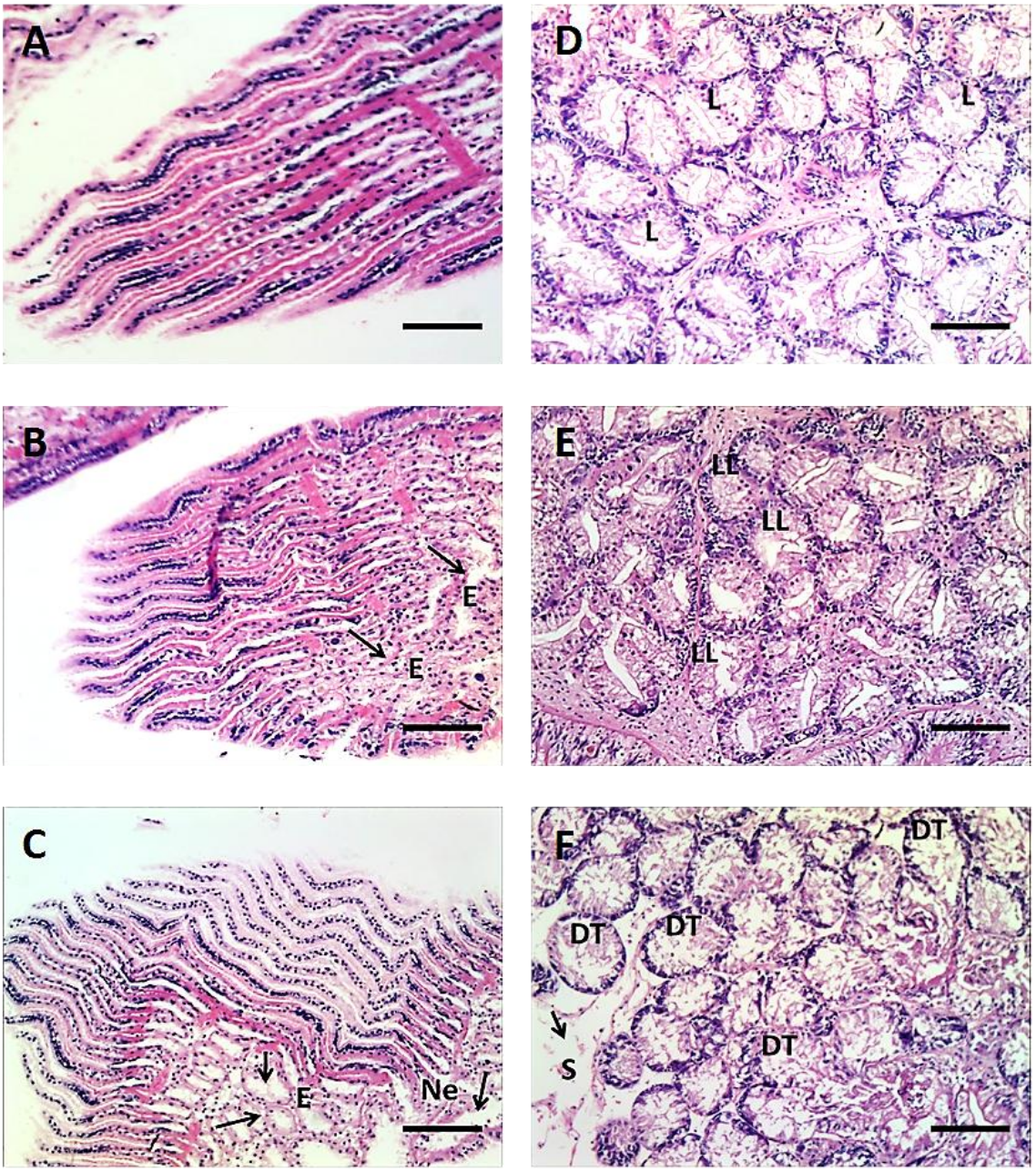

Figure 4.2. Histological sections of *A. umbonella* gills and digestive glands from animals that were acutely exposed to engineered nanoparticles (0.05 mg/l n-Ag and n-TiO_2) for 48 hours. (A) Gill structure of control, (B) gills after exposure to n-Ag, and (C) n-TiO_2, (D) digestive gland structure of control, digestive gland after exposure to (E) n-Ag and (F) n-TiO_2. Ne = necrosis; E = erosion; L = normal tubule with intact lumen; LL = loss of lumen; DT = degeneration of tubule; S = sloughing and loss of tubule structure. Scale bar: 100 µm.

n-TiO_2 showed sloughing of the epithelia (S) and deposition (D) in n-Ag exposed gills. In some regions of the gills exposed to n-TiO_2 swollen eroded gill filaments (SF) and necrotic erosion (NE) were observed. The appearance of ghost cells (G) and heavy deposits (probably metal) were common alterations observed in the gills on exposure to both types of nanoparticles (Figure 4.3 B-E). The damage was more severe in n-TiO_2 exposed gills with substantial deposition in the tissues (Figure 4.3 F).

Digestive gland

The normal structure of the clam's digestive gland consists of digestive tubules (digestive diverticula) formed by a single layer of ciliated epithelial cells, with an almost occluded lumen. The normal intertubular tissue is formed by a few fibrocytes and haemocytes (hyalinocytes). In specimens from control animals, digestive tubules showed normal round/oval structure, lined by a columnar epithelium; there was no evidence of haemocyte infiltration, necrosis or other damage (Figure 4.2 D, Figure 4.4 A, E and H).

In the digestive gland, acute exposure to n-Ag resulted in degeneration of the epithelium of the digestive tubule, and loss of the lumen with cellular damage (Figure 4.2 E). After chronic exposure the damage was aggravated with haemocytic infiltration, disintegration of the lumen, and erosion of the digestive duct. Only a few intact tubules were observed and many were under the process of degeneration (Figure 4.4 B, C and I).

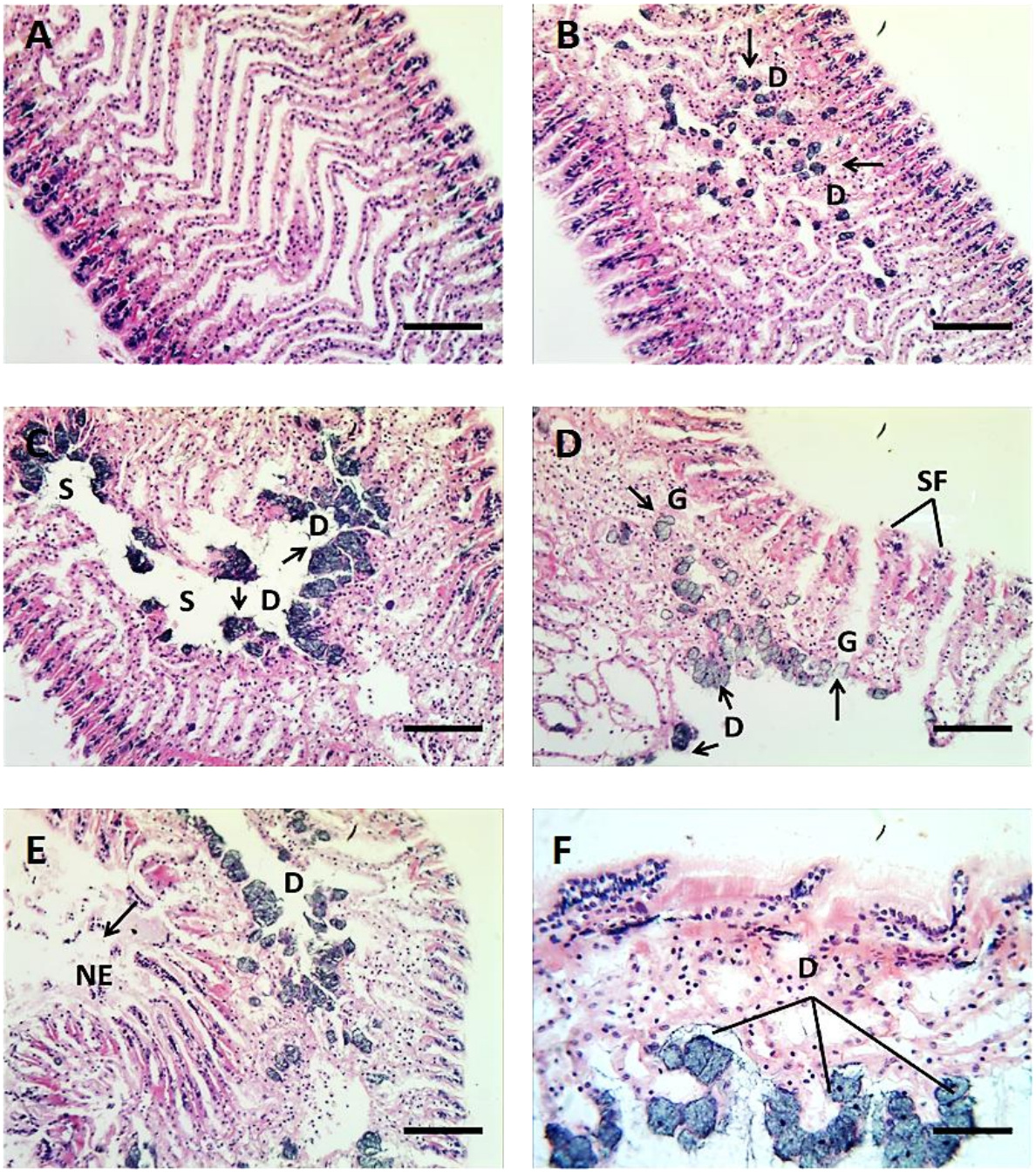

Figure 4.3. Histological sections of *A. umbonella* gills from animals that were chronically exposed to engineered nanoparticles (0.05 mg/l n-Ag and n-TiO_2) for four weeks. (A) Gill structure of control, gills after exposure to n-Ag (B & C), and n-TiO_2, (D-F). D = deposition; S = sloughing; G = ghost cells; SF = swollen and eroded gill filaments; NE = necrotic erosion. Scale bar: 100 µm (A, B, C, D & E); 50 µm (F).

Exposure to n-TiO_2 resulted in more drastic effects on the digestive gland even in acute exposure as loss of tubule structures, sloughing of the intertubular tissue (S) and extensive degeneration of the digestive tubules (DT) were observed (Figure 4.2 F). After chronic exposure, the histological pattern reflected severe tubular damage, the complete loss of the tubule epithelium and collar, probably caused by necrosis. Complete loss of structure, sloughing within the tubules with poor inflammatory response and loss of the lumen (LL) in many of the tubules showing extensive degeneration of the digestive tubules (Figure 4.4 D and G) was also evident. Ghost cells in the villi of the digestive surface along with probable deposits of n-TiO_2 (Figure 4.4 J), vacuolization, pyknosis (Pyk) and erosion of the digestive epithelium and loss of villi of the digestive duct were also detected (Figure 4.4 F and J).

4.3.2 *Asaphis violascens*

The histopathological response of *A. violascens* after acute exposure to n-Ag and n-TiO_2 was investigated to determine whether differences existed in its response compared with *A. umbonella*. Since the response of n-Ag and n-TiO_2 in the two species was not different, for chronic exposure other nanoparticles were selected. Consequently, two other nano-metal oxides, n-ZnO and n-CuO were used and *A. violascens* was exposed at 0.05 mg/l for four weeks to study the chronic toxicity of these metal oxides. The data are described below.

4.3.3 Acute exposure of *A. violascens* to n-Ag and n-TiO_2

Gills

In the control group, the gills exhibited well-preserved histological structures. The gill lamellae were intact with rich lamellar foliation and the gill filaments were covered with ciliated epithelia on their external surfaces (Figure 4.5 A)

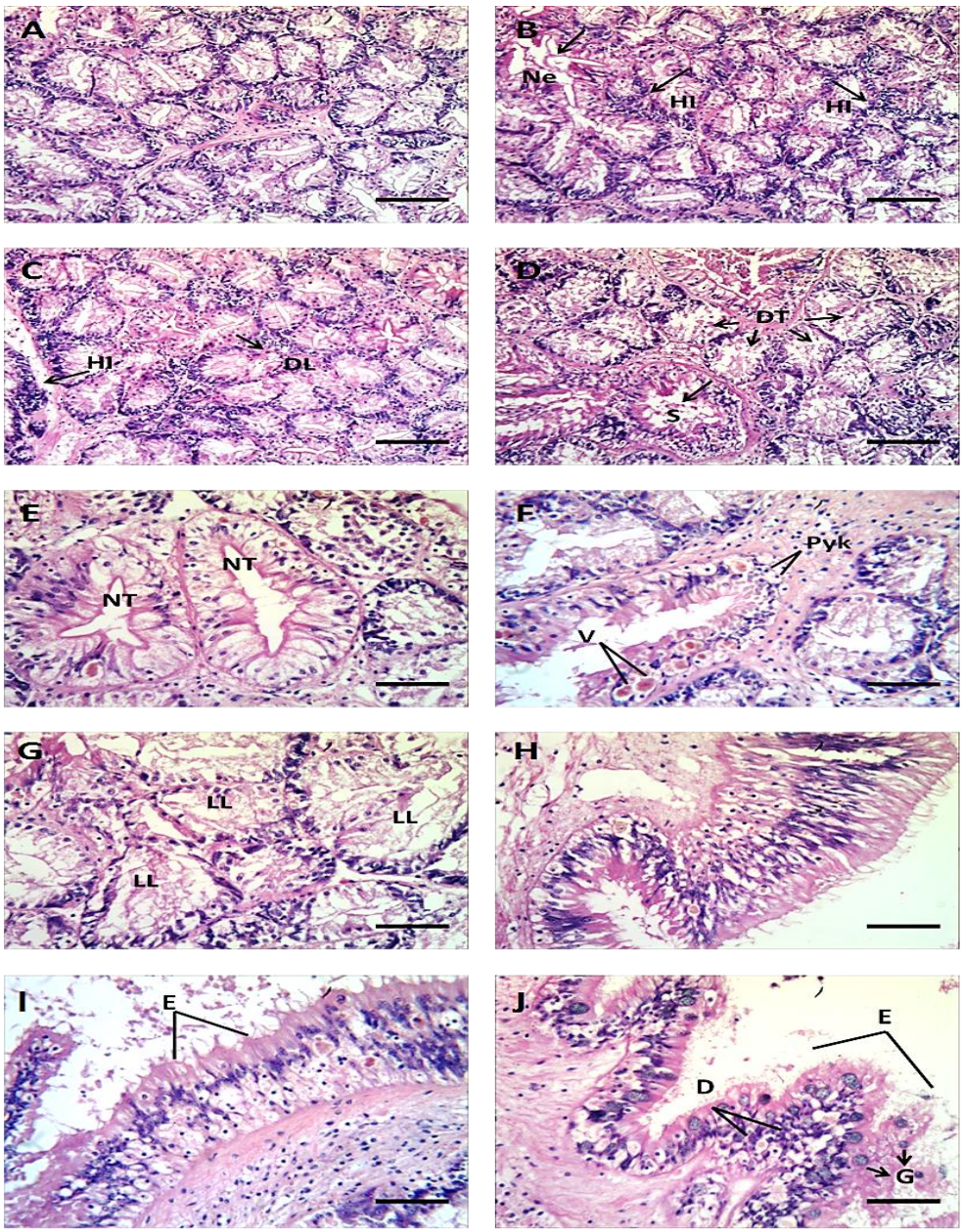

Figure 4.4. Histological sections of *A. umbonella* digestive glands from animals that were chronically exposed to engineered nanoparticles (0.05 mg/l n-Ag and n-TiO_2) for four weeks. (A, E & H) Digestive gland structure of control, (B, C & I) digestive glands after exposure to n-Ag, and (D, F, G & J) n-TiO_2. HI = haemocytic infiltration; E = erosion; Ne = necrosis; DL = disintegration of lumen; DT = degeneration of tubule; S = sloughing and loss of tubule structure; NT = normal tubule; Pyk = pyknosis; V = vacuolation; LL = loss of lumen; E = erosion; G = ghost cells; D = deposition. Scale bar: 100 µm (A, B, C, & D); 50 µm (E, F, G, H, I & J).

observed The gills from acute exposure to n-Ag and n-TiO_2 exhibited minor histological alterations. There was a moderate influx of haemocytes in the entire specimen of the gills studied indicating an inflammatory response, but no particular modification of the tissue was (4.5 B and C). There was no erosion of the gill epithelium and necrosis contrary to that found in *A. umbonella* after acute exposure.

Digestive gland

In control animals, the digestive tubules showed well preserved normal round/oval structures, with brush borders of columnar epithelium and no evidence of haemocytic infiltration, necrosis or other damage (Figure 4.5 D).

In the digestive gland of animals acutely exposed to n-Ag, degeneration of the epithelium and lumen with cellular damage were observed along with widespread intertubular neoplasia and hyperplasty (Figure 4.5 E, F and G). Necrotic changes were also observed with hypertrophy of the epithelium cells and the lumen with cellular debris. In the digestive gland of animals acutely exposed to n-TiO_2, pyknosis was observed in the epithelial cells of the digestive tubules, complete loss of tubular epithelium without cellular inflammatory response and therefore no haemocytic infiltration was observed (Figure 4.5 H and I).

4.3.4 Chronic exposure of *A. violascens* to n-ZnO and n-CuO

Gills In the control animals, well-preserved histological structures were found in the gills; the lamellae were intact with rich lamellar foliation and the gill filaments were covered with ciliated epithelia on their external surfaces (Figure 4.6 A and B).

Chronic exposure to n-ZnO caused pathological changes in the gill structure with epithelial erosion (EE), exfoliated lamellae (EL), haemocyte infiltration (HI), and necrosis (Ne). Some depositions in the region of the gill filaments and a loss of definition of the ciliated epithelium were also detected (Figure 4.6 C and D).

The gills after chronic exposure to n-CuO in particular showed necrosis (Ne), degeneration, and extensive areas with high pigmentation (Pg) (Figure 4.6 E and F). In general, exposure to n-ZnO and n-CuO induced histological changes in the gills with progressive changes in the epithelial cells and in the cilia, with reduction of contacts between adjacent filaments with increasing haemocytic infiltration.

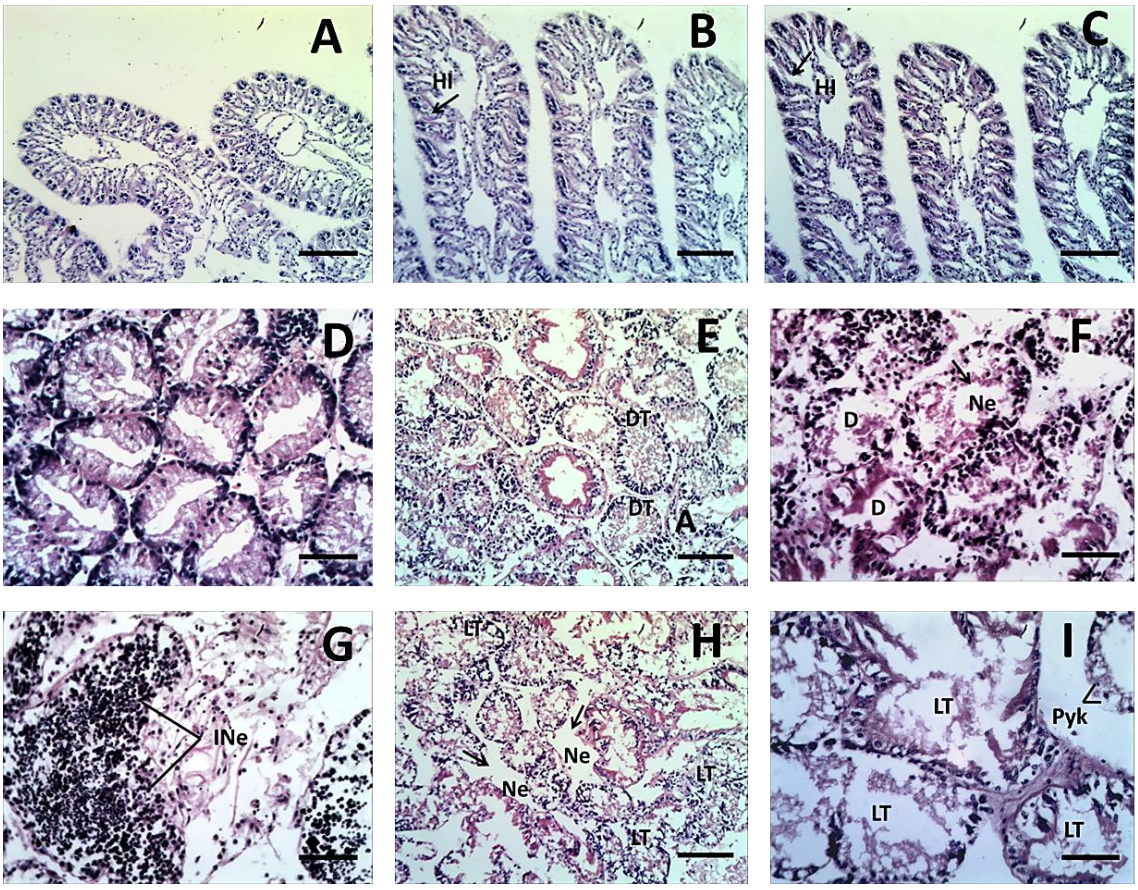

Figure 4.5. Histological sections of *A. violascens* gills and digestive glands from animals that were acutely exposed to engineered nanoparticles (0.05 mg/l n-Ag and n-TiO_2) for 48 hours. (A) Gill structure of control, (B) gills after exposure to n-Ag, and (C) n-TiO_2, (D) digestive gland structure of control, digestive gland after exposure to n-Ag (E, F &G) and n-TiO_2 (H & I). HI = haemocyte infiltration; DT = degeneration of tubule; LT = loss of tubule structure; Ne = necrosis. Scale bar: 100 µm (A, B, C, D, E, F & H), 50 µm (G & I).

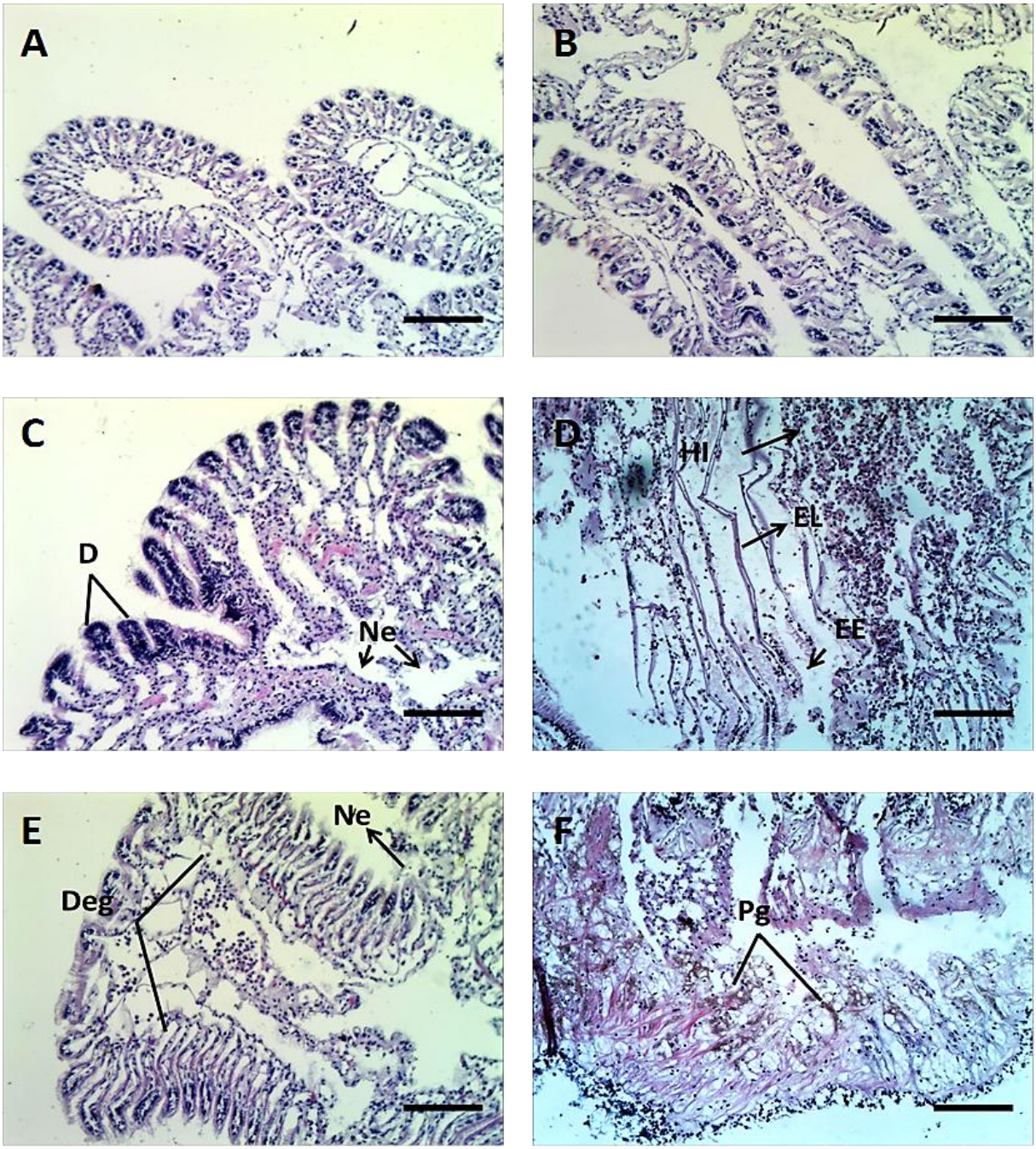

Figure 4.6. Histological sections of *A. violascens* gills from animals that were chronically exposed to engineered nanoparticles (0.05 mg/l n-ZnO and n-CuO) for four weeks. (A & B) Gill structure of control, gills after exposure to n- ZnO (C & D), and n- CuO, (E &F). Ne = necrosis; HI = haemocytic infiltration; EL = exfoliated lamellae; EE = epithelial erosion; Deg = degeneration; Pg = pigmentation. Scale bar: 100 µm.

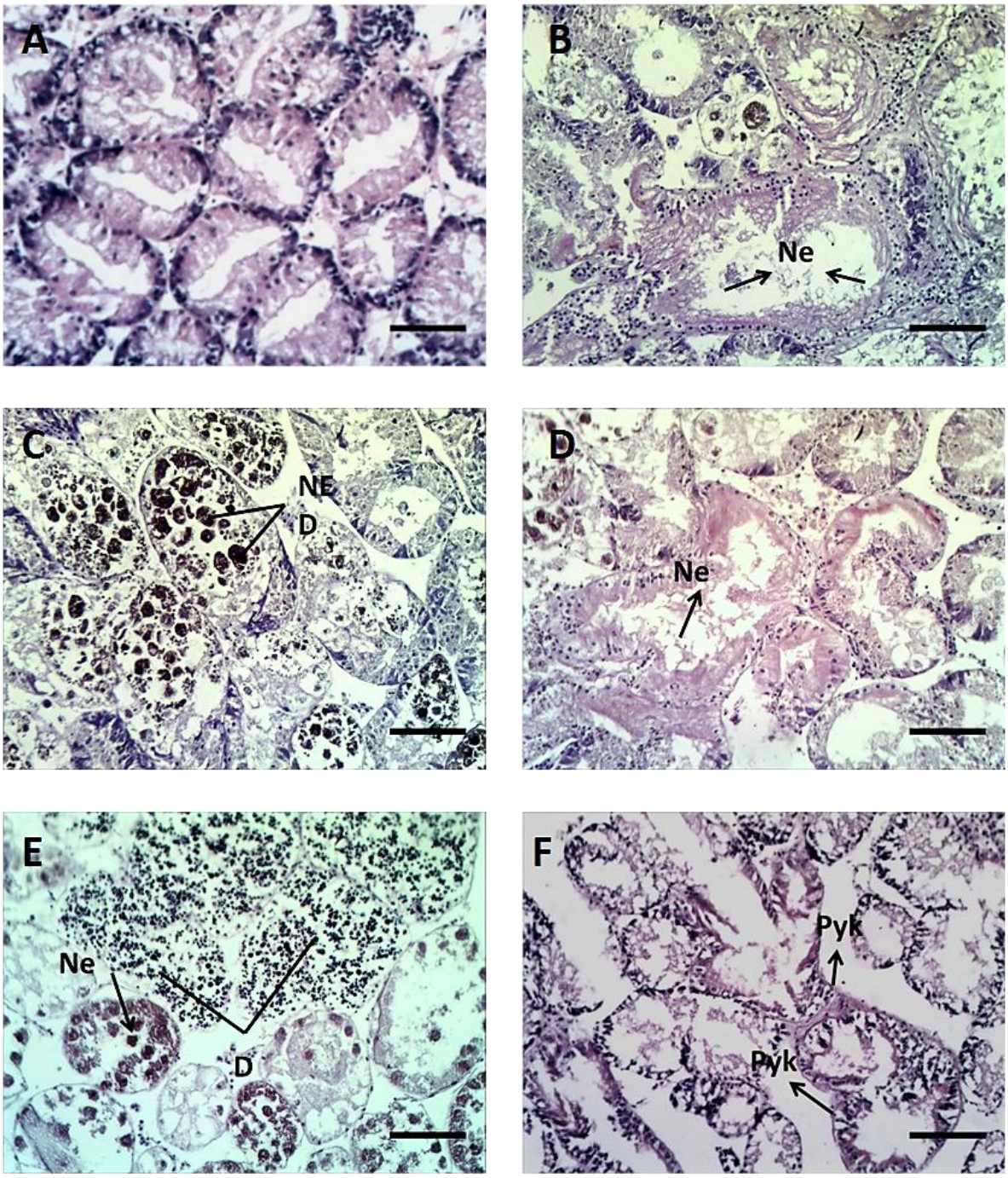

Figure 4.7. Histological sections of *A. violascens* digestive glands from animals that were chronically exposed to engineered nanoparticles (0.05 mg/l n- ZnO and n- CuO) for four weeks. (A) Digestive gland structure of control, (B C) digestive glands after exposure to n-ZnO, and (D, E & F) n-CuO. Ne = heavy necrosis; Nf = focal necrosis; D = deposition; Pyk = pyknosis. Scale bar: 100 µm.

Digestive gland. In control animals, digestive tubules showed well preserved normal round/oval structures, with brush borders of columnar epithelium and no evidence of haemolytic infiltration, necrosis or other damage (Figure 4.7 A).

Exposure to n-ZnO and n-CuO caused similar histological changes in the digestive gland. The degeneration of the digestive gland cells increased in severity after chronic exposure. There appeared to be severe tubular damage, with complete loss of the tubule epithelium and collar, possibly caused by necrosis. Focal damage was also observed in damaged tissues of the digestive gland exposed to n-ZnO (figure 4.7B and C).

Chronic exposure to n-CuO resulted in focal damage and tissue necrosis. The exposure caused erosion of the tubular epithelium and epithelial necrosis within the digestive tubules. Few intact tubules were observed and many were under the process of degeneration. Pyknosis, complete loss of the digestive tubules and inter-tubular neoplasia was also observed in the digestive gland cells with poor inflammatory response (Figure 4.7 D, E and F).

4.4 Discussion

Histological changes in gills and digestive glands of bivalves have been shown to be responsive to a wide variety of pollutants since these organs play an important role in respiration, food collection and digestive processes (El-Shenawy et al., 2009; Usheva et al., 2006). The gills are arguably the tissues that are most rapidly affected by waterborne contaminants. In filter feeders, feeding and respiration are gill dependent processes with the gills functioning as a first line of defence against environmental pollutants that includes particle rejection, psuedofaeces formation, and mucous secretion (El-Shenawy et al., 2007; Janssen et al., 1992). Reduced filtration rate and valve closure are other defensive mechanisms against environmental stress. In chapter 2 it was observed that chronic exposure to nanoparticles caused reduced clearance rates with valve opening severely affected at higher exposure

concentrations. Since the gills and digestive glands are the main sites of particle uptake in bivalves, histopathological effects of nanoparticle exposure were consequently examined in these target tissues. All the gill and digestive gland samples showed histopathological changes associated with nanoparticle exposure but the abnormalities were more severe after chronic exposure.

The gill plates and filaments are made up of loose connective tissues with interlamellar tissue. Acute exposure to n-Ag and n-TiO_2 at 0.05 mg/l, in general, caused minor histological changes in gill structure. In *A. umbonella*, erosion and necrosis of the gill filament epithelium were also seen. No such lesions were observed in *A. violascens* thereby suggesting that *A. violascens* was more resilient under acute exposure. However, influx of haemocytes into the gills of *A. violascens* was observed indicating an inflammatory response, but no particular tissue modification was detected. Changes in the lamellar region and increased haemolytic infiltrations have also been observed in gill tissues of mussels exposed to hexavalent chromium (Ciacci et al., 2012) and bivalves exposed to organophosphate pesticides (El-Shenawy et al., 2009). Reported electron microscopic studies with nano-glass particles showed that nanoparticles are taken up in the gill epithelial cells where larger fibrils are more often found, suggesting material is first sorted and transported at the gills either by a simple diffusion pathway or by the process of endocytosis (Koehler et al., 2008). The entry of nanoparticles possibly triggers an inflammatory reaction with an influx of haemocytes since haemocytic infiltrations (both focal and diffuse) constitute repair processes following tissue damage (Ruiz et al., 2015).

On chronic exposure, the histopathological symptoms in the gill epithelial structure were aggravated. There were some common changes in the gills after chronic exposure to n-Ag and n-TiO_2 including sloughing of the epithelia and the appearance of ghost cells and heavy deposits (probably metal) were common alterations. Swollen eroded gill filaments and necrotic erosion were observed in some regions of gills exposed to n-TiO_2. The damage was more severe in n-TiO_2 exposed gills with substantial deposition in the tissues. The effect of n-Ag and n-TiO_2 after

chronic exposure may be summarized as heavy deposition, epithelial exfoliation, necrosis and epithelial erosion as common occurrences. Comparing these symptoms with chronically exposed *A. violascens* to n-ZnO and n-CuO revealed that accumulation/deposition of n-ZnO also occurred in the gills after chronic exposure. In mussels, copper accumulation following exposure to 32 µg/l concentrations for five days caused severe abnormalities in different organs, such as swelling in the adductor muscle, erosion of the gill cilia and necrosis in the digestive tubules (Al-Subiai et al., 2011). In a study conducted by Trevisan et al. (2014), n-ZnO was initially incorporated in the gills and digestive gland, but was preferably accumulated in the digestive gland after 96 hours of exposure. Some other studies suggest that the uptake of nanoparticles occurs preferentially in the digestive organs of bivalves (Al-Subiai et al., 2012; Garcia-Negrete et al., 2013; Joubert et al., 2013; Moore, 2006); however, it has also been confirmed that gills can also be a main target for nanoparticles (Koehler et al., 2008; Trevisan et al., 2014).

The presence of pigmented brown cells in *A. violascens* gills after chronic exposure to n-CuO is substantiated by similar findings by Hu et al. (2014), wherein brown cell accumulation was also seen in the gills, mantle, digestive tubules and sinuses of mussels exposed to n-CuO at various concentrations after four hours. Such pigmentation has been linked to the accumulation of granules thought to affect protein turnover (Lowe and Clarke, 1989). Higher levels of "dark" residual bodies were also observed within the tissue of species sampled from polluted sites and the authors linked it to heavy metal, and organic pollutant exposure (Domouhtsidou and Dimitriadis, 2001). Increased observation of pigmented cells due to n-CuO exposure could therefore be consistent with altered lysosomal structure and function. Several authors suggest that lysosomes are implicated in non-specific protein turnover and contribute to proteolytic breakdown of ingested foreign matter (Hu et al., 2014; Moore, 1990; Moore et al., 2009).

The digestive gland is an important organ involved in the basic process of subcellular digestion. It is covered by a squamous epithelium resting on a layer of

fibrous connective tissue. The epithelium largely contains digestive cells and also secretary cells. Several histopathological studies have shown that epithelial cells lining the tubules of the digestive gland are sensitive targets of environmental contaminants (Cajaraville et al., 1990; Moore, 1985). The digestive gland has been known as a target organ for contaminant effects because this organ plays a major role in contaminant uptake, intracellular food digestion and metabolism of inorganic and organic chemicals (El-Shenawy et al., 2009; Rainbow, 2002; Usheva et al., 2006).

In *A. umbonella*, the digestive glands acutely exposed to n-Ag displayed degeneration of the epithelium and lumen with cellular damage observed along with widespread intertubular neoplasia and hyperplasty. Necrotic changes were also observed with hypertrophy of the epithelium cells and lumen with cellular debris. Upon chronic exposure to n-Ag the digestive gland exhibited tubule damage and the complete loss of tubule epithelium.

On acute exposure to n-TiO_2, pyknosis was observed in the epithelial cells of digestive tubules, with complete loss of tubular epithelium without a cellular inflammatory response - therefore no haemocytic infiltration was observed. The symptoms observed after acute exposure were also visible after chronic exposure. In addition, chronic exposure to n-TiO_2 also caused an inflammatory response with epithelial necrosis and vacuolization. Recently, D'Agata et al. (2014) observed vacuolation of the digestive tubules in *Mytilus galloprovincialis* exposed to nano and bulk forms of TiO_2 after four days of exposure; however, the histopathological changes were more severe with bulk TiO_2. In bivalves, the vacuolization of digestive cells is considered a common response to metal toxicity associated with cellular detoxification processes (El-Shenawy et al., 2009; Rubio et al., 1993). In another study, mussels exposed to 100 µg/l of n-TiO_2 for acute exposure (96 hours) showed no morphological alterations in the digestive tubes as observed under electron microscopy but nanosized particles were detected in the microvilli and within the lysosomal vacuoles of the digestive cells (Barmo et al., 2013). *In vitro* exposure of haemocytes presents a similar picture (Ciacci et al., 2012). These observations

suggest that nanoparticle transfer from the digestive tract to the haemolymph and into the haemocytes does take place (Browne et al., 2008). It has been reported that nanoparticles are not accumulated in the soft tissues of *Mytilus* but get filtered and excreted in pseudofaeces (Montes et al., 2012).

The common response observed with both n-Ag and n-TiO_2 in the digestive gland was that the epithelium of channels and tubules, connective muscle and nervous tissues, were damaged and some were under the process of degradation. Exposure to n-ZnO and n-CuO caused similar histological changes in the digestive gland of *A. violascens*. The most essential pathology was disruption of the integrity of the digestive tubule, the epithelium of tubules and channels as a result of desquamation of digestive cells. Loss of definition of digestive tubules by necrosis may result in the dysfunction of the gland, thereby exerting deleterious effects on the organism. The desquamation of digestive cells in bivalves under the impact of various pollutants is an often recorded pathology, both in experimental conditions and under chronic anthropogenic pollution (Berthou et al., 1987; Cajaraville et al., 1990; Syasina et al., 1997; Usheva et al., 2006). As a rule, that phenomenon is accompanied by an increase in the number and size of vacuoles in digestive cells. Many authors relate the occurrence of abnormal vacuoles in cells of the molluscan digestive gland to a sharp increase in volume of lysosomes impacted by pollutants (Lowe and Fossato, 2000; Moore, 1979, 1985; Moore, 1988). The increase of vacuolization of digestive gland cells is one of the most usual phenomena recorded in bivalves under the effect of xenobiotics of organic (Neff et al., 1987; Zupan and Kalafatić, 2003) and inorganic (Bright and Ellis, 1989; Wedderburn et al., 2000) natures.

The histopathological examination showed alterations in the morphological structures of gills and digestive glands both after acute and chronic exposure of bivalves to the nanoparticles used in this study. The exposure time was an important factor as bivalves exposed for a longer time period in chronic exposure showed severe alterations in their tissues. Accumulation of nanoparticles in different tissues and structural alterations deter the normal functioning of the cells, which leads to

organ dysfunction, disease or death due to the inability of the organism to cope with the stress caused by nanoparticles. In this study, both gills and digestive gland were affected due to nanoparticle exposure; therefore, these two organs can be used as target organs in determining the toxic effects of nanoparticles on marine bivalves.

Chapter 5. Synthesis

This study relates to the emergence of engineered nanoparticles (ENPs) as contaminants in the marine environment. When this study was initiated (2011), few research papers were available on ENP toxicity. Classical toxicity testing protocols were adopted in most of the studies with few modifications concerning the nature and behaviour of ENPs in aqueous media. The regulatory machinery of the OECD and the US EPA formed a consortium of scientists to evaluate the appropriateness of their standard ecological test guidelines for testing nanoparticles. The review concluded that existing protocols could not be used *in toto* for evaluating nanoparticle safety, and exploratory research is needed to either modify the existing protocols or to draft altogether new protocols (OECD, 2012; USEPA, 2009). Recently, environmentally relevant test conditions for the evaluation of ENPs ecotoxicity testing have been thoroughly debated and reviewed (Holden et al., 2016; Montaño et al., 2014; Selck et al., 2016). However, the basic requirements for any test guidelines remain the same, e.g., selection of exposure levels; running simultaneous controls alongside exposed groups; selection of healthy and viable test organisms; and relevant endpoints that indicate ENP toxicity mechanisms. At the time of project conception, the most active group publishing on ENP toxicity was Canesi and co-workers (2008, 2010 a, b); as such, the current study adopted similar protocols. The data presented in this thesis were generated using uniform test conditions. The seawater was filtered to 0.45μm to remove particulate matter, and water quality was kept constant regarding pH, salinity, and temperature for all exposure studies. The exposure durations were fixed at 48 hours for acute exposures and four weeks for chronic exposures.

5.1 Nanoparticles Used in this Study

The nanoparticles studied for their toxicological effects were mainly silver (n-Ag) and titanium dioxide (n-TiO_2), and nano- and bulk- zinc oxide (n-ZnO and b-ZnO) and copper oxide (n-CuO and b-CuO). Metal oxide nanoparticles were chosen

because of their large-scale industrial production, common occurrence in consumer products, and occurrence as industrial pollutants, and their potential toxicity (Abdel-Khalek et al., 2015; Melegari et al., 2013). All nanoparticles, without any surface modifications, were procured from a reputable international company, Sigma-Aldrich, USA, through a local chemical supplier.

5.2 Exposure Concentrations

After mixing a known concentration (1 mg/ml) of ENPs into suspension through sonication, the final exposure concentrations were adjusted to 0.05, 0.2, 1.0 and 2 mg/l. However, the concentration in the medium was not determined pre- and post- organismal exposure due to non-availability of the relevant laboratory equipment. The behaviour of the particles in the prepared medium was examined by dynamic light scattering (DLS). As reported (Chapter 2), a phenomenon of agglomeration was observed and the collective particle size in suspension increased. Agglomeration was also evidenced using transmission electron microscopy (TEM). The agglomeration and settling of nanoparticles in aqueous media are well documented (Fabrega et al., 2011; Shang et al., 2014). However, in my test system, a gentle stream of air was passed through the exposure vessel that helped keep the particles in suspension.

The complexity of ENP behaviour in aquatic medium requires multiple methods to determine accurate concentrations to define dose metrics. Suspended ENPs undergo a variety of modifications such as agglomeration, state of surface oxidation, and dissolution; each particle state may have different toxicological properties. It requires excessive efforts using expensive methods to fully understand the phenomenon of each modification state, i.e., ageing and quantitative determination. Montaño et al. (2014) summarised various measured ENP properties based on current analytical approaches, and highlights the technical obstacles to accurate detection and characterization (Table 5.1). In another recent study, it has been suggested that the diameter of spherical ENPs used to assess dose metrics has no

Table 5.1. Need for improved analytical approaches to characterise ENPs. Source: (Montaño et al., 2014). TEM, transmission electron microscopy; SEM, scanning electron microscopy; sp-ICP-MS, single particle inductively coupled plasma–mass spectrometry; DLS, dynamic light scattering; Fl-FFF, flow-field-flow fractionation; Sed-FFF, sedimentation field-flow fractionation; ENPs, engineered nanoparticles; NNPs, naturally occurring nanoparticulate matter; FFF, field flow fractionation; NTA, nanoparticle tracking analysis; EDX, elemental X-ray analysis; OES, optical emission spectroscopy; XAS, X-ray absorption spectroscopy.

Measured property	**Current analytical approaches**	**Obstacles to accurate detection and characterization**	**Potential need**
Particle size	TEM, SEM, sP-ICP-MS, UV-vis, DLS, Fl-FFF, Sed-FFF	Introduction of artifacts from sample drying (TEM/SEM); No elemental specificity (DLS); Elemental specificity: Inability to differentiate between ENPs and NNPs of similar elemental composition (sP-ICP-MS, TEM, SEM); Obstructed by the high background of natural particles (sP-ICP-MS, TEM, SEM, DLS, FFF)	Analysis of samples in situ with minimal sample preparation; Elemental specificity to differentiate between dissimilar nanoparticles; Requires another measured property to differentiate between particles of similar elemental composition
Surface groups	NMR spectroscopy, FTIR spectroscopy, zeta potential	The original coating may have been replaced or overcoated in the environment (all current approaches); Ensemble techniques unable to characterise individual particle populations without prior fractionation steps (all current approaches)	Ability to differentiate between different particle populations in situ; Knowledge of how surface groups are attached may help determine if original coating
Particle number concentration	sp-ICP-MS, NTA	Unable to distinguish aggregates from single particle without parallel imaging or sizing technique	Requires knowledge about aggregation state of ENPs
Elemental composition	EDX, sp-ICP-MS, ICP-MS, ICP-OES, XAS	Unable to distinguish aggregates from single particle without parallel imaging or sizing technique; May require acidification, eliminating particle integrity (ICP-MS, ICP-OES); Sample preparation may alter sample representativeness; The concentration of ENP in a sample may be too low (XAS)	Requires knowledge about aggregation state of ENPs Improve detection levels for X-ray based spectroscopy
General considerations			
Mass detection limit	ENPs are expected to enter into the environment at very low concentrations (nanograms per litre)		
Size detection limit	Most nanoparticles are between 1 and 100 nm (much smaller than 20 nm)		
Aggregation state	Need the ability to discern aggregated from single particle material.		
NNPs	The concentration of NNPs in the environment are several orders of magnitude above that of ENPs (milligrams per litre *v.* nanograms per litre); Some NNPs have similar elemental composition and morphologies to ENPs; NNPs tend to be very polydisperse and can interact with ENPs in the environment		

relevance for non-spherical ENPs (Hua et al., 2016). The authors considered volume, surface area, or particle number, and demonstrated that volume (irrespective of the ENP composition) was the most appropriate dose metric for most of their nine test organisms, but the surface area was found to be important for three of the organisms, showing that different organisms respond to different ENP physical states.

Numerous studies are now available where dissolution, particle characterization in tissues, and the ageing of ENPs, have been investigated (Table 1.7). In other studies, the toxicity of ENPs was compared with their ionic form to shed light on the difference in the toxicity mechanism (Wang et al., 2016). The literature is full of explorative studies conducted in different laboratories using their method of choice. To confirm the exposure and internalisation of ENPs most studies utilised measures of pre- and post-exposure concentrations in the medium. However, this does not necessarily ensure an accurate measure of particle uptake by the organism. In other studies, total metal content in the tissue was determined, but this suffers from the drawback that the ratio of ionic and particulate forms is not differentiated; however, it confirms internalisation during exposure. Microscopic identification of particles has also been done to show internalisation. The question remains as to what proportion of ENPs gets transformed in an aqueous medium, are dissolved (i.e., changes to an ionic state) and how much enters the organism and in what form. Chemical estimation of total metal content in tissues only confirms that exposure is there but cannot ascertain the exposure dose. Study on the bioaccumulation of total metals in the gills and digestive gland of bivalve exposed to n-Ag and n-TiO_2 revealed that a very small fraction of exposure concentration of nanoparticles was accumulated in tissue (Chapter 2). The accumulation was concentration dependent, but the level of total silver accumulated was far more than total titanium that is attributed to the nature of the two types of nanoparticles in the aqueous medium. Therefore, a different methodology is needed to correctly identify the effective concentration and the form in which nanoparticle reaches the target tissues and produce its effect. Landsiedel (2016) concludes that in spite of studies on

exposure conditions and the revision of international test protocols, no single physical or chemical material property of a nanoparticle perfectly correlates with its observed biological effects. Therefore, in the present study varying concentrations made in the exposure medium reflects an exposure effect response and can define in totality that a specific concentration of ENPs present in the surrounding water irrespective of its physical state can cause significant functional changes in an exposed organism.

5.3 Predicted Environmental Concentrations (PECs)

Analytical data on the environmental concentration of ENPs is not yet available, even from those countries where their use is well documented. Exposure modeling has been used to determine their probable environmental concentration (PEC). PEC values are required for environmental risk assessment. Gottschalk et al. (2013) reviewed several modeling studies to predict environmental concentrations and acknowledged the difficulties and information gaps regarding ENP production and distribution to products leading to uncertain release assessments. The PEC values are derived from; a probabilistic material flow model, production and use estimates, the release of nanoparticles per year, as well as transfer coefficients for release and mass transfer at all stages of the ENP lifecycle (Sun et al., 2014). The lifecycle-based mass transport model tracked the mass of ENPs through all technical compartments, as well as their flows between technical and natural compartments. However, there are inherent difficulties in direct validation of modelled predicted concentration because in most of the modelling studies, standard handling processes are considered, and accidental release is not accounted for; secondly, methods are not available to distinguish and quantify ENPs in environmental samples containing naturally occurring nanoscale materials (Von der Kammer et al., 2012). The PEC values in seawater calculated in different countries are different and far less than the concentrations used in the majority of exposure studies. The total metal concentrations used in many exposure studies are not environmentally relevant;

nevertheless, when organisms are exposed to realistic environmental metal concentrations, their defence mechanism may be different or may not even be triggered (Vale et al., 2014). The ENP PECs of sediment has always projected to be higher than surface waters; therefore, using bivalves as test organisms justifies the use of higher test exposure concentrations since water close to bottom contains suspended sediment causing exposure to bottom-dwelling organisms (Gottschalk et al., 2013; Selck et al., 2016).

The basic objective of ecotoxicological assessments is to gather knowledge about the harmful effects of the test material, if any, on a representative biological subject. In most cases, several concentrations are tested to determine toxicity and the target of toxic reactions. In this study, bivalves were exposed to multiple concentrations and the functional activity of the organisms, and the parameters of oxidative stress were examined. No effect was observed at 0.01 mg/l in our preliminary experiments (Appendix 1). Therefore further study was conducted at exposure concentrations ≥0.05 mg/l.

5.4 Confirmation of Exposure through Biological Response Assessment

As discussed above, the dose-response metrics, though important in exposure assessment, remain a scientific challenge in nanoparticle toxicity assessment. Therefore, in my system the observed biological effects were the measure of exposure assessment to the collective ENP states present in the exposure medium; the effective environmental concentration was, therefore, the concentration that affected the organism. Selck et al. (2016) stated that an alternative to measuring the test substance itself in and on the organism is to determine its presence indirectly from the biological responses of the whole organism, or preferably key target organs/cellular compartments. This approach requires some prior knowledge for the selection of target biomarkers or behavioural responses. It is known from the literature that nanoparticles are mainly taken up by the gills and then transported to the digestive

gland or digestive system, where disaggregation/ disagglomeration may occur (Canesi et al., 2002). Additionally, the gills being in contact with the exposure medium may receive a direct effect. Therefore, the target organs chosen for this study were the digestive gland and the gills. Concerning the selection of target biomarkers, the extensive evidence is available from the literature that nanoparticles cause oxidative injury in cells, tissues, and organs. Therefore, membrane damage was examined by lipid peroxidation (LPO) and, as a part of the cellular response to exposure insult, the activity levels of enzymes involved in oxidative defence were simultaneously monitored. A non-destructive, whole organism response to exposure was determined by measuring the functional activity of the organism. Since the data were from single species toxicity testing experiments in controlled environments, other confounding factors were eliminated that may be present in mesocosm and actual field scenarios. The advantage of this system is the availability of toxicity data of the test material (ENPs in the present study) in the aquatic medium, irrespective of its physical state. To support this contention, an appraisal of the merits (or otherwise) of laboratory, mesocosm and field studies will not be out of place.

5.5 Laboratory vs. Mesocosm vs. Field Studies

As discussed above, the environmental behaviour and fate of ENPs differ compared to other contaminants (e.g., metals, PAHs, and organic pesticides). Classical toxicity testing methods approved for regulatory purposes have been used in most of the studies. So far, concerning environmental safety, specific methodologies are not yet available for testing ENPs. Therefore, to better understand the real effects of nanoparticles in the environment, studies with conditions that approach environmentally realistic situations are necessary. Experimental exposures are typically done in highly controlled systems in the laboratory (as in the current thesis) or the open air in natural weather conditions (Bour et al., 2015). Studies that integrate complex model systems incorporating environmental conditions (such as microcosms or mesocosms) with ecotoxicity studies of ENPs are rare.

To more realistically assess the toxicity of nanoparticles, environmentally relevant approaches may include exposure via the trophic route under laboratory conditions. These experiments would allow us to determine the potential role of trophic transfer (accumulation/transformation) in affecting toxicity in food chains. Another advantage of these experiments is the accurate calculation of trophic transfer factors (TTFs) due to tight controls of the quantities of food ingested and excreted. Further complexity should be built into future ecotoxicology trials, including multispecies exposures. Simultaneous exposure of several species might lead to complex toxicity responses that are less likely to occur during single-species experiments; for example, filter-feeding bivalve molluscs delivering concentrated ENPs to the sediment via pseudofaeces that may then be ingested by deposit feeders. Competition for food/nutrients (Debenest et al., 2011) and the production of toxins that might affect other species (Campos et al., 2013) or the establishment of a "defence system" at the community level in algal or microbial communities should also be considered (Garay-Narvaez and Ramos-Jiliberto, 2009). Moreover, such experiments under laboratory conditions, in contrast to field studies, allow for the control of abiotic factors. Outdoor exposure systems are a further step closer to realistic environmental conditions. Specific designs and real climate conditions make them more realistic, and these can be performed at larger scales compared to laboratory studies. As described earlier "mesocosms are limited, to more or less closed experimental systems at an intermediate scale between the microcosm of the lab and the full complexity of the real world, the ecosystem" (Odum, 1984). The spontaneous variability of climatic factors to which mesocosms are exposed (François, 2007), makes them more similar to realistic conditions than microcosms. Thus, mesocosms can be representative of natural environments at an intermediate scale of complexity that is between laboratory experiments and natural ecosystems. However, these studies offer less control and can be more difficult to conduct, mainly due to the abiotic factors, which might lead to low repeatability. The exposure time in mesocosms typically varies from a few days to several months, making these studies

difficult to perform and too long to be considered for standardisation. Subsequently, data are very scarce from such studies (Gottschalk and Nowack, 2011).

5.6 Selection of Test Species

The two bivalve species (*Amiantis umbrella* and *Asaphis violascens*) were used as test organisms due to their ability to filter and accumulate suspended particles from the surrounding water. Understanding the fate and effect of chemicals, such as nanoparticles, can be used to assess the health of ecosystems and to provide early warning of adverse environmental change (Burger, 2002). In addition, the amount of bio-accumulated pollutants is a concern as these edible bivalves are consumed by humans and wildlife. Information regarding legacy contaminants, such as metals, is extensively available (Burger and Gochfeld, 2004). However, there is a paucity of literature on the effect of emerging contaminants, e.g., nano-metals, especially for the biota of coastal regions threatened by the development and industrialisation of the region.

Bivalve molluscs are abundant in aquatic benthic systems and tolerate a wide range of environmental conditions. They have the ability to accumulate organic and metallic pollutants to concentrations several orders of magnitude above those in the ambient environment and are capable of metabolising many of these contaminants (Bryan and Gibbs, 1983; Bryan and Hummerstone, 1977). Their filter feeding ability adds greatly to their ecological significance in that bivalves are important calcium and carbon accumulators that link primary producers (bacteria and phytoplankton) with higher organisms in aquatic food chains. Further, by continuously filtering the water column they provide an important ecosystem service by removing suspended particulates, e.g., particulate organic matter and toxic phytoplankton (Canesi et al., 2012; Rocha et al., 2015). As such, bivalves have been used as bioindicators in different international monitoring programs such as the Mussel Watch (USA) since the 1960s. Such programs provide information on both the current status and long-

term trends which allows evaluation of species, population, and ecosystem effects (Burger, 2006).

After Moore (2006), bivalve molluscs were recognised as a unique and well-suited target group for nanotoxicology as they are well suited to take advantage of the behaviour of ENPs in aqueous media. ENPs agglomerate and in some scenario form aggregates that settle to the benthos. Bivalves are suspension or deposit feeders, with some being able to utilise both feeding methods (Arapov et al., 2010). The phenomenon of particle selection has been described in several studies but the basic question as to why some particles are ingested and others are rejected unresolved. It is generally assumed that selection is based on particle size (Defossez and Hawkins, 1997; Lehane and Davenport, 2002), shape (Bougrier et al., 1997), nutritive value (Hawkins et al., 1998; Hawkins et al., 1996; MacDonald and Ward, 1994; Prins et al., 1991) or in response to chemical components on the particle surface (Yahel et al., 2009). Particles greater than 6 μm are captured with >90% efficiency, 1 μm particles with 15-50% efficiency depending on species, whereas smaller particles are captured with an efficiency that decreases with decreasing size (Kach and Ward, 2008; Riisgård, 1988; Ward and Kach, 2009; Ward and Shumway, 2004).

Considering their benthic habitat, the selection of bivalves for testing ENP toxicity is, therefore, more appropriate compared to pelagic organisms (Canesi et al., 2012; Selck et al., 2016). Bivalves are considered a key target group for nanoparticle toxicity for two main reasons: (i) their filter-feeding habit that is characterised by processing large volumes of water (Canesi et al., 2012; Moore, 2006); and (ii) the potential for direct uptake by endocytotic and phagocytotic processes in digestive-related organs e.g., the digestive gland, in epithelial boundaries such as the mantle and gills, and in immunological cells such as the haemocytes.

Mussels (*Mytilus* spp.) are the best-studied bivalve group within the nanoparticle literature. *Mytilus*, which belongs to the subclass Pteriomorphia, currently has 83 papers, with 12 on *Crassostrea* spp. (the second most studied

Pteriomorphid). It is also to be mentioned that *Mytilus, Crassostrea*, etc. represents cold/temperate climatic conditions and other bivalves especially warm water species (including clams and cockles) have rarely been examined. The clam *Amiantis umbonella* (Lamarck, 1818) and the cockle *Asaphis violascens* (Forskal, 1775) belong to the subclass Heterodonta (Figure 5.1). This subclass contains 50% of all bivalve species, and its most important order is the Veneroida, which includes clams and cockles. The use of 'model' bivalves such as *Mytilus* for ecotoxicology undeniably has its place and value; however, given the unique environmental conditions of the Persian Gulf, it would be inadvisable to assume that data generated from *Mytilus* or *Crassostrea* can be directly transposed into a Gulf scenario. This study assumes significance because the test organisms were from hot climatic conditions and their response to the nanoparticles may be different than that observed in the organism from temperate climatic conditions. There may be several environmental factors that may differ in cold and warm conditions like dissolution rate of nanoparticles in warm water, but in experimental studies, these factors were kept constant as assays were conducted under uniform conditions. Therefore, this thesis has given due consideration to the need to utilise bioassay organisms that are adapted to Gulf conditions. *A. umbonella* and *A. violascens* are abundant in the coastal regions of the Gulf (and by extrapolation are presumably well suited to the environment). Both species are widely distributed in Asia; especially in coastal India, Pakistan, Mauritius, the Arabian Gulf, Qatar, and Kuwait. They live and feed at a sediment depth of 7-8 cm, typically in fine sediments, and are quiet easily observed and accessed during low tide. Despite their ecological importance practically no information is available on their physiological and metabolic functions other than scattered reports addressing their reproduction and spawning season (Al-Mohanna et al., 2003; Saeedi et al., 2010). Also, they have not previously been used as ecotoxicology bioassay organisms. Therefore, to study the potential toxic effects of nanoparticles in Gulf waters, these two prevalent yet understudied species were selected to generate ecosystem-relevant baseline information on nanoparticle toxicity and the responses of enzymatic proteins involved in addressing oxidative stress. This study was conducted

as a forerunner to future toxicogenomic investigations. To the best of the author's knowledge, the present study is the first report on the toxic effects of nanoparticles using marine bivalves species from this region.

The Heterodonta comprises more than twenty genera, of which only *Dreissena*, *Corbicula*, and *Venerupis* (later *Ruditapes*) have been studied about nanoparticles. Only sixteen papers are available at the time of writing on nanoparticle toxicity to heterodont species. The bivalves belonging to the different subclasses are closely related in their functional responses; however, from an evolutionary viewpoint they will have potentially crucial differences in their genomes, and for this reason, it is important to study and understand the differences and similarities in their genomic make-up. Considering the National Centre for Biotechnology Information (NCBI) database for genomic/proteomic studies, a considerable amount of information exists for *Mytilus* sp. More than 65,000 expressed sequence tags (ESTs) and 13,000 protein sequences are available; however, only three protein sequences exist for *Asaphis* with no ESTs or protein sequences available for *Amiantis*.

A variety of nanoparticles (most commonly n-TiO_2) have been used to study toxic effects in different veneroid species (Table 5.2). However, the species used in this study have not previously been examined. Zebra mussels (*D. polymorpha*) exposed to n-TiO_2 at low concentrations (7-120 µg/l via water; 4-830 µg/l via food) for one hour were examined for Their capacity to assimilate n-TiO_2 from their diet.

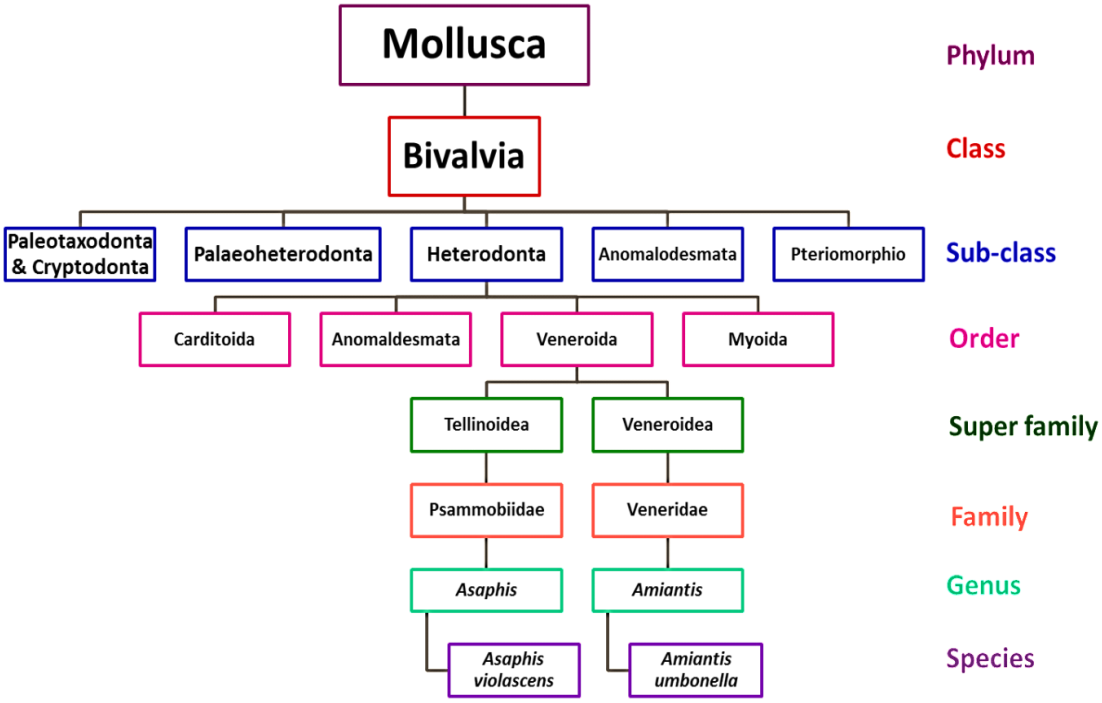

Figure 5.1. A simplified phylogenetic tree of the bivalve subclass Heterodonta concerning the species used in this study.

The assimilation efficiency of n-TiO_2 by mussels from their diet was very low suggesting that nanoparticles mainly accumulate in their gut (Bourgeault et al., 2015). In an earlier *D. polymorpha* study, a 24 hours exposure to n-TiO_2 at 0.1, 1, 5 and 25 mg/l resulted in the inhibition of phagocytosis activity at 0.1 mg/l and above, with the internalisation of nanoparticles into the haemocytes (Couleau et al., 2012). *D. polymorpha* when exposed to n-CeO_2 led to decreases in the size of the lysosomal system, catalase (CAT) activity and LPO in the digestive glands and also negatively impacted haemolymph ion concentrations (Garaud et al., 2015). The author further assessed the effects of chronic (three weeks) exposure to citrate- coated ci-CeO_2 and bare ba-CeO_2 using an integrated multi-biomarker approach (Garaud et al., 2016). Mussels accumulated ci-CeO_2 three times more than ba-CeO_2, with pi-glutathione-S-

transferase mRNA, CAT activity and the lysosomal system being impacted, indicating that mussels exposed to both forms of n-CeO_2 were stressed.

Electron micrograph sections have shown *R. philippinarum* to accumulate gold nanoparticles (n-Au) within either the digestive gland or gill tissues, at different concentrations after 28 days of exposure (Garcia-Negrete et al., 2013). Also in *R. philippinarum,* studied the acute and sub-chronic toxicity of n-Au at 0.75 µg/l was studied (Volland et al., 2015). The particles were readily taken up by the digestive gland and gills and exerted oxidative stress measured by phase II antioxidant enzymes and quantitative PCR gene expression analysis. The data showed that this exposure level caused a low magnitude response as the oxidative damage was not initiated. The authors suggested that n-Au is not toxic to *R. philippinarum* under the conditions of the study.

In another *R. philippinarum* study, Marisa et al. (2015) investigated the effects of n-TiO_2 on haemocytes at 0, 1 and 10 µg/ml were investigated, and a decreased phagocytic index was observed, suggesting that nanoparticles can interfere with cell membranes and their functions (Marisa et al., 2015). Oxidative stress in *R. philippinarum* exposed to n-ZnO at 1 and 10 mg/l concentration was also studied for seven days, and the haemolymph, gills and digestive gland were evaluated for biochemical and cellular responses (Marisa et al., 2016). The results showed that n-ZnO, at concentrations close to predicted environmental levels, significantly affected various tissue parameters. Significant increases in CAT and superoxide dismutase (SOD) activities and a decreasing trend of GST activity indicated the involvement of oxidative stress.

Table 5.2. Summary of engineered nanoparticle studies on the sub-class Heterodonta. ↑ = increase in biomarker measure, ↓ = decrease in biomarker measure.

ENP type	Species	Test Conc.	Exposure period	Effects	Reference
n-TiO_2	*Dreissena polymorpha*	7-120 µg/l-4-830 µg/l	1 hour	ENP accumulation in gut	(Bourgeault et al., 2015)
n-TiO_2	*Ruditapes philippinarum*	0, 1, 10 µg/ml		↓ Phagocytosis; interference with cell membranes and their functions	(Marisa et al., 2015)
n-TiO_2	*D. polymorpha*	0.1, 1, 5 and 25 mg/l	24 hours	↓ Phagocytosis; ↑ ERK1/2, ↑ p38 phosphorylation (5 and 25 mg/l)	(Couleau et al., 2012)
n-ZnO	*R. philippinarum*	1 and 10 mg/l	7 days	↑ CAT and SOD activities and ↓ GST activity	(Marisa et al., 2016)
n-Au	*R. philippinarum*	6, 30 µg/l	28 days	Sub-cellular distribution: hetero-lysosomes in the digestive gland	(Garcia-Negrete et al., 2013)
n-Au	*R. philippinarum*	0.75 µg/l		Accumulation in gills and digestive gland exerted oxidative stress	(Volland et al., 2015)
n-diamond	*Corbicula fluminea*	0.01, 0.1, 1 and 10 mg/l	7 and 14 days	↑ GST and ↑ CAT activities; ↑ LPO; histological alterations of the digestive gland	(Cid et al., 2015)
n-Au	*C. fluminea*	2-8 mg/l	12-180 hours	NPs undergoing extracellular digestion process. Faeces with nanoscale aggregates and free NPs	(Hull et al., 2011)
n-Au	*C. fluminea*	1.6×10^3-1.6×10^5 NP/cell	7 days	↑ Oxidative stress; ↑ MT; gene expression changes of CAT, SOD, GST and cytochrome C oxidase subunit-1 in gills and visceral mass	(Renault et al., 2008)
n-TiO_2, and Cd	*C. fluminea*	0.1-1 mg/l	10 days	Co-exposure (n-TiO_2^+ Cd_2^+): ↓ free Cd levels (FW); ↑ CAT; ↑ oxidative stress (O); ↑ tissue damage (DG). No effects in SOD, GST, and Cd accumulation	(Vale et al., 2014)
ci-CeO_2, ba-CeO_2	*D. polymorpha*	1 mg/l	21 days	↑ CAT activity and the lysosomal system being impacted	(Garaud et al., 2016)
n-CeO_2	*D. polymorpha*	10 and 100 µg/l	24 and 96 hours	↓ in the size of the lysosomal system, CAT activity and LPO in digestive gland; negatively impacted haemolymph ion concentrations	(Garaud et al., 2015)

Exposure to n-diamond in *Corbicula fluminea* at 0.01, 0.1, 1 and 10 mg/l concentrations caused an increase in GST and CAT activities after seven and fourteen days of exposure respectively at 0.1-1 mg/l. An increased trend in LPO was seen for the different tested concentrations along with histological alterations of the digestive gland (Cid et al., 2015). Hull et al. (2011) exposed *C. fluminea* to n-Au which were either retained within the digestive tract or excreted in their faeces suggesting a significant role of biotransformation and biodeposition in the fate and transport of persistent nanoparticles in aquatic systems. A report on the effect of n-TiO_2 on the toxicity of Cd present in the environment was studied in *C. fluminea,* and activation of CAT activity was observed while SOD and GST activities remained unchanged. No clear-cut results were obtained on LPO although cell damage and other morphological changes in the digestive gland were registered indicating inflammatory reactions (Vale et al., 2014).

5.7 Gross Toxicological Effects

The gross toxicological effect of ENPs in the surrounding medium was measured as the functional activity of the whole organism by measuring the volume of water cleared by filtering suspended particles, in the present case unicellular algae. As filter-feeding bivalves process large volumes of water and thus exert a considerable impact on their environment (Charles et al., 1999). Their filter feeding ability adds greatly to their ecological significance, and hence any influence on their functional activity will considerably reduce their ecological importance. The clearance rate of unexposed control bivalves was found to be dependent on the size of the animals; therefore, for the exposure study, the size of the exposed animals and controls was kept comparable. Exposure to nanoparticles caused a concentration dependent decrease in clearance rate. The response of the two species was not different and exposure at 0.05 mg/l for 48 hours caused no effect on clearance rate, but concentrations ≥1mg/l caused a decline in clearance rates. Chronic exposure at 0.05 mg/l for four weeks significantly decreased the clearance rate in response to n-

Ag, n-TiO_2 and n-CuO (Chapter 2). The inhibitory effect on the clearance rate of n-Ag was severe in comparison to the n-TiO_2. Buffet et al. (2013a) also observed no effect after early days of exposure to n-Ag, but after 10 days of exposure, a significant decrease in clearance rate was registered compared to controls. The effect of n-Ag on the functional activity of bivalves observed in my study was similar to that reported by Buffet et al. (2013a) and differences in the degree of effect are attributed to the exposure concentrations used and also the differences in test species. A comparison of the effects of nano- and bulk- ZnO and CuO on clearance rate was also made, with both CuO forms significantly reducing the clearance rate, whereas both ZnO forms were innocuous. The differences observed between the bulk- and nano-forms observed in my study may be related to their solubility. It has been reported that n-CuO does not readily dissolve (Baek and An, 2011; Mortimer et al., 2010) and its toxicity is caused by the nanoparticles and not the ion, suggesting a nano-specific toxic effect (Hanna et al., 2013; Midander et al., 2009). In contrast, n-ZnO rapidly dissolves in seawater (Miller et al., 2010); therefore the effect observed in my study may be due to Zn^{2+}. Few reports are available on n-ZnO effects on bivalves. Recently, Hanna et al. (2013) reported that n-ZnO increased mussel respiration rate after chronic exposure and their survival decreased.

The present data ratified the hypothesis that ENPs present in the marine water will influence the functional activity of bivalves. It is surprising that measuring clearance rate as an index of nanoparticle toxicity has not received the attention of many authors. Whereas on the basis of the present study it is suggested that clearance rate may indicate the degree of interference in the physiological function of the exposed organism, it can differentiate toxic and non-toxic particles and can be used for early detection of possible hazards. In toxicology and ecotoxicology any parameters useful in early detection are important from the viewpoint of regulatory intervention in environmental management. The influence of functional activity changes on the metabolism of vital organs such as the gills and digestive glands and

tissue damage was further examined after acute and chronic exposure as discussed in the following section.

5.8 Oxidative Stress

It is well documented that during normal metabolic functions oxygen free radicals and non-radicals (commonly referred to as reactive oxygen species, ROS) are produced. The basic mechanism of energy production in cells utilises molecular oxygen through the process of oxidative phosphorylation, ultimately reducing oxygen to water. The non-reduced oxygen forms superoxide (O_2^{-*}) that can form the hydroxyl radical (HO*), which is the most effective among the physiologically relevant ROS such as hydrogen peroxide (H_2O_2), peroxyl radicals (ROO^-), nitrogen oxide (NO*) among others. Their regulated production and maintenance of redox homeostasis are essential for which biological systems developed enzymatic and non-enzymatic antioxidant mechanisms. When the balance between generation and the neutralisation of ROS is disturbed, oxidative injury can occur (Vale et al., 2016).

Preliminary studies have revealed that bivalves chosen for this study are equipped to combat ROS. The gills and digestive gland contained demonstrable activity of CAT and glutathione peroxidase (GPx) that can detoxify H_2O_2. The tissue contained glutathione reductase (GR) and glutathione-S-transferase (GST) can regenerate reduced glutathione (GSH) which can directly combat ROS and also participate in glutathione-dependent enzyme activities. Metallothionein, a low molecular weight cysteine-rich protein that plays an important role in the sequestration of metals and ROS scavenging (Viarengo et al., 2007), was present in appreciable concentrations in the digestive glands. These parameters have been extensively used as biomarkers of oxidative stress. Therefore, this battery of enzymes was primarily used in this study to assess oxidative stress produced in response to nanoparticle exposure.

The mechanism of nanoparticle toxicity is not yet fully understood, but the most supported mechanism is oxidative stress. It is well established that nano-metals and nano-metal oxides with redox characteristics can produce ROS through Fenton reactions ($Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + *OH + :OH^-$), Fenton-like reactions ($Cu^+ + H_2O_2 \rightarrow Cu^{2+} + *OH + OH^-$; or $Ag + H_2O_2 \rightarrow Ag^+ + *OH + OH^-$), or the Haber-Weiss cycle reaction ($Fe^{3+} + *O^-_2 \rightarrow Fe^{2+} + O_2$; or $Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + *OH + OH^-$), yielding hydroxyl radicals (Fu et al., 2014). The Haber-Weiss reaction generates hydroxyl radicals (*OH) from H_2O_2 and also superoxide anion radicals ($*O^-_2$). Therefore, their role in exerting toxicity is expected, and the capacity of the exposed organism to mitigate their effects determines the extent of the damage.

In recent years, the potential ecotoxicological risks of ENPs to aquatic organisms have frequently been reviewed, and the information is being generated about their mode of action and biological risk (Rocha et al., 2015). Some mechanisms of toxicological damage have been identified, including ROS generation, protein misfolding, membrane damage and direct physical damage. Mostly the toxic effects of nanoparticles are attributed to the generation of reactive radicals causing oxidative stress at the cellular level (Al-Subiai et al., 2012; Canesi et al., 2015). Therefore, in the present study biomarkers were examined as indicators of cellular injury and the mechanism of cellular defence in response to oxidative stress produced by ENP exposure (Bodin et al., 2004; Porte et al., 1998; Roméo et al., 2003). Since oxidative stress is tissue-specific, biomarker levels in the gills and digestive glands were determined separately. A summary of the responses observed in the various biomarkers in both species after acute and chronic exposure is given in Appendix IV. The levels of selected biomarkers in the two bivalve species differed in their basal antioxidant enzyme activity levels. In the gills, the activity of CAT, GPx, and GR were lower whereas GST was higher in *A. violescens.* The pattern was the same in the digestive gland except for CAT which was similar in both species. The levels of antioxidant enzyme defence were lower in the digestive gland than in the gills in both species indicating a reduced capability of the digestive gland to cope with oxidative

stress, as reported in the marine mussel, *Perna viridis* (Cheung et al., 2001). GST was the most active enzyme in both the gills and digestive glands of both species and may be a common feature of bivalves as the same observation was reported for *M. edulis* (Fitzpatrick et al., 1995). This is probably an adaptation to deal with the metabolic disposition of a wide range of xenobiotics that bear electrophilic sites by catalysing conjugation with GSH (Jena et al., 2009).

Among the four types of ENPs used in this study n-TiO_2 did not dissolve whereas n-Ag, n-ZnO, and n-CuO are known to dissolve and produce their ionic form. ENP exposure resulted in concentration dependent effects on biochemical parameters which were not linear and the response in the gills, and digestive gland was not always similar. The difference in response may be due to the behaviour of nanoparticles in the exposure medium, for instance, their agglomeration or dissolution state. It has been observed that a high silver ion fraction in n-Ag suspensions is unable to produce measurable toxicity in human A549 lung cells (Beer et al., 2012). In another study it has been shown that Ag^+ released from the surface of n-Ag in the presence of water can react with molecular oxygen and generate superoxide radicals and other ROS, ultimately causing apoptosis (Lubick, 2008). There is no consensus about the toxicity of n-Ag since some studies have suggested that any toxicity is due to dissolved Ag^+ ions, whereas whereas others support that n-Ag particles are directly responsible (Ali et al., 2014; Schultz et al., 2014). Coated n-Ag particles have been used to determine whether n-Ag particles are the cause of toxic effects (Schultz et al., 2012), with similar toxic effects obtained as with Ag^+ (Luoma, 2008). Kumar et al. (2014) used coated n-Ag and concluded that toxicity arose from ROS generated from the particulate form. These studies suggested a thorough physicochemical characterization of the nanoparticles in the exposure media and simultaneous exposure to suitable controls containing ionic Ag may shed light on the form of Ag responsible for the observed toxic effects. Even though the majority of studies were unable to demonstrate if n-Ag toxicity is because of its particulate or

ionic form it has been established that the presence of n-Ag in the water poses a high risk to the resident biota (Vale et al., 2016).

The toxicity of n-TiO_2 is associated to its particle size; therefore, the degree of agglomeration in aqueous media may determine its size and surface properties and thus the levels of *in vivo* ROS production (Ekstrand-Hammarström et al., 2012). It has been reported that n-TiO_2 has low toxicity to aquatic organisms (Gottschalk et al., 2013) and at 1 mg/l concentration rainbow trout can avoid oxidative stress by scavenging ROS species (Federici et al., 2007). At higher exposure concentrations ROS generation is decreased due to agglomeration and low bioavailability to the organism (Dalai et al., 2013) whereas at low exposure concentrations (0.1–1 mg/l) biochemical disturbances and pathological changes in several organs of rainbow trout were observed (Boyle et al., 2013). Tissue damage was mainly attributed to the internalisation of n-TiO_2 and its accumulation in different organs leading to physical stress as reported for the digestive gland of bivalves (Vale et al., 2014). In my exposure conditions, the most consistent biochemical response was observed at the lowest exposure concentration (0.05 mg/l). At this concentration the clearance rate was unaffected, but cellular responses were initiated as LPO was increased both by n-Ag and n-TiO_2 in *A. umbonella.* Nanoparticle toxicity is attributed to their small size and large surface area (Xiong et al., 2011). Electron microscopy studies with n-glass particles showed that nanoparticles are taken up in the gill epithelial cells where larger fibrils are more often found, suggesting material is first sorted and transported at the gills either by a simple diffusion pathway or by endocytosis (Koehler et al., 2008). The entry of nanoparticles possibly triggers an inflammatory reaction with an influx of haemocytes since haemocytic infiltrations (both focal and diffuse) constitute repair processes following tissue damage (Ruiz et al., 2015). Membrane disruption may be the result of physical damage due to the particle size and surface properties, or due to a chemical mechanism by producing ROS (Elsaesser and Howard, 2012). It was substantiated by erosive and necrotic histological changes observed in the gill filament epithelium. There was no increase in LPO in *A. violascens,* and histological

examination revealed only an influx of haemocytes into the gill without any structural damage. This shows clear species variation in response nanoparticle assault, with *A. violascens* the more resilient of the test species.

LPO was the most obvious effect observed in the gills and digestive gland on exposure to n-Ag and n-TiO_2. Simultaneously, the process of activation of antioxidation enzymes was also initiated. CAT in n-TiO_2 exposed animals, and GPx in the n-Ag treatment were enhanced in the gills as well as the digestive glands of *A. umbonella* but insufficiently so to protect against membrane damage. When antioxidant defences are unable to cope with the generation of oxy-radicals, there is an imbalance between the production and removal of oxidants, a situation described as oxidative stress resulting in membrane LPO (Barata et al., 2005; Halliwell and Gutteridge, 1986; Livingstone et al., 1990). At higher exposure concentrations the two enzymes known as primary scavengers of peroxides were inhibited compared to controls, and LPO persisted at higher levels. This indicated that a change in enzyme levels might increase or decrease depending on the exposure concentration, but once membrane damage is started, it continues and takes longer to repair (Vale et al., 2014), potentially leading to cell death. Normally, ROS damage membrane lipids and produce lipid hydroperoxides which trigger an adaptive response in cells and/or cause cell death (Girotti, 1998). Phospholipid hydroperoxidase can be directly detoxified by phospholipid hydroxyperoxides-GPx or classical-GPx or by phospholipase-A2 (Ursini et al., 1991), affecting the ultimate formation of malonaldehyde that was detected as TBARS in the present study.

Nanoparticles are generally considered more toxic in comparison to their bulk state due to their small size and greater surface area providing enhanced surface activity and the ability to penetrate the cell (Xiong et al., 2011). However, about oxidative stress a comparison of n-CuO and n-ZnO with bulk CuO and b-ZnO revealed a much more complex picture. Exposure to n-ZnO caused no change in CAT activity in the gills, but a slight inhibition was observed in the digestive gland, whereas with b-ZnO a significant increase in CAT was found in the digestive gland

compared to controls. Decreased GPx and GR activity was the main effect observed in the exposed animals; however, the response was tissue-specific and opposite in the gills and digestive gland with both nano and bulk ZnO. Similar responses were also observed in *C. gigas* by Trevisan et al. (2014), reporting inhibition in GR activity at 4 mg/l both in gills and digestive gland with no change in CAT compared to controls; although GPx was increased in gills after 48 hours of exposure but remained unchanged in the digestive gland. Inhibition of GR has also been detected in various organisms including mussels (Franco et al., 2006; Trevisan et al., 2014), fish (Franco et al., 2008b) and rats (Franco et al., 2008a; Maris et al., 2010), suggesting a key role for this enzyme in the mechanism of zinc toxicity. Alterations in GR activity can cause a disturbance in the reduction of GSSG under oxidative conditions, thereby increasing the susceptibility of the organism to oxidative damage (Mitozo et al., 2011). GST activity did not show any changes compared to controls in the n-ZnO exposed animal which is similar to the findings of Buffet et al., (2012), where n-ZnO particles did not affect GST activity in the whole tissue of the clam *Scrobicularia plana*; however, b-ZnO showed a significant inhibition in the exposed gills. When the defence mechanism is not efficient, it may be revealed by TBARS, as gills exposed to n-ZnO in this study also showed significant induction of LPO, whereas the levels in the digestive gland were similar to controls; however, LPO was significantly induced in the exposed tissues by b-ZnO. Oxidative stress of n-ZnO was observed in various organs of carp exposed for 14 days to as high as 50 mg n-ZnO/l causing an increase in LPO and a decrease in GSH (Hao and Chen, 2012) and a concentration dependent effect was observed in zebrafish embryos on SOD, CAT, GPx, with an increase in LPO (Brun et al., 2014; Zhao et al., 2013). In bivalves, only a few reports are available on n-ZnO toxicity despite soluble zinc toxicity being well documented (Devos et al., 2012; Fathallah et al., 2010; Franco et al., 2006; Trevisan et al., 2014). Mussels can efficiently filter out suspended n-ZnO particles and can excrete them in pseudofaeces, but still, a high fraction accumulates in the soft tissue (Montes et al., 2012). Slowed shell growth, increased mortality, zinc accumulation, and increased respiration rates were observed in mussels exposed to n-ZnO, which indicates a high

energy demand to cope with excessive zinc uptake (Hanna et al., 2013). These data are further supported by behavioural disturbances and increased CAT activity in marine clams (Buffet et al., 2012) and an increase in LPO and metallothionein levels in freshwater mussels (Gagne et al., 2013b). Exposure to n-ZnO led to an increase of LPO in the bivalve digestive gland (Gagne et al., 2013b), whereas in exposed gastropods protein and GSH decreased and SOD and CAT inhibited (Fahmy et al., 2014).

The toxicity of n-ZnO can be related to the accumulation of nanoparticulate or soluble Zn^{2+} ions, therefore, the toxicity may be the function of both particulate n-ZnO and the ionic fraction (Bondarenko et al., 2013; Brunner et al., 2006; Franklin et al., 2007; Heinlaan et al., 2008). In aqueous media, the particles agglomerate and the increase in particle size increases dissolution (Brun et al., 2014). Dissolution of n-ZnO can also occur after internalisation of stabilised or polymer coated particles (Merdzan et al., 2014). Several reports support that the toxicity of n-ZnO is due to the released Zn^{2+} ions (Xie et al., 2011; Yu et al., 2011) whereas others supported that toxicity is the function of the particle (Zhu et al., 2008).

The environmental hazards of n-CuO are poorly investigated and based on available information it is concluded that it provokes ROS toxicity mediated by Cu^{2+} (Kahru and Dubourguier, 2010). The presence of n-CuO (0.1 mg Cu/l) induced the formation of superoxide anions, hydrogen peroxide and single-stranded DNA in different recombinant luminescent *E. coli* mainly because of dissolved ions (Bondarenko et al., 2012). However, all Cu forms; n-CuO, bulk CuO and Cu salt are capable of inducing ROS, but a larger induction was obtained in the presence of n-CuO, which could not be explained just by the released Cu^{2+}, probably because n-CuO is not fully dissolved and particulate form simultaneously exists to exert its effect (Mortimer et al., 2010). In another study, higher ROS formation and toxicity were obtained in the presence of coated n-CuO compared to non-coated n-CuO mainly due to its internalisation in cytosolic membrane structures (Perreault et al., 2014).

The activity of CAT in exposed gills and digestive glands was more than in controls, but the increase was significant only in the digestive gland of clams treated with b-CuO; however, LPO was not significantly changed in either nano and bulk CuO exposures. Gomes et al. (2012) reported that *M. galloprovincialis* exposed to n-CuO showed an increase in CAT in the digestive gland, as the first response within three days after exposure, whereas GPx was higher in exposed animals, but the increase became significant in long-term exposure by that time CAT returned to control levels. In the present study, after a four-week exposure CAT was comparable to controls but GPx in the gills was significantly increased, but was decreased in the digestive gland. LPO was not observed in the present exposure to n-CuO and b-CuO. Buffet (2011) found an increase in CAT and GST in n-CuO exposure without any change in LPO.

Significant changes were seen in GPx and GR indicating that the target of n-CuO toxicity was the glutathione cycle. Cysteine thiol groups present in proteins and low molecular mass thiols react fast with oxidising species and thus contribute to antioxidant defence (Hansen et al., 2009). These reduced proteins (enzyme protein in the present case) may signal more widespread toxicity affecting overall protein synthesis or increased degradation rate of protein as reported for n-Ag by McCarthy et al. (2013). Some of these aspects may be understood by proteomic studies that enable broad comparison by simultaneously examining hundreds of proteins (Kultz et al., 2007).

Another defensive mechanism initiated on exposure to nanoparticles was the induction of low molecular weight metal scavenging proteins (metallothioneins, MT), which play important roles in sequestering toxic or exogenous metals (Polizzi et al., 2014). In general, exposure to nanoscale metals and metal oxides induces MT which increase with increasing concentration or increasing exposure period at low exposure concentrations as observed in the present study and for n-Ag exposure to *M. galloprovincialis* (Gomes et al., 2014b); n-CuO exposure to *M. galloprovincialis* (Gomes et al., 2012; Gomes et al., 2011); n-ZnO exposure to the freshwater mussel

Elliptio complanata (Gagne et al., 2013b); and Co^{+2}-containing nanoscaled polymeric complex exposure to the swan mussel *Anodonta cygnea* (Falfushynska et al., 2012). The role of MT in handling nanoparticle related metals in bivalves has been studied, and the detoxification mechanism is either by controlling its intracellular availability or by detoxifying excessive metal concentrations (Bebianno et al., 2004; Regoli and Principato, 1995). Therefore, protection of the system was expected because of increased binding of nanometals with this soluble protein (Gomes et al., 2014a; Gomes et al., 2014b).

Comparing acute and chronic responses revealed interesting effects. In acute exposures to n-Ag and n-TiO_2 for 48 hours even though antioxidant enzyme activity was increased, it was not enough to protect the tissue from oxidative assault. The inhibition of enzyme activity after chronic exposure was attributed to the persistent presence of nanoparticles in the medium causing disruption of catalytic activity in the gills. Similarly, the inhibition of LPO may be due to alternative pathways that utilise LPO products as the metabolic adjustment under stress (Chapter 3). Histological changes in the gills commonly observed after chronic exposure were necrotic changes in the lamellar region and increased haemocyte infiltrations and in the digestive glands where tubule damage, complete loss of tubule and epithelium necrosis were also observed. Thus, the present study revealed that the tested nanoparticles exerted concentration-dependent effects after acute exposure on antioxidant enzymes and enhanced metallothionein levels as a defence to combat ROS production. However, despite these metabolic adjustments, it afforded insufficient protection against LPO during acute exposure. However, during chronic exposure, some of the metabolic effects were mitigated, but interestingly the cytological damage was not repaired.

Thus, data with n-Ag and n-TiO_2 exposure revealed that the most effective concentration was the lowest concentration and an increase in concentration produced an irregular response in oxidative defence mechanisms and tissue damage. It is considered that the toxicity caused by ROS is reflected in an increase in antioxidant enzymes to ameliorate oxidative stress; however, with excessive ROS production due

to the higher exposure concentration the antioxidant defence mechanism is reduced due to exhaustion of the detoxification mechanism (Jacobson and Reimschuessel, 1998; Valavanidis et al., 2006). This contention is further supported by the observations in the present study that antioxidant enzyme activity increased in acute exposure and was depleted with time in chronic exposure, reflecting not only exposure to ROS but also its toxicity.

Conclusions

The present study demonstrated that the presence of nanoparticles in the surrounding water exerted deleterious effects on the metabolism of *A. umbonella* and *A. violascens*. The range of concentrations used (0.05 to 2.0 mg/l) was not fatal (at least over the timescales explored); however, the animals' functional activity was reduced. The effect of nanoparticles on clearance rate was concentration- and time-dependent. The response in levels of various biomarkers (CAT, GPx, GR, GST, LPO, MT) were not linear to the exposure concentrations and the response in two tissues (gills and digestive glands) was not always similar. The effect of nanoparticles on the different organs depends on the physicochemical properties like solubility and agglomeration/aggregation that governs bioavailability, uptake, accumulation and toxicity (Bhatt and Tripathi, 2011; Scown et al., 2010). The inhibition in enzyme activity in chronic exposure was attributed to the persistent presence of nanoparticles in the medium causing disruption of catalytic activity in the gills. Similarly, the inhibition in LPO may be due to alternative pathways to utilise LPO products as metabolic adjustments under stress. Severe histological changes in the gills (necrotic changes in the lamellar region and increased haemocytic infiltrations) and the digestive glands (tubule damage, complete loss of tubule and epithelium necrosis) after chronic exposure were observed. Thus, the present study revealed that the tested nanoparticles exerted concentration-dependent effects in acute exposure on antioxidant enzymes and enhanced metallothionein levels as a defence to combat ROS produced by the nanoparticles. These metabolic adjustments could not protect

cells from LPO reactions in acute exposures. However, during chronic exposure at low concentration some of the apparent metabolic effects were mitigated but histological damage was not repaired. To the best of the author's knowledge this is the first metabolic study on these species suggesting that these species may be applied further in the elucidation of the mechanism of action of the ever increasing number and types of ENP introduced into the aquatic realm by human activities.

Future Recommendations

1. Published studies concern different types of nanoparticles and different biological models, which prevent direct comparison of the results and make it difficult to establish general trends regarding nanoparticle toxicity. Many uncertainties and artefacts can occur and complicate comparisons for nanoecotoxicity studies (Petersen et al., 2014). Indirect toxicity is a possibility in complex mesocosm systems, where species are simultaneously exposed. Even if the species have no direct interactions (such as predator-prey relationships), it can be hypothesised that in response to nanoparticle exposure one species might produce substances that are potentially toxic or repellent to another species. Modifications of the media resulting from degradation of organic tissues can also lead to toxicity in other species (Colman et al., 2014). In such scenarios, whether the observed toxicity is a direct consequence of nanoparticle exposure or is a case of indirect toxicity becomes extremely difficult to answer. It is difficult to define a distinct limit between complex and simple systems because a range of complexity exists across each experimental setup. Classically, it is recommended under toxicity testing protocols that experimental toxicity testing should be done separately with individual species in order to get a definitive answer regarding safety of exposed species against a test chemical (OECD, 1993) and these protocols are being modified to suit the nanoparticle toxicity tesing (OECD, 2012). However, a minimum three species representing an ecological hierarchy should be chosen to draw conclusions regarding environmentally safe concentrations for regulatory purposes. The author also suggests that classically tested methods should

be used to generate baseline information that will open avenues for further detailed studies.

2. In spite of studies on exposure conditions and revision of international test protocols, no single physical or chemical material property of nanoparticles perfectly correlates with their observed biological effects Landsiedel, (2016). It requires extensive research on physicochemical characterization, its transformation in aquatic media, dissolution kinetics and uptake kinetics in the organism to precisely demonstrate the behaviour and effects of ENPs in the environment.

3. Another gap in the literature that needs attention is to determine the toxic effects of ENPs at the ontogenetic level in aquatic invertebrates. A few studies are available in relation to nanoparticle toxicity (nano silver only) on the early life stages of marine organisms (mainly fish) (Browning et al., 2013; Massarsky et al., 2013; Ribeiro et al., 2014). The authors of these studies suggest that the presence of nanoparticles in the exposure medium has shown developmental abnormalities in tested organisms. Therefore, it is important to study the toxic effect of ENPs on the early stages of the life cycle of marine coastal organisms as they are more prone to the toxic effect of nanoparticles than the juvenile or adult stages.

4. Among several other concerns regarding nanoparticle toxicity, information regarding multiple stressors present in a given environment is very limited - this is required for accurate risk assessment. In estuaries and other coastal ecosystems, there is a growing concern regarding the presence of nanoparticles and microplastics along with legacy pollutants which may influence the behaviour, fate and toxicity of nanoparticles or other contaminants (Ferreira et al., 2016). Aquatic ecosystems are expected to accumulate nanoparticles because freshwater/saltwater interactions facilitate their precipitation (Burns et al., 2013; Ward and Shumway, 2004). Microplastics originating from plastic litter in the marine environment are considered a class of marine pollutants of high concern considering their threat to biota, especially filter feeders (Murray and Cowie, 2011; Ward and Shumway, 2004;

Wright et al., 2013). The European Marine Strategy Framework Directive (MSFD, 2008/56/EC) suggests that the fate, behaviour and toxicity of microplastics should be further investigated under the scope of international regulations. The problem of plastic production, use and disposal along with the increasing production and application of nanoparticles are creating new challenges of the interactions of multiple pollutants before environmental scientists and regulatory authorities to protect the environment and human health. Such interactions have recently started to be investigated but knowledge on the biological and ecological effects of multi-stressors remains limited (Luís et al., 2015; Oliveira et al., 2013). It is therefore required to make serious attempts in future to examine the effects of multiple stressors of special relevance in highly dynamic environments of anthropogenically impacted regions, such as estuaries and other coastal areas.

5. To date, post-genomics techniques have been applied in the field of nanoparticle risk assessment to a limited extent (Jia et al., 2013; Kobeissy et al., 2014). Furthermore, the integration of multi-omics data has only recently been performed (Bartel et al., 2015; Cooney et al., 2016; Decourcelle et al., 2015; Diez et al., 2015; Eichner et al., 2014; Fagerberg et al., 2014; Kutmon et al., 2014; Meierhofer et al., 2014). The proteome and metabolome are directly interconnected as protein levels influence the metabolic profile of a cell system and metabolites' concentration may affect protein expression. Therefore, keeping in mind recent progress in bioinformatics, it is suggested that instead of stressing on single protein or enzymes an integrated approach that combines proteomics and metabolomics is required to be used in future studies. It will provide a more comprehensive understanding of specific biological effects of potential toxicants, including nanoparticles.

Appendix I. Preliminary Studies on Lipid Peroxidation and Antioxidant Enzymes in Two Species of Marine Bivalves

Introduction

This section contains preliminary data on the basal biochemical activities in two varieties of bivalve molluscs collected from coastal areas of Kuwait. The bivalves were chosen as test organisms to examine the toxicity of nano-metal oxides. Therefore, the chosen biochemical parameters were based on the available information on the action of nano-metal oxides on biological systems. Exposure to nano-metal oxides exerts oxidative stress by generating reactive oxygen species (ROS) in an exposed organism that are subsequently detoxified by anti-oxidant enzymes. Bivalves are known to have the ability to detoxify ROS (Bigot et al., 2009; Solé et al., 2010). Catalase is one of the primary enzymes that degrade hydrogen peroxide produced in the cell. The other enzyme that catalyzes organic and inorganic peroxides is selenium dependent glutathione peroxidase (Se-GPx), the other isoform of which (selenium independent GPx) only reduces organic peroxides (Bigot et al., 2009). Glutathione reductase (GR) is another antioxidant enzyme that maintains the balance of reduced and oxidized glutathione (GSH/GSSG) in favour of its reduced form GSH which acts as a soluble antioxidant by itself and also plays a role as a cofactor in other enzymatic activities (Solé et al., 2010). The thiobarbituric acid reactive substances (TBARS) assay can be used as a gross biomarker of oxidative injury, for example the presence of malondialdehyde (MDA) in tissues indicates membrane lipid peroxidation (Bergayou et al., 2009; Parvez et al., 2006).

A comparison of the basal levels of biomarkers of oxidative stress with the levels obtained after exposure reflect the degree of exposure and indicate the presence of harmful concentrations of a toxicant in the exposure medium. Therefore, before exposure to the selected nano-metals it was felt necessary to determine the range of various enzymes known to play important roles in dealing with oxidative stress in the

organism. The enzymes standardized in this study were, catalase (CAT; one of the primary enzymes that degrade hydrogen peroxide produced in cells), glutathione reductase (GR; an important antioxidant enzyme that maintains the balance of reduced and oxidized glutathione (GSH/GSSG) in favour of its reduced form, GSH, which acts as a soluble antioxidant by itself and also plays a role as a cofactor in other enzymatic activities), glutathione peroxidase (GPx; catalyzes peroxides), glutathione S-transferase (GST; an enzyme of Phase II metabolism that is involved in the metabolism of glutathione which plays an important role in non-enzymatic antioxidant system and fights oxidative stress), and lipid peroxidation (LPO) in gills and digestive gland of the two bivalve species, *Amiantis umbonella* and *Asaphis violascens*. In addition, metallothionein was also determined as a measure of metals scavenging in the tissues. Metallothioneins are also known to play an important role in quenching the effects of ROS alongside the battery of antioxidant enzymes in the cell.

Materials and Methods

The animals were collected from the coastal areas of Kuwait during low tide and transported to the laboratory in boxes previously filled with sea water from the sampling site (Figure 1). The bivalves were weighed (whole animal weight, soft tissue weight, shell weight), measured (shell length and shell width) and dissected to separate the gills and digestive gland. Morphometric data were used to calculate health condition index. Twenty-five *A. umbonella* and 20 *A. violascens* were kept in separate aquaria in the bioassay laboratory and maintained with a clean seawater supply. Similarly, the two species were exposed to n-Ag and n-TiO_2 in separate aquariums at 0.01 mg/l concentration for 48 h. The water quality and conditions were similar to that as described in chapter 2 (section 2.2). The clearance rate was measured as given in section 2.2. For biochemical analysis, animals were dissected with the gill and digestive gland from five animals pooled by tissue type to get enough tissue for homogenization and enzyme assays that constituted one sample of

each. Thus, five samples of *A. umbonella* and four samples of *A. violascens* for both control and exposed animals were analyzed for biochemical parameters. The method of homogenate preparation, medium of homogenization, total protein estimation, antioxidant enzymes (catalase, glutathione peroxidase, glutathione reductase, glutathione S-transferase), lipid peroxidation and metallothionein determination was as described in chapter 3 (section 3.2).

Figure 1. Doha sampling site (A-C), Nuwaiseeb sampling site (D-G,), selection of bivalves for experiments (H & I).

Statistical analysis

Data were analyzed using statistical package Minitab 17. All data are presented as mean ± standard deviation (SD). Statistical analysis of the results was

carried out by one-way ANOVA, after the data had been checked for assumptions of normality and homogeneity (Leven's test). Fisher's multiple comparison test was used to differentiate between the groups of data and only $P<0.05$ was accepted as significant.

Results and Discussion

Morphometric measurements

The specimens of *A. violascens* were larger than *A. umbonella* (Figure 2A). There were significant differences ($P > 0.05$) in all morphometric parameters between the two species (Figure 2A).

Clearance rate

Clearance rate (CR) was estimated as the volume of water cleared of particles per unit time by measuring the reduction of particles in a known volume of water. In the present study removal of unicellular algae from the medium was used as a measure of particles removed. The concentration of algae used was selected based on a trial experiment in which no pseudofaeces was produced and more than 90% of particles were trapped during 24h. The CR for *A. umbonella* and *A. violascens* was 90.9±14.4 and 125.2±18.0 ml/h/g fresh weight respectively (Figure 2B). The data showed that CR of *A. violascens* was significantly higher than *A. umbonella* ($DF = 1$, $F = 10.73$, $P = 0.01$).

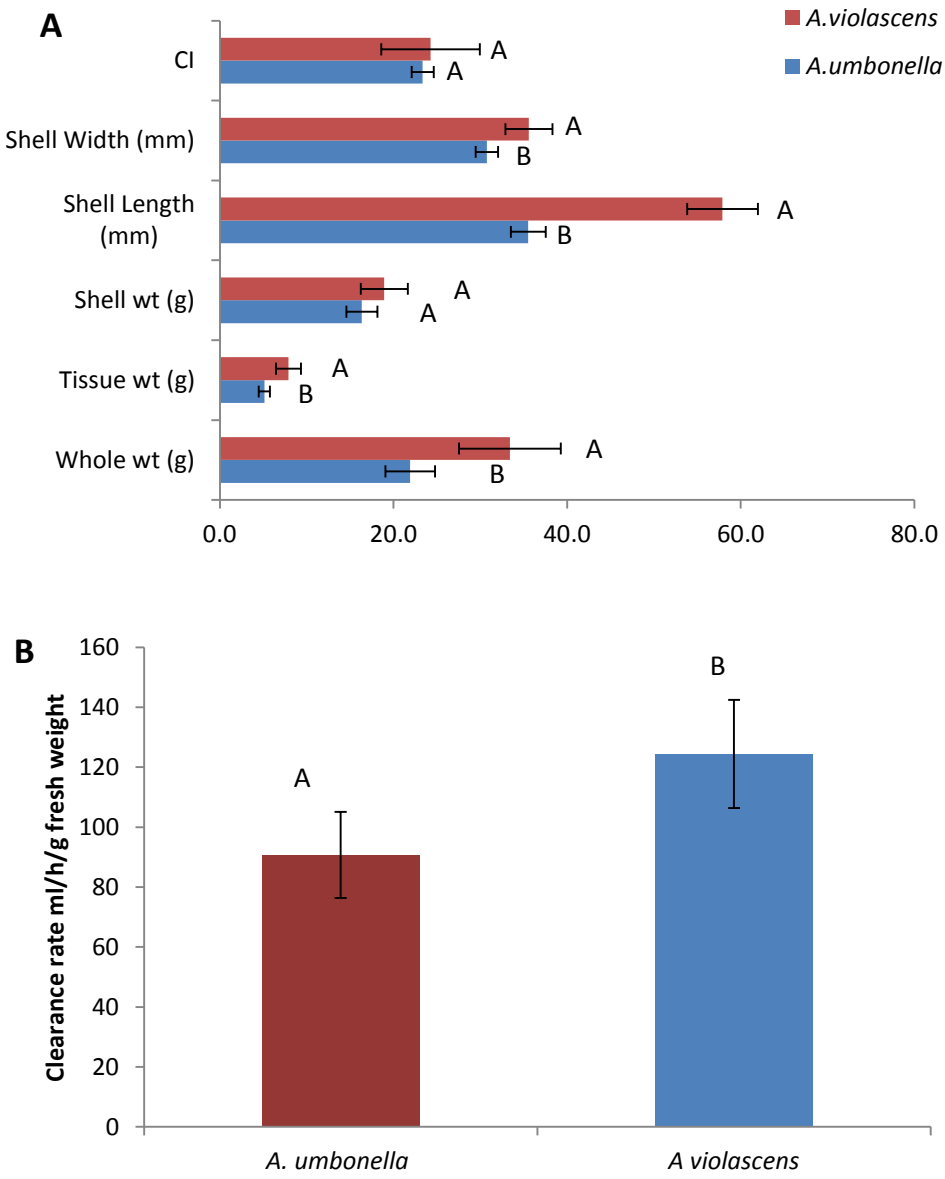

Figure 2. Comparison of morphometric measurements (A), and clearance rate (B) in the two species of bivalves. Means that do not share a letter are significantly different, n = 5 (*A. umbonella*, tissue of five animals pooled to constitute one sample); n = 4 (*A. violascens*, tissue of four animals pooled to constitute one sample).

Protein content in tissues

The protein content in the gills was lower than in the digestive gland in both species. Protein in the gills of *A. violascens* was 11.17±2.05 mg/g fresh weight which was higher than *A. umbonella* (9.17±0.98 mg/g fresh weight), but not significantly so (DF = 1, F = 3.83, P = 0.091). The protein content in the digestive gland of *A. umbonella* was 45.0±2.37, which was significantly higher than in *A. violascens* (36.13±4.57 mg/g fresh weight; DF = 1, F = 14.35, P = 0.007) (Figure 3A & B).

There was no significant change observed in the exposed group of animals compared to controls in both the species (*A. umbonella*, gills: DF = 2, F = 0.6, P = >0.05, digestive gland: DF = 2, F = 0.4, P = >0.05; *A. violascens*, gills: DF = 2, F = 0.09, P = > 0.05, digestive gland: DF = 2, F = 0.2, P = >0.05).

Catalase activity

The CAT enzyme activity in gills was 58.46±21.7 and 115.8±16.15 µmol/min/mg protein and 28.55±5.97 and 65.36±15.53 µmol/min/mg protein in the digestive gland in *A. umbonella* and *A. violascens* respectively. The pattern of activity showed that gills contained higher CAT compared to the digestive gland. *A. violascens* showed high CAT in both gills and digestive gland tissues compared to *A. umbonella* (gills DF = 1, F = 19.18, P = 0.003; digestive gland DF = 1, F = 24.34, P = 0.02) (Figure 3C & D).

There was no significant change observed in the exposed group of animals compared to controls in both the species (*A. umbonella*, gills: DF = 2, F = 0.01, P = >0.05, digestive gland: DF = 2, F = 0.11, P = >0.05; *A. violascens*, gills: DF = 2, F = 0.03, P = > 0.05, digestive gland: DF = 2, F = 0.01, P = >0.05).

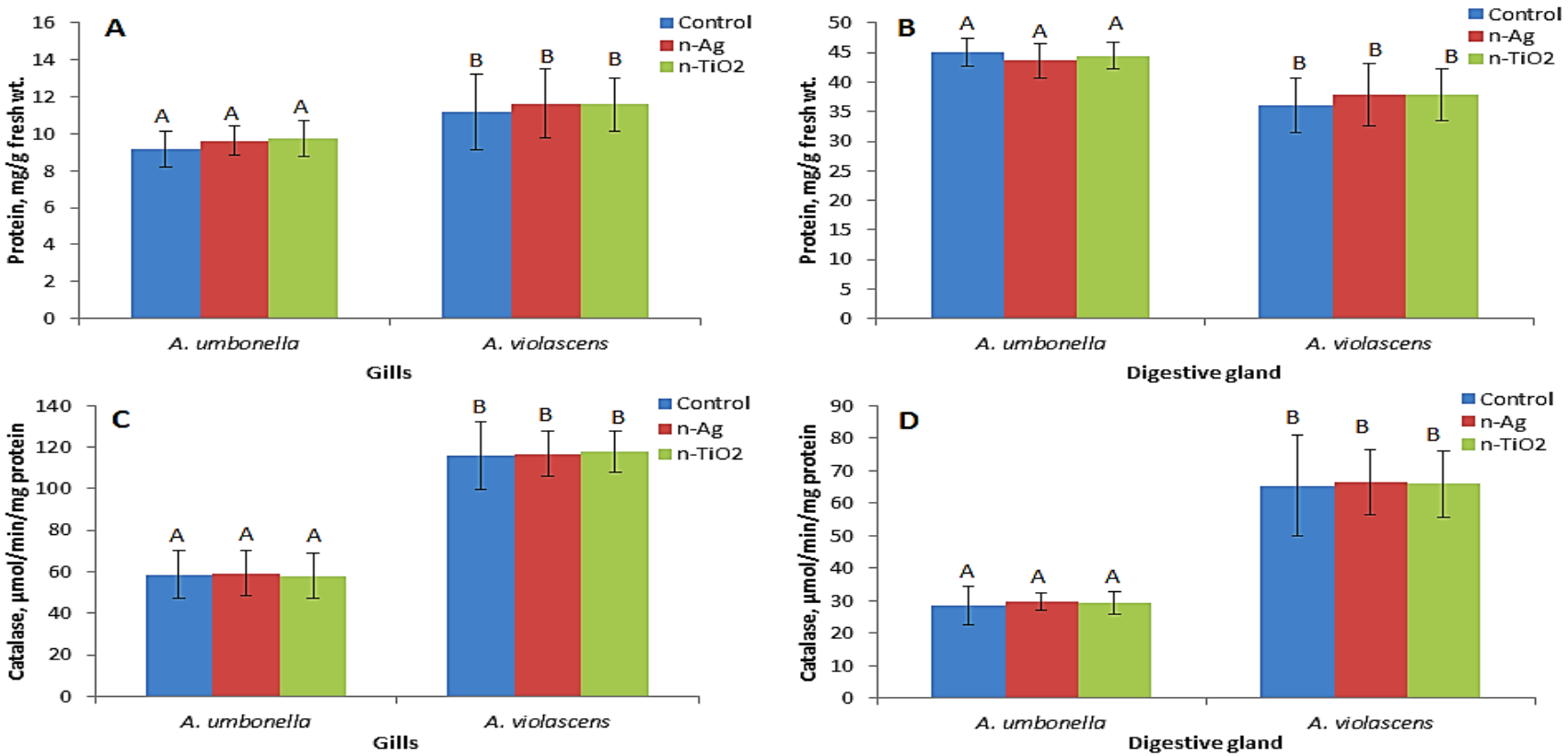

Figure 3. Antioxidant enzymes in gills and digestive glands of *A. umbonella* and *A. violascens* that were acutely exposed to engineered nanoparticles (n-TiO_2 & n-Ag) at 0.01 mg/l concentration for 48 hours. A, B = total protein content; C, D = catalase activity. Means that do not share a letter are significantly different (*A. umbonella* n = 5; *A. violascens* n = 4).

Glutathione peroxidase

The GPx activity in the gills and digestive gland of *A. umbonella* was 14.8±3.7 and 20.4±2.94 nmol/min/mg protein respectively. In *A. violascens* GPx activity in the gills and digestive gland was 20.75±3.59 and 11.66±2.4 nmol/min/mg protein respectively. GPx activity in the gills was not significantly different between the two species (DF = 1, F = 2.45, P = 0.1); however, in the digestive gland the activity of GPx in *A. violescens* was significantly less than in *A. umbonella* (DF = 1, F = 22.95, P = 0.002) (Figure 4A & B).

There was no significant change observed in the exposed group of animals compared to controls in both the species (*A. umbonella*, gills: DF = 2, F = 0.02, P = >0.05, digestive gland: DF = 2, F = 0.05, P = >0.05; *A. violascens*, gills: DF = 2, F = 0.18, P = > 0.05, digestive gland: DF = 2, F = 0.2, P = >0.05).

Glutathione reductase

Gill tissue contained higher glutathione reductase (GR) activity than the digestive gland in both bivalves. The GR activity in the gills was 23.25±3.54 and 10.0±1.83; whereas it was 17.78±1.86 and 10.70±1.66 nmol/min/mg protein in the digestive gland of *A. umbonella* and *A. violascens* respectively. GR activity was significantly lower in the gills and digestive gland of *A. violascens* compared to *A. umbonella* (gills DF = 1, F = 45.39, P <0.001; digestive gland DF = 1, F = 35.14, P = 0.001) (Figure 4C & D).

There was no significant change observed in the exposed group of animals compared to controls in both the species (*A. umbonella*, gills: DF = 2, F = 0.01, P = >0.05, digestive gland: DF = 2, F = 0.09, P = >0.05; *A. violascens*, gills: DF = 2, F = 0.04, P = > 0.05, digestive gland: DF = 2, F = 0.38, P = >0.05).

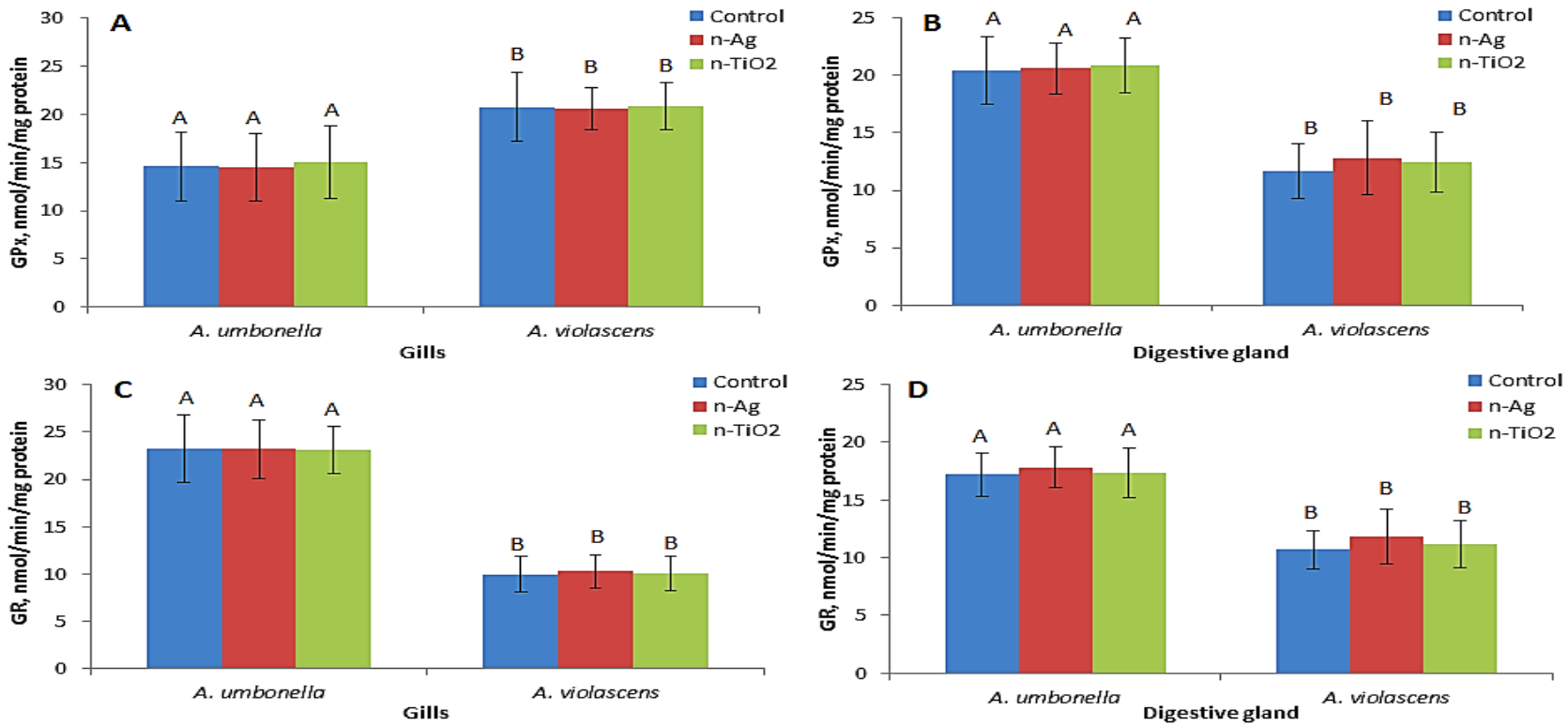

Figure 4. Antioxidant enzymes in gills and digestive glands of *A. umbonella* and *A. violascens* that were acutely exposed to engineered nanoparticles (n-TiO_2 & n-Ag) at 0.01 mg/l concentration for 48 hours. A, B = glutathione peroxidase (GPx); C, D = glutathione reductase (GR). Means that do not share a letter are significantly different (*A. umbonella* n = 6; *A. violascens* n = 5).

Glutathione S-transferase (GST)

Glutathione S-transferase (GST) enzyme activity in the gills was 1520±280 and 1870±410 and 790±30 and 1140±300 nmol/min/mg protein in the digestive gland of *A. umbonella* and *A. violascens* respectively. GST activity in the gills of *A. umbonella* and *A. violascens* was higher than in the digestive gland, with digestive gland GST activity of *A. violascens* significantly higher than *A. umbonella* (DF = 1, F = 6.69, P = 0.03) (Figure 5A & B).

There was no significant change observed in the exposed group of animals compared to controls in both the species (*A. umbonella*, gills: DF = 2, F = 0.0, P = >0.05, digestive gland: DF = 2, F = 0.32, P = >0.05; *A. violascens*, gills: DF = 2, F = 0.0, P = > 0.05, digestive gland: DF = 2, F = 0.0, P = >0.05).

Lipid peroxidation (LPO)

Membrane lipid peroxidation (LPO) was determined as the concentration of thiobarbituric acid reactive (TBAR) substances. The LPO in the gills and digestive glands differed significantly between species. The LPO levels in the gills were 71.24±8.81 and 20.28±2.88 nmol/min/mg protein and 20.14±5.68 and 11.63±3.21 nmol/min/mg protein in the digestive glands of *A. umbonella* and *A. violascens* respectively. *A. umbonella* gills LPO was three times higher than in *A. violascens* (DF = 1, F = 120.39, P < 0.001). The digestive gland LPO was also significantly higher in *A. umbonella* (DF = 1, F = 7.04, P = 0.03) (Figure 5C & D).

There was no significant change observed in the exposed group of animals compared to controls in both the species (*A. umbonella*, gills: DF = 2, F = 0.09, P = >0.05, digestive gland: DF = 2, F = 0.02, P = >0.05; *A. violascens*, gills: DF = 2, F = 0.12, P = > 0.05, digestive gland: DF = 2, F = 0.07, P = >0.05).

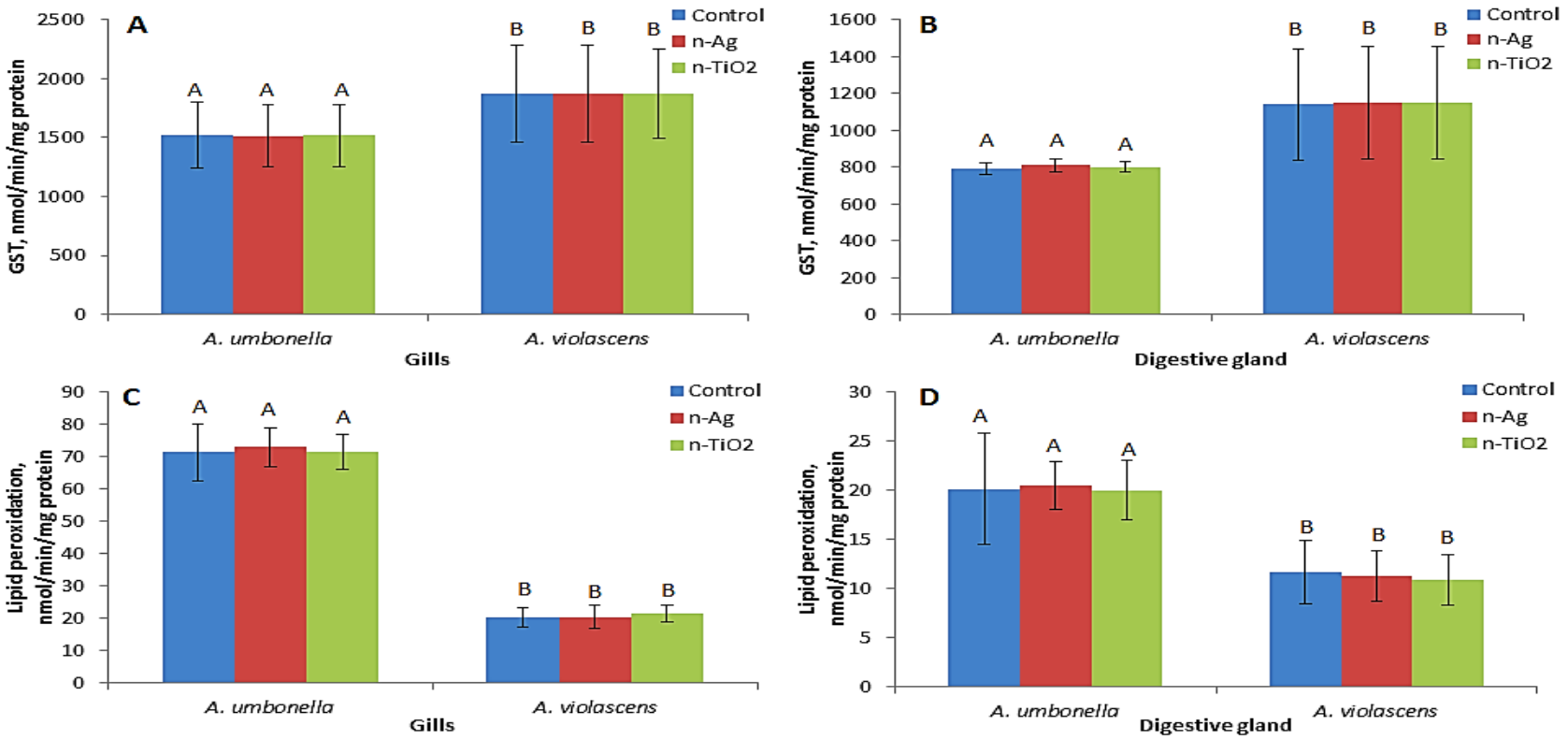

Figure 5. Antioxidant enzymes in gills and digestive glands of *A. umbonella* and *A. violascens* that were acutely exposed to engineered nanoparticles (n-TiO_2 & n-Ag) at 0.01 mg/l concentration for 48 hours. A, B = glutathione-S-transferase activity; C, D = lipid peroxidation levels. Means that do not share a letter are significantly different (*A. umbonella* n = 6; *A. violascens* n = 5).

Metallothionein

The metallothionein levels in the gills were 55.5±17.2 and 39.3±3.8 µg/g fresh weight in *A. umbonella* and *A. violascens* respectively (Figure 6A & B). The difference in the gills metallothionein between two species was not significant. Metallothionein levels in the digestive glands were 225±33 and 650-106.5 µg/g fresh weight in *A. umbonella* and *A. violascens* respectively. A significant difference was observed between the digestive gland metallothionein levels in the two species with *A. violascens* containing significantly higher metallothionein levels (DF = 1, F = 55.36, P < 0.001).

There was no significant change observed in the exposed group of animals compared to controls in both the species (*A. umbonella*, gills: DF = 2, F = 0.01, P = >0.05, digestive gland: DF = 2, F = 0.01, P = >0.05; *A. violascens*, gills: DF = 2, F = 0.13, P = > 0.05, digestive gland: DF = 2, F = 0.01, P = >0.05).

Summary and conclusions

This section contains basal activity data on the antioxidant enzyme activity in two species of control and exposed bivalves. In genereal no significant (P < 0.05) effects on any of the parameters were observed due to exposure to nanoparticles at the tested concentration. Therefore, higher concentrations were used in other experiments. The levels of antioxidant enzymes indicate the ability of the organisms to withstand oxidative stress. The biochemical parameters chosen for this study have been extensively used as biomarkers of oxidative stress. The two species showed some differences in the condition index, clearance rate and the enzymes of oxidative stress. The clearance rate for *A. violascens* was significantly higher, possibly due to the larger size of *A. violascens*. The protein levels were the same in the gills of both species but significantly higher in the *A. umbonella* digestive gland. The basal levels of GPx, GR, and LPO were significantly lower in the *A. violascens* digestive gland.

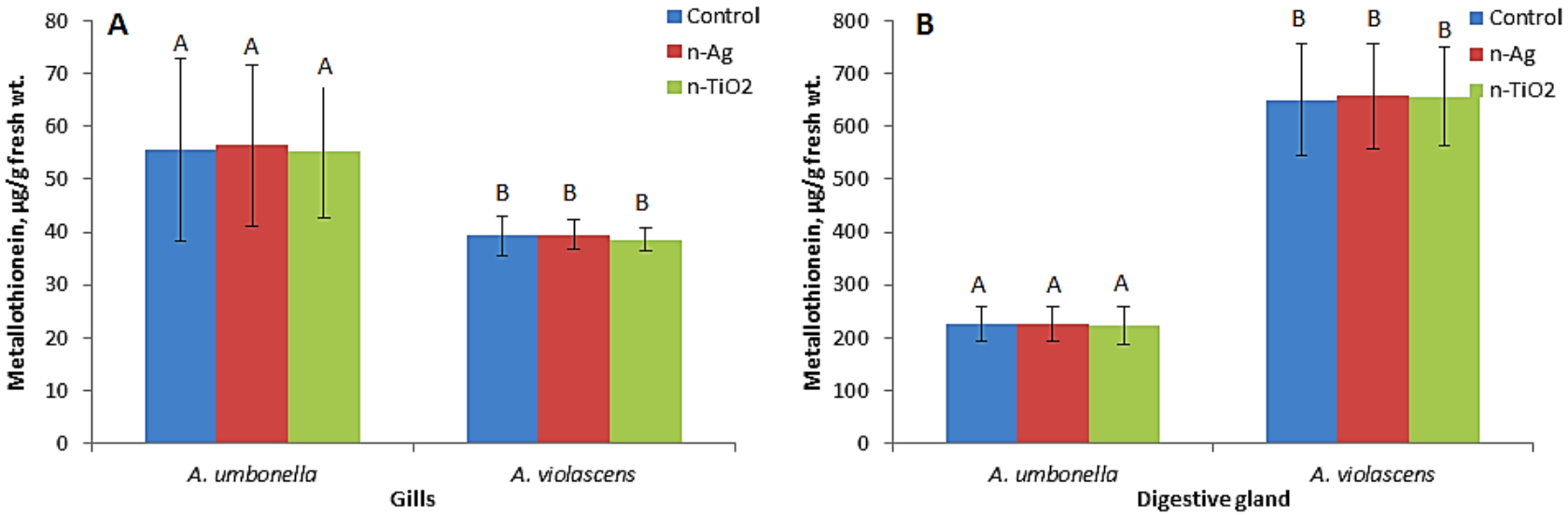

Figure 6. Antioxidant enzymes in gills and digestive glands of *A. umbonella* and *A. violascens* that were acutely exposed to engineered nanoparticles (n-TiO_2 & n-Ag) at 0.01 mg/L concentration for 48 hours. A, B = metallothionein levels. Means that do not share a letter are significantly different (*A. umbonella* n = 6; *A. violascens* n = 5).

The gills were in general more metabolically active compared to the digestive gland as most of the enzymes had higher activity levels. The gills respond to, and may therefore be considered as being representative of the ambient oxidative stress levels in the external environment as they filter large volumes of water and are in continuous contact with waterborne contaminants, whereas the digestive gland represents the metabolic activity inside the organism in response to the ingestion of particulates. There was not much variation observed in the gill tissue of the two species. Tissue protein levels, GPx, GST, and LPO were the same, however, CAT, GR, and LPO showed different activity levels. In terms of the digestive gland, all biomarkers (including metallothioneins) differed between species, but not consistently so.

Appendix II. Effect of Nanoparticles on the Protein Profile of the Gills and Digestive Glands

Introduction

The increasing industrial use of metallic nanoparticles during the last decades poses a potential threat to the environment and in particular to organisms living in the aquatic environment (Joo and Zhao, 2017). Given the size of nanoparticles, it is possible that they can encounter different cell types and may also translocate across membrane barriers via phagocytosis, macropinocytosis or endocytosis. Once taken up, nanoparticles can accumulate in the lysosomes, intracellular vacuoles, or cytoplasm (Saptarshi et al., 2013). Aquatic ecosystems are considered a final destination for many chemicals, including nanoparticles, but information about the fate of nanoparticles in such environments is still scarce (Scown et al., 2010).

The toxic potential of test chemicals in experimentally exposed organisms is determined by the effects on physiological activity or the expression of specific biomolecules. In this sense, the activity of an array of enzymes involved in oxidative stress defence, energy metabolism and xenobiotic detoxification are estimated, in addition to the levels of cellular stress derived metabolites such as lipid peroxides (Lima et al., 2007; Sarkar et al., 2006). Previous chapters have examined particle clearance rate as a measure of functional activity, and antioxidant defence enzyme activity in response to reactive oxygen species (ROS) production. Injury occurs when cellular antioxidant defences are overcome, and a state of oxidative stress ensues (Lesser, 2006). Exposure to ROS can cause a range of reversible and irreversible modifications of protein amino acid side-chains (Ghezzi and Bonetto, 2003). The proper redox status of cells is regulated by the ratio of reduced (GSH) to oxidized (GSSG) glutathione and NADPH is oxidized to NADP+ to maintain this ratio in favour of GSH (Schafer and Buettner, 2001). If the ratio excessively favours GSSG, apoptosis may be triggered (Matés and Sánchez-Jiménez, 2000).

Recently, the search for novel biomarkers has been focused on the application of omics methodologies especially the organism's proteome and the detection of changes in the level of individual proteins/peptides in response to environmental stressors (Campos et al., 2012). Proteomics (study of proteomes and their functions) and metabolomics (study of metabolites present within organism, cell or tissue) can provide a more robust approach for the assessment of stress since it will not only identify single protein markers, but also generate protein patterns that react robustly and specifically to particular pollutants (Gioria et al., 2016; Knigge et al., 2004). In this chapter the protein profiles of gill and digestive gland tissues of bivalves exposed to nanoparticles were examined using one dimensional polyacrylamide gel electrophoresis separation. The objective was to determine whether bivalves, after exposure to nanoparticles, may adjust to stress at the protein expression level.

Materials and Methods

Nanoparticles

Nanoparticles used for protein separation were commercially procured, and the exposure protocols were the same as described in Chapter 2 (section 2.2).

Experiments with *Mytilus edulis*

Mytilus edulis of length 4.9-5.5 cm were collected from the marine laboratories of Newcastle University, and were exposed to n-TiO_2 and n-Ag at 1 mg/l concentration. The exposure was conducted for 48 h, seperately for the two nanoparticles with fifteen animals in each with continuous aeration. Controls were treated identically but were not exposed to any treatment. The treatment was renewed daily with the required concentration of nanoaprticles. Temperature was maintained at 16±1 ºC and the salinity was 33-34 ppt. No mortalities were observed under any of the conditions used. After the exposure period the gills and digestive glands were dissected for each exposure three groups were made with three animals in each group.

The pooled samples were homogenised in 10 mM TRIS-HCl (pH 7.2), containing 0.5 M sucrose, 0.15 M KCl, 1 mM ethylenediaminetetraacetic acid (EDTA), and 1 mM phenylmethylsulfonyl fluoride (PMSF) using a manual Teflon Potter-Elvehjem homogeniser. Protein concentration in the homogenate of gills and digestive glands was quantified using bovine serum albumin (BSA) as a calibration standard (Bradford, 1976).

Gill and digestive gland samples were heat denatured and loaded alongside protein molecular mass markers (Bio-Rad, catalogue no. 161-0373) into wells embedded within 4-12 % Bis-TRIS high performance pre-cast gels (Invitrogen, Nu-PAGE Novex Bis-TRIS gels, Carlsbad CA, lot no. 5050972) into Nu-PAGE SDS running buffer. Gel electrophoresis was carried out at 4 ºC using an Invitrogen Xcell sure lock Novex Mini-Cell system at a constant voltage of 111 V until samples entered the resolving gel, then 180 V for 45 min or until the dye front reached the bottom of the gel. Protein bands were visualised by colloidal coomassie brilliant blue (CBB) staining.

Experiments with *Amiantis umbonella* and *Asaphis violascens*

The gills and digestive gland homogenates of animals chronically exposed for four weeks (as detailed in chapter 2) were used for protein separation by gel electrophoresis. The tissue homogenates were mixed with equal volumes of double strength sample buffer containing 62.5 mM TRIS-HCl pH 6.8, 2% SDS, 25% glycerol, 0.01% bromophenol blue, 5% 2-mercaptoethanol made in deionized water which was supplied by Bio-Rad. The homogenate in sample buffer was heat denatured at 95 ^{o}C for five minutes, and the supernatant was used for protein separation. Electrophoresis was done using the Bio-Rad mini-Protean system. Precast TRIS-HCl gels were purchased from Bio-Rad with preformed sample wells and stacking gel for separation of proteins by molecular weight. The gel dimension was 6.8 x 8.6 cm (H x W) and contained 10 wells.

Three samples (25 µg) each of gill and digestive gland tissues from control and treated animals were loaded in two gels and ran simultaneously. In each plate a standard mixture of protein molecular mass markers (Promega, catalogue no. V8491) was also loaded. The running buffer which contained 25 mM TRIS, 192 mM glycine, 0.01% SDS, pH 8.3 made in deionized water was obtained from Bio-Rad. Electrophoresis was carried out at 4 ^{o}C at a constant voltage of 111 V until samples entered the resolving gel, then 160 V for 45 minutes or until the dye front reached the bottom of the gel. Protein bands were visualized by colloidal Coomassie brilliant blue (CBB) staining.

Destaining

Coomassie-stained gels were placed in destaining solution in a dish kept on a shaker. The destaining solution was made by mixing 450 ml ethanol, 100 ml glacial acetic acid and 450 ml distilled water. The destaining solution was changed twice to remove excess stain and get clear bands.

Image analysis

Coomassie-stained gels were scanned and quantification of protein bands was performed with a calibrated imaging densitometer (Chemidoc MP, Imaging system; Bio-Rad, USA).

Statistical analysis

Data were analyzed using statistical package Minitab 17. All data are presented as mean ± standard deviation (S.D.). Statistical analysis of the results was carried out by one-way ANOVA, after the data had been checked for assumptions of normality and homogeneity (Levene's test). Fisher's multiple comparison test was used to differentiate between the groups of data and only $P<0.05$ was accepted as significant.

Results

Protein profile of *Mytilus edulis* after n-TiO_2 and n-Ag treatment

Exposure to n-Ag and n-TiO_2 caused changes in the protein profile of gills and digestive glands of mussels. In gills, the intensity of 13 protein bands was different compared with controls, and some of the proteins were significantly decreased. Only four proteins were above the levels found in controls, however, the change was not significant, except for n-TiO_2 at ≤ 50 kDa (DF = 2; F = 12.80; P = < 0.001) (Figure 1 A and B).

In general, nine proteins in digestive glands showed difference in exposed tissues compared to non-exposed mussel (Figure 2 A and B). Exposure to n-Ag caused three proteins to be upregulated and six proteins to be downregulated, whereas in exposure with n-TiO_2 four proteins were upregulated and five were down regulated. There were some common effects of both types of nanoparticles while change in some proteins was specific to the type of nanoparticles used. In the digestive glands a protein in the region of ≤75 kDa was specifically increased in the n-Ag treatment whereas n-TiO_2 treatment caused a specific decrease in this protein; however the change was not significant (DF = 2; F = 4.33; P = 0.069). N-TiO_2 specifically increased (2.2-fold) the proteins at ≤15 kDa (DF = 2; F = 65.07; P = < 0.001) and at 10 kDa (DF = 2; F = 49.45; P = < 0.001), while the treatment with n-Ag caused a lowering in proteins of the equivalent molecular weights.

Protein profile of *Amiantis umbonella* after n-TiO_2 and n-Ag treatment

Exposure to n-Ag and n-TiO_2 changed the protein profile of both the gills and digestive glands of *A. umbonella*. In the gills the intensity of eight protein bands was different in the nanoparticle exposed group compared to the control animals. There was a general up-regulation of protein expression in both of the exposed groups. Out

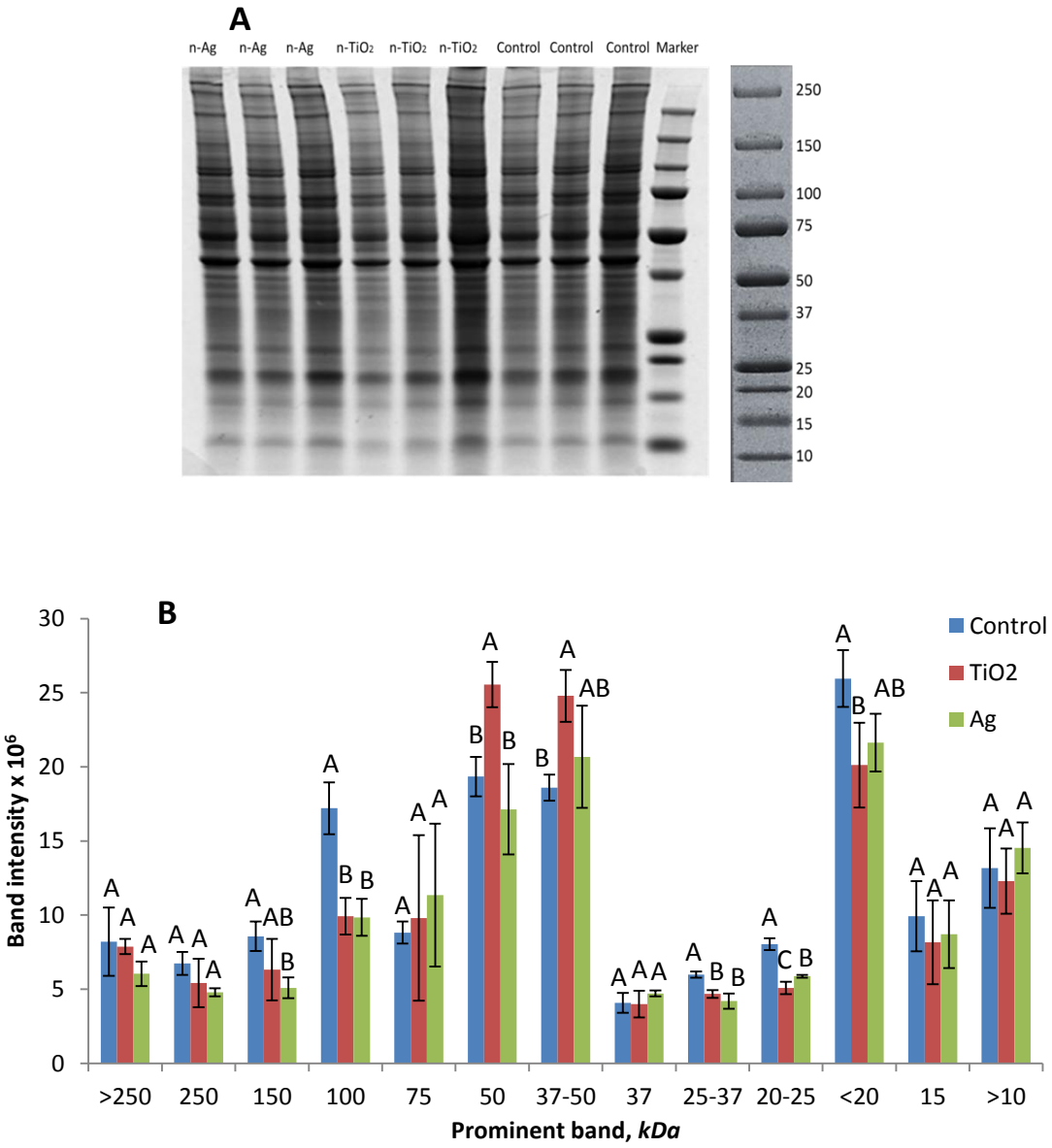

Figure 1. A) Gels showing protein expression in gills of *M. edulis.* B) Protein band intensity in the gills of *M. edulis* after 48 h exposure to n-Ag and n-TiO_2.

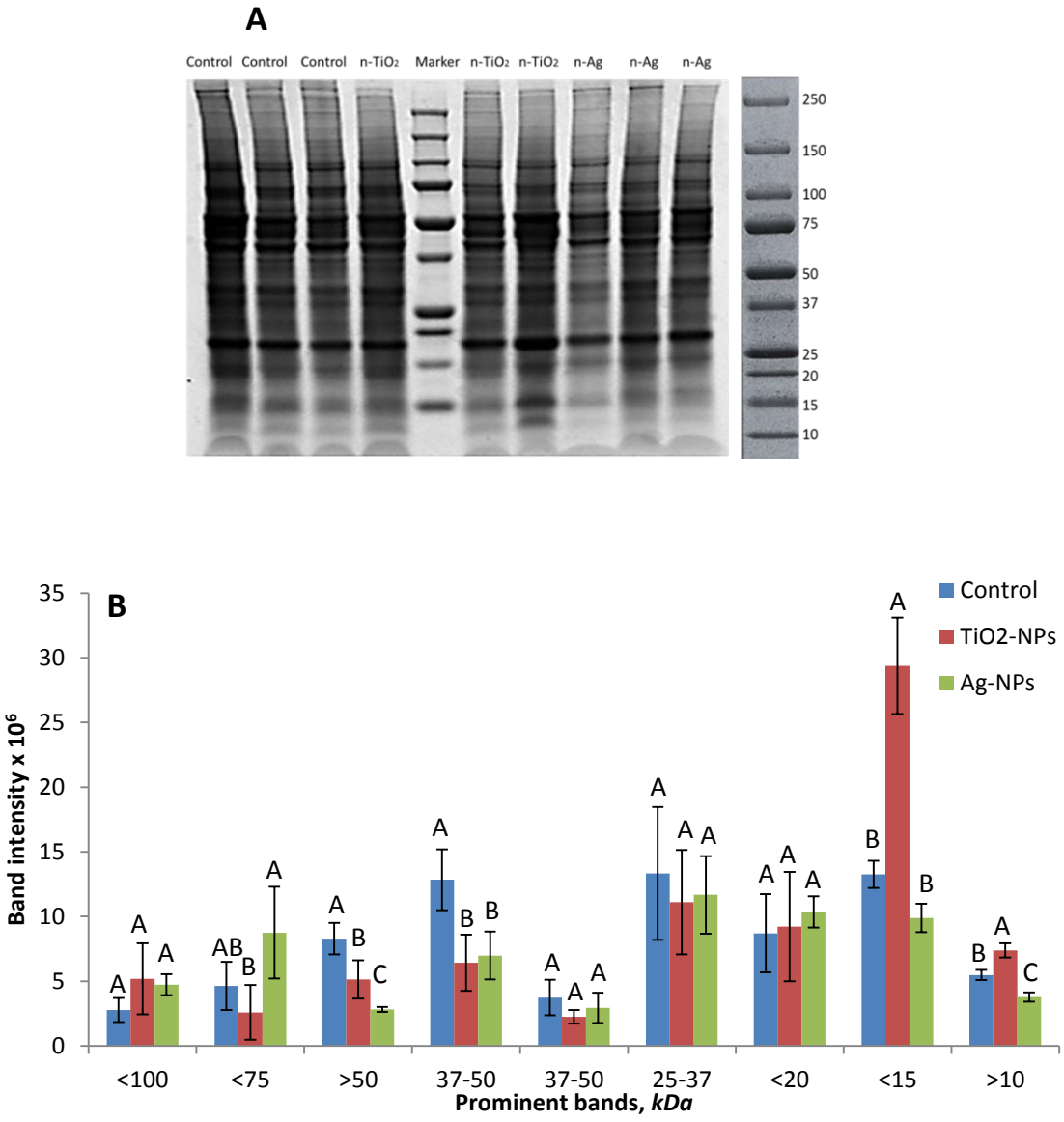

Figure 2. A) Gels showing protein expression in the digestive gland of *M. edulis.* B) Protein band intensity in the digestive gland of *M. edulis* after 48 h exposure to n-Ag and n-TiO_2.

of eight bands the increase in some bands was higher in n-TiO_2 exposed animals and some were equally affected compared to control animals (Figure 3 A and B).

In gill tissues of exposed animals a significant increase in the intensity of protein bands was observed at 225 kDa (DF = 2, F = 22.5, P = 0.001), and at <100 kDa (DF = 2, F = 13.90, P = 0.006) compared to the controls. The increase in the band intensity in n-TiO_2 treated gills was higher than n-Ag at 150-225 kDa but the difference was not statistically significant in comparison to controls (DF = 2, F = 0.79, P = 0.495). Two bands were found between 35-50 kDa in treated gills. Both bands showed a significant increase in intensity in treated gills. The intensity of the first band was lower than the second band observed between 35-50 kDa but treatment with n-TiO_2 and n-Ag significantly increased protein in the first (DF = 2, F = 23.63, P = 0.001) and also in the second band (DF = 2, F = 8.79, P = 0.016). Between the two treatments the increase was higher with n-TiO_2 than n-Ag, but not significantly so.

As observed in the gills, the digestive glands of treated animals also showed differences in eight proteins compared to the controls (Figure 4 A and B). The change in the protein bands at 225 kDa and in first band between 35-50 kDa was similar to that observed in the gills after exposure to n-TiO_2 and n-Ag. The increase in band intensity was significantly higher than controls at 225 kDa (DF = 2, F = 8.94, P = 0.016) and the first band between 35-50 kDa (DF = 2, F = 6.49, P = 0.032). The second band between 35-50 kDa was not changed in the n-TiO_2 treatment but increased significantly in the n-Ag treatment (DF = 2, F = 18.28, P = 0.003) compared to controls. The other protein band that showed a significant increase in the n-Ag treatment was at 75 kDa (DF = 2, F = 7.44, P = 0.024) whereas, the increase in the n-TiO_2 treatment was not significant. Thus, two protein bands in the n-TiO_2 and four proteins in the n-Ag treatment were significantly increased in the digestive gland of exposed animals.

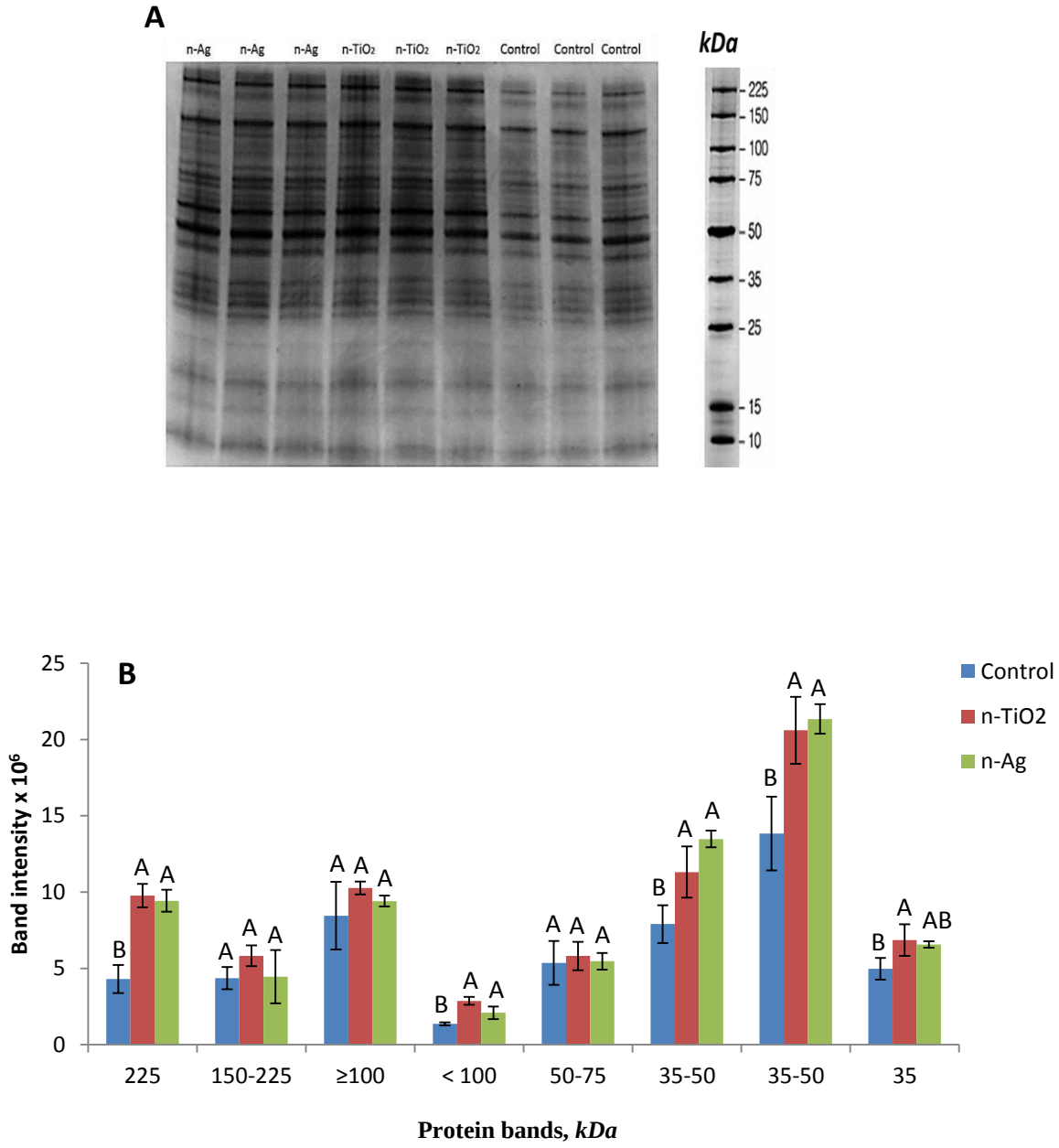

Figure 3. A) Gels showing protein expression in gills of *A. umbonella* B) Protein band intensity in the gills of *A. umbonella* after chronic exposure to n-Ag and n-TiO_2.

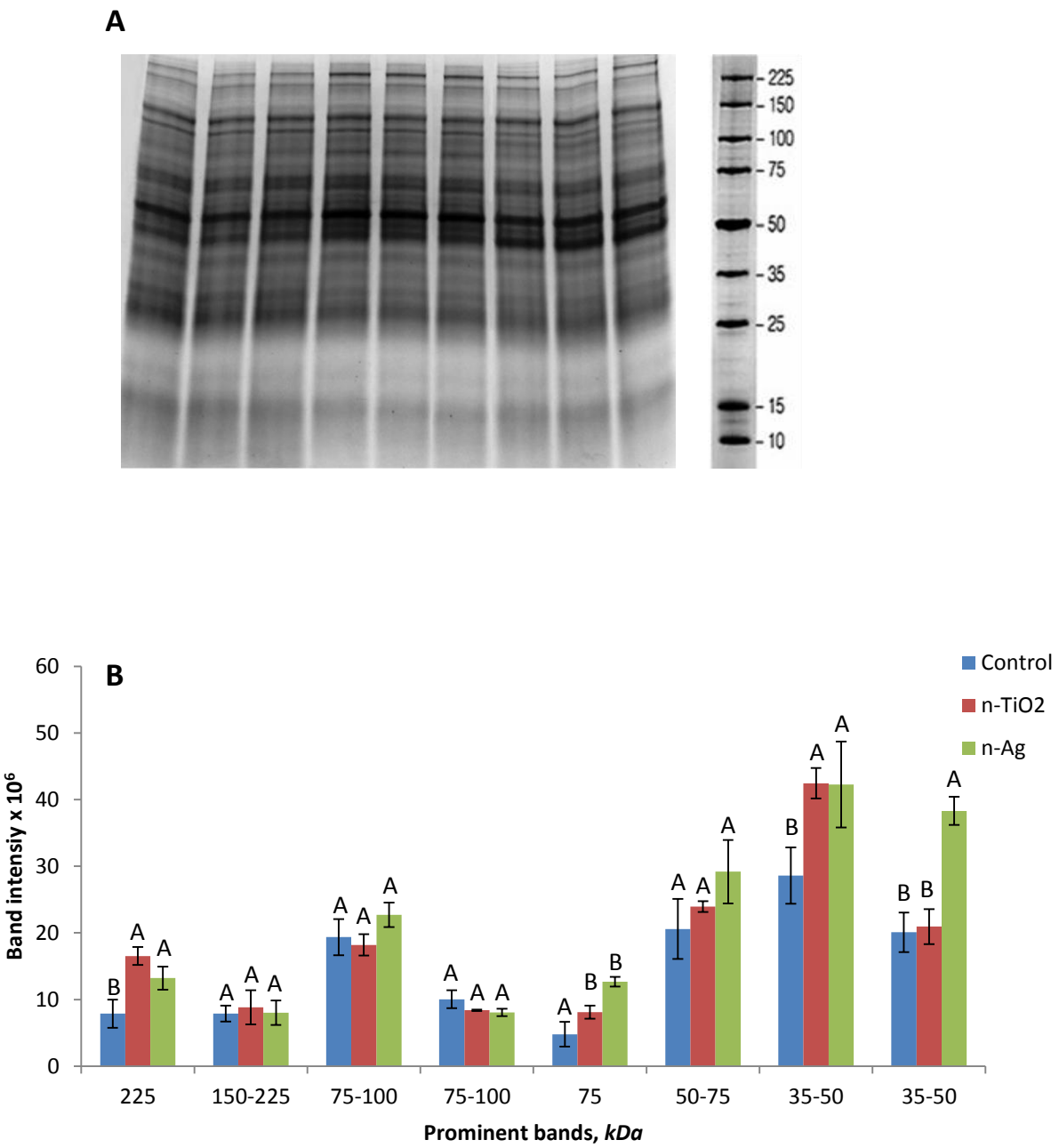

Figure 4. A) Gels showing protein expression in digestive gland of *A. umbonella* B) Protein band intensity in the digestive gland of *A. umbonella* after chronic exposure to n-Ag and n-TiO_2.

Between 75-100 kDa, the protein was decreased in both of the exposed groups compared to controls but the change was not significant (DF = 2, H = 4.36, P =

0.113) (DF = 2, F = 2.52, P = 0.113). However, it indicates that this protein tends to be down-regulated due to the exposure to these nanoparticles. The increase and decrease in intensity was considered as the up and down regulation of protein in broader terms.

Protein profile of *Asaphis violascens* after nano- and bulk CuO and ZnO treatment

Further investigation was conducted on the protein expression profile of nano- and bulk- CuO and ZnO treatment in *Asaphis violascens.* The gill tissues of treated animals showed differences in five protein bands compared to the controls (Figure 5 A and B). The protein band at 150 kDa showed significant decreases in the gills of animals exposed to b-ZnO, n-CuO and b-CuO but not in the n-ZnO treatment where the decrease was not significant (DF = 4, F = 16.10, P = < 0.001). Proteins at <100 kDa and 50-75 kDa were significantly decreased in n-CuO and b-CuO and n-ZnO and b-ZnO treatments compared to the controls (DF = 4, H = 10.63, P = 0.031).

The lower molecular weight protein in the gills between 35-50 kDa was significantly increased in the n-ZnO treatment but not in the b-ZnO treatment, while for animals exposed to n-CuO and b-CuO the increase in the 35-50 kDa protein was significant (DF = 4, F = 5.28, P = 0.015). Another protein at 35 kDa was increased significantly in the b-ZnO treatment but not in the n-ZnO treatment, thus showing a difference in response of nano and bulk ZnO. The same protein at 35 kDa was significantly increased by n-CuO and b-CuO treatments (DF = 4, F = 19.28, P = < 0.001).

It was interesting to find that in the digestive gland only three prominent protein bands were different in the nano and b-ZnO and CuO treated animals as compared to five protein bands observed in the exposed gill tissues (Figure 6 A and B). However, the three protein bands which showed different expression in the treated digestive glands have also shown different expression patterns in the gills. The protein at 150

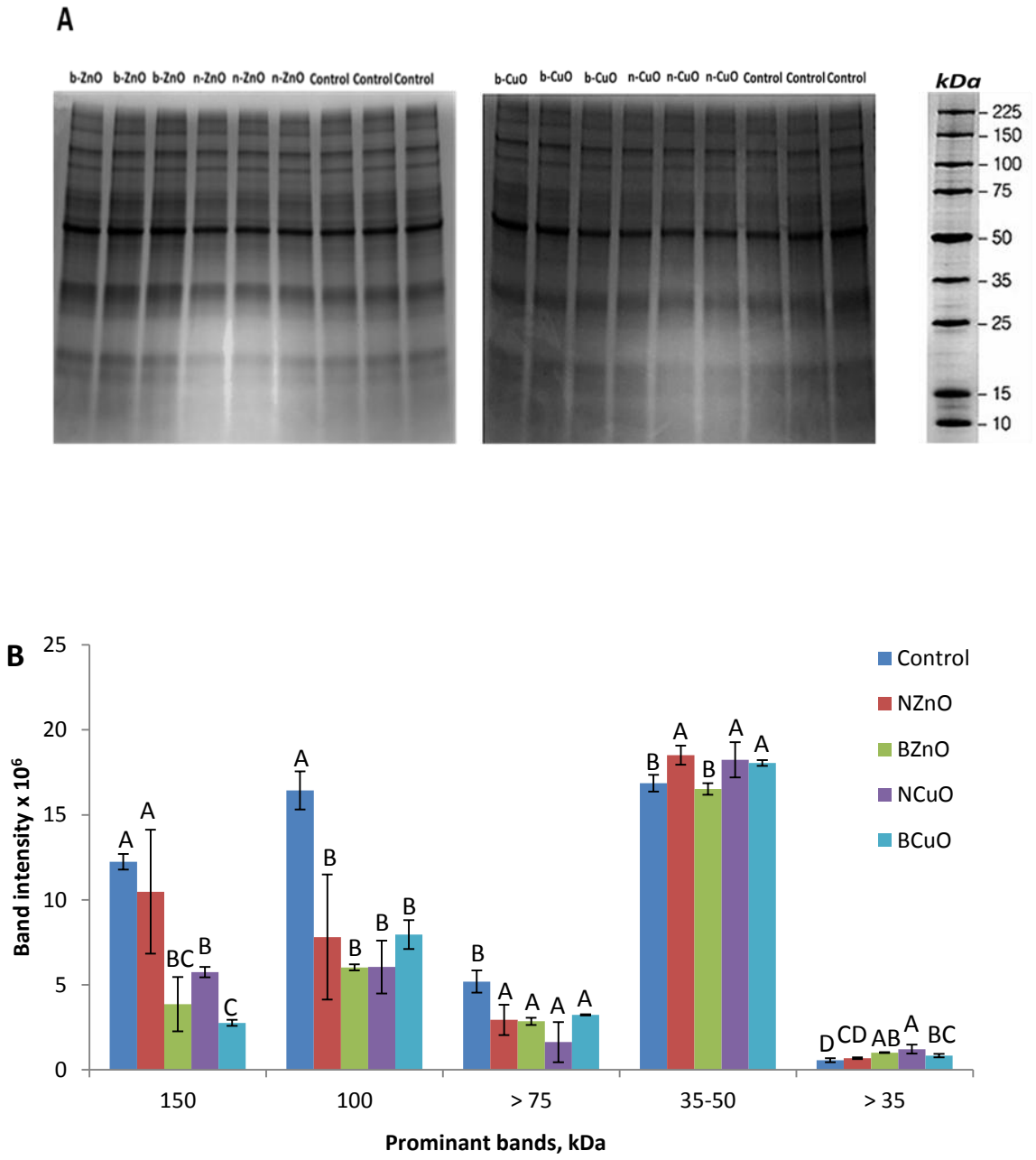

Figure 5. A) Gels showing protein band intensity in the gills of *A. violascens* B) Protein band intensity in the gills of *A. violascens* after chronic exposure to nano and bulk ZnO and CuO.

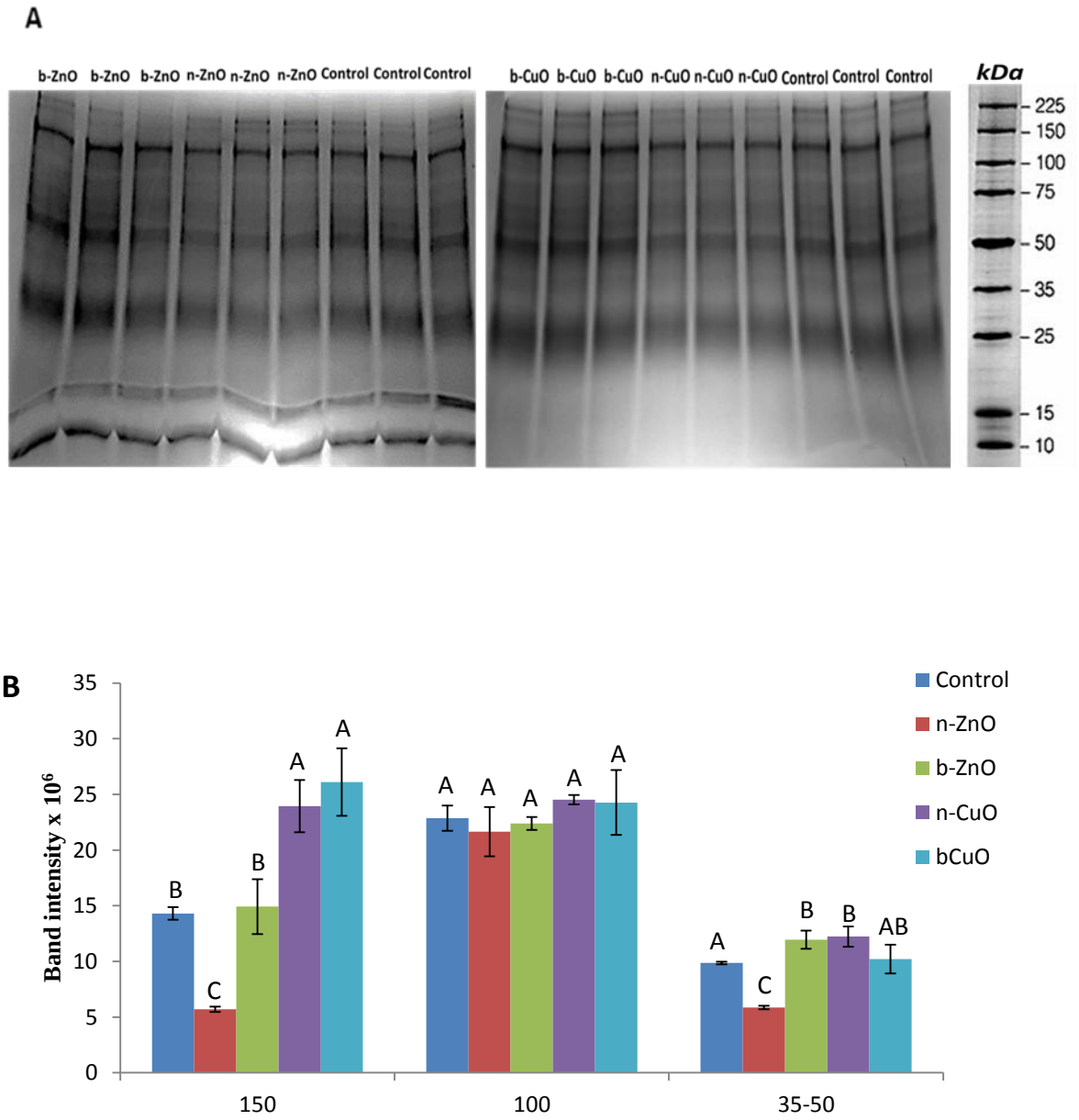

Figure 6. A) Gels showing protein band intensity in the digestive glands of *A. violascens*. B) Protein band intensity in the digestive gland of *A. violascens* after chronic exposure to nano and bulk ZnO and CuO.

kDa showed significant inhibition in the digestive gland in the n-ZnO treatment while b-ZnO caused no change. In the n-CuO and b-CuO treatments the protein at 150 kDa was significantly upregulated (DF = 4, F = 95.62, P = < 0.001). This effect was different from their effect on the gills and also from the effect of ZnO on the digestive gland. The protein at 100 kDa showed insignificant changes in both nano and bulk ZnO and CuO treatment in the digestive gland (DF = 4, F =1.39, P = 0.304), whereas in the gills the proteins significantly decreased in all exposures compared to controls. The protein band between 35-50 kDa was significantly decreased by n-ZnO treatment compared to controls (DF = 4, F = 10.40, P = 0.034).

Discussion

Biological tissues and cells express a characteristic subset of proteins encoded within the genome. The array of expressed proteins in a given biological system under specific conditions is called the proteome. The proteome is highly dynamic. Exposure to chemicals can alter the profile of proteins in exposed tissues by altering protein structure (which is called post-translational modification) or by changing the expression level of specific proteins (known as the protein expression signature). Thus, proteomics provides a potentially highly-sensitive means of detecting effects as well as offering potential insights into toxicity mechanisms (Heijne et al., 2005).

In this study protein separation by gel electrophoresis was done from gill and digestive gland tissues as major sites of oxidative stress. This study, by necessity, stopped at 1D-gels as a 2D-gel electrophoretic system and the software for spot detection was unavailable. It was also not possible to contract work externally due to a lack of funds. However, some interesting observations were made based on 1D-gel separation.

The intention of the author by conducting this study was to open an avenue for further research on the application of proteomics in nanotoxicology since few reports are available for marine organisms. Despite its limitations, this study has

provided insight that bivalves exposed to nanoparticles undergo changes in protein profiles in response to stress. The synthesis of some proteins was upregulated while others were downregulated. Proteins in the range of 35-50 kDa were upregulated in n-Ag, n-TiO_2, n-ZnO, n-CuO, b-CuO, and b-ZnO in the gills but in the digestive gland n-ZnO was an exception. The response of higher molecular weight proteins (225 and 100 kDa) was different in the n-Ag and n-TiO_2 treatments from that of the n-ZnO and n-CuO treatments. However, the present study identified differences in eight bands in the gills and digestive glands after n-Ag and n-TiO_2 treatment, out of this significant increase was a common response of n-TiO_2 and n-Ag in four proteins, whereas one protein at 35 kDa showed a different response to the two nanoparticle exposure. The animals exposed to n-CuO, n-ZnO and b-CuO and b-ZnO showed differences in five bands in the gills and three bands in the digestive gland compared to the controls. In the nano and bulk ZnO and CuO treatment the higher molecular weight proteins were downregulated whereas low molecular weight proteins in general were upregulated. These data showed differences in response of animals exposed to different types of nanoparticles and also in the nano and bulk states of the particles.

In the literature there is limited information available on the protein expression profile in nanoparticle-exposed tissues. Gomes et al. (2013b) conducted a comparative study on the effect of n-Ag and Ag+ exposure in the *Mytilus galloprovincialis* proteome. The authors observed that n-Ag and Ag+ affected a similar cellular pathway with a common response mechanism across the cytoskeleton and cell structure, stress response (HSP70), and energy metabolism. However, a different mechanism was found for the toxicity of n-Ag and Ag+ as different sets of protein were differentially expressed in the gills and digestive gland. This observation indicated that the release of ions from n-Ag was not the only factor in n-Ag toxicity. It was mediated by an oxidative stress signal transduction pathway leading to apoptosis. The study established the role of n-Ag and Ag+ in inducing oxidative stress and helped in the elucidation of their modes of toxicity. In another study Gomes et al. (2014a) examined the effect of n-CuO and Cu^{2+} on *M. galloprovincialis*

and observed major alterations in protein expression profiles in both the gills and digestive glands. Some of the common observations with both Cu forms were similar to that found with both Ag forms, like the cytoskeleton and cell structure, stress response, and energy metabolism. However, different response in protein expression profiles was related to the marked effect by n-CuO on oxidative stress and apoptosis and proteolysis, whereas Cu^{2+} were found associated with adhesion and mobility. The conclusion with the n-CuO and Cu^{2+} exposure experiment was also the same as observed for n-Ag and Ag^{+}, suggesting that the effect of nano structure was different compared to the ionic form. Two dimensional electrophoresis could have revealed exactly how the protein profiles changed after nanoparticle exposure. A 2-D separation of mussel protein after n-Ag and ionic–Ag caused 129 and 83 differentially expressed proteins with twofold or higher changes compared with controls in the gills and digestive glands exposed to n-Ag, respectively; whereas, Ag^{+} exposure generated fewer protein modifications with 106 in the gills and 48 in the digestive glands (Gomes et al., 2013b). This study showed that silver in its nano-state caused more changes in protein expression than its ionic state. The present exposure study established that exposure to nanoparticles produced changes in protein profile. This study opens an avenue for further proteomic research on different types of nanoparticles and their biological effects on marine biota using bivalves as test organisms.

These approaches were used previously to detect new biomarkers in mussels in response to conventional contaminants, such as metals (Apraiz et al., 2009; Shepard et al., 2000) and citrate gold nanoparticles (Tedesco et al., 2010a, b). It is considered that proteomics applied to nanotoxicology may help in understanding the mode of action and mechanism of toxicity of different types of nanoparticles in aquatic biota (Gomes et al., 2014a). Some of the initial studies on proteomic analysis in bivalves (*Mytilus edulis*) were conducted in laboratory conditions in response to metal copper, polychlorinated biphenyls (PCBs) and salinity stress (Shepard and Bradley, 2000; Shepard et al., 2000). The authors compared protein expression

signatures as obtained by the separation in 2-D gel electrophoresis after treatment with test compounds. These studies formed the basis of further investigation in field experiments and other bivalve species such as *M. galloprovincialis* (López et al., 2001), *Chamaelea gallina* (Rodríguez-Ortega et al., 2003), *Ruditapes decussatus* (Chora et al., 2008), *Unio pictorumoysters* (Marie et al., 2010), *Perna viridis* (Leung et al., 2011), *Saccostrea glomerata* (Thompson et al., 2011) and *Dreissena polymorpha* (Riva et al., 2011). Shepard et al. (2000) could separate 500–600 protein spots and discriminated unique protein expression signatures using 2D gel electrophoresis and quantitative analysis of protein abundance by gel image analysis. These results emphasized the importance of the method and further accuracy was obtained in coupling the 2D electrophoretic separation of protein with mass spectrometry. The analysis of proteins by peptide mass fingerprinting and liquid chromatography coupled to tandem MS (LC-MS/MS) followed by a homology search in the bioinformatics database SWISS-PROT and TrEMBL lead to unambiguous identification of proteins (Rodríguez-Ortega et al., 2003). The methods currently in use and the methods under trial, along with unexplored promising methods in proteomic research are given in Figure 7.

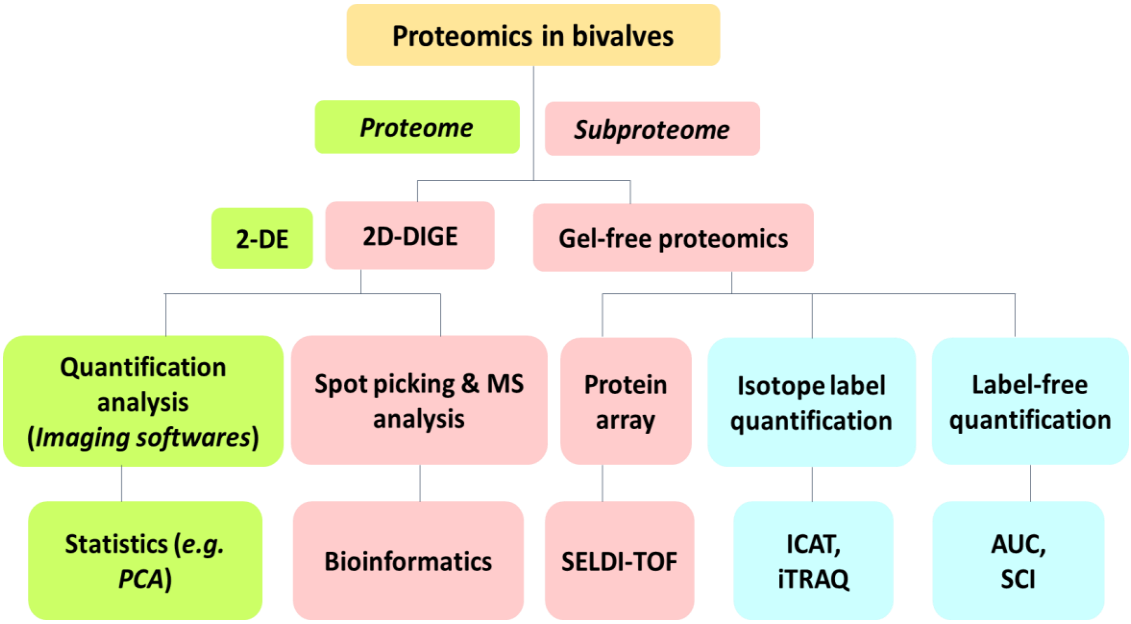

Figure 7. Overview of traditional (green boxes), emerging (pink boxes), and unexplored (blue boxes) proteomic techniques in bivalves (from Campos et al., 2012). 2DE = two dimensional gel electrophoresis of protein; 2D-DIGE = fluorescence difference gel electrophoresis; MS analysis = mass spectrometric analysis; SELDI-TOF = weak cation-exchange protein chip arrays combined with surface enhanced laser desorption/ionization time-of-flight mass spectrometry; ICAT = Isotope coded affinity tag; iTRAQ = isobaric tag for relative and absolute quantitation; SCI = Spectral counting index; PCA= Principal component analysis.

Appendix III: Summary Tables

Table 1. A summary of the effects of acute exposure to nanoparticles on functional activity and oxidative stress in *A. umbonella* and *Asaphis violescens* as determined in this thesis. CR = clearance rate, CAT = catalase, GPx = glutathione peroxidase, GR = glutathione reductase, GST = glutathione-S-transferase, LPO = lipid peroxidation, MT = metallothionein, G = gills, DG = digestive gland.

Parameters	NP	Tissue	***Amiantis umbonella***, exposure concentrations, mg/l					***Asaphis violescens***, exposure concentrations, mg/l			
			0.05	0.20	1.0	2.0		0.05	0.20	1.0	2.0
CR	n-Ag										
	$n\text{-}TiO_2$										
Protein	n-Ag	G									
		DG									
	$n\text{-}TiO_2$	G									
		DG									
CAT	n-Ag	G									
		DG									
	$n\text{-}TiO_2$	G									
		DG									
GPx	n-Ag	G									
		DG									
	$n\text{-}TiO_2$	G									
		DG									
GR	n-Ag	G									
		DG									
	$n\text{-}TiO_2$	G									
		DG									
GST	n-Ag	G									
		DG									
	$n\text{-}TiO_2$	G									
		DG									
LPO	n-Ag	G									
		DG									
	$n\text{-}TiO_2$	G									
		DG									
MT	n-Ag	G									
		DG									
	$n\text{-}TiO_2$	G									
		DG									

Significant increase = ; V significant increase = ; V significant decrease = ;
No change =

Table 2. A summary of the effects of chronic exposure to nanoparticles on functional activity and oxidative stress in *A. umbonella* and *Asaphis violescens* as determined in this thesis. CR = clearance rate, CAT = catalase, GPx = glutathione peroxidase, GR = glutathione reductase, GST = glutathione-S-transferase, LPO = lipid peroxidation, MT = metallothionein, G = gills, DG = digestive gland.

Parameters	**Tissue**	**Chronic exposure to nanoparticles at 0.05 mg/L**					
		n-Ag	*n-TiO_2*	*n-ZnO*	*b-ZnO*	*n-CuO*	*b-CuO*
CR							
Protein	G						
	DG						
CAT	G						
	DG						
GPx	G						
	DG						
GR	G						
	DG						
GST	G						
	DG						
LPO	G						
	DG						
MT	G						
	DG						

Significant increase = ; V significant increase = ; Significant decrease = ; V significant = ; N =

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