CHAPTER 1 - Introduction

OVERVIEW OF NANOSCIENCE AND NANOMATERIALS

Nanotechnology is the art and science of manipulating matter at the atomic or molecular scale and holds the promise of providing significant improvements in technologies, including industrial manufacturing, human health, medicine, personal care, and even environmental protection (Gaunch et al., 2009, Martin et al., 2009, Recillas et al., 2010, Machado et al., 2013). Nanotechnology uses nanomaterials (NMs) that are most commonly defined as having at least one dimension between 1 and 100 nm (OECD, 2009).

Over a thousand consumer products are currently listed to contain NMs, including sunscreens, paints, semiconductors, fabrics, cosmetics, ceramics & composite materials, etc. (PEN, 2012), with global markets expected to reach over $1 billion by 2015 (Nel et al., 2006, Defra, 2007). As the use of NMs for manufacturing increases (Aitken et al., 2004, Royal Society, 2004), a similar trend is expected also for their environmental concentrations in different compartments.

Among nanomaterials are nanoparticles (NPs) that are normally found in nature, as produced by natural phenomena, mainly terrestrial weathering and mineral formation processes within soil. The total NPS mass distribution at Earth level is not known to date, but is expected that deep ocean sediments could be the main reserve (Poulton and Raiswell, 2005; Raiswell et al., 2006). Recently, the global budget for terrestrial sinks and budgets has been evaluated by Hochella et al. (2012). The global terrestrial reservoir of naturally occurring inorganic nanoparticles has been recently estimated in 10^{7-8} Tg (Teragram, 10^{12} g), most of which is represented by clays. Metal oxides (in particular iron oxide), carbonates, sulphates and sulphides provides a much lower mass contribution, but even more relevant

from a geological and ecological point of view, as those materials enter the mineral/nutrient biogeochemical cycling. There is a large flux of natural NMs to the continental shelves and ocean margins, mainly from rivers. This flux accounts for 10^{3-4} Tg and about 90% of that is deposited at estuaries and just off coastal areas for aggregation and sedimentation processes caused by sudden salinity change. Other important sources of natural NMs for the marine environment is represented volcanic activities, atmospheric dust and continental glaciers (in Antarctica) which contributes to fertilization (e.g. with iron) and primary production of Northern and Southern hemispheres oceans.

Along with natural nano-products are engineered nanomaterial (ENMs) and/or nanoparticles (ENPs) of human origins. These are manufactured to exhibit novel properties with specific and improved functionality and increased efficiency (Navarro et al., 2008). They can be exploited in a wide range of applications such as electronics, mechanics, medicine, cosmetics, renewable energies, environmental remediation, etc. (De et al., 2008, Ghosh and Paria, 2012). The use of ENPs has grown dramatically in the last decade and will continue growing in coming years (Aitken et al., 2006, Royal Society, 2004).

ENPs are usually classified based on their chemical composition into carbon-containing (organic) and metal-based (inorganic) materials (Nowack et al., 2007, Peralta-Videa et al., 2011). Among inorganic ENPs, OECD highlighted four metal or metal oxide ENPs with high interest due to their inherent properties, widespread use and commercial importance, namely cerium oxide (CeO_2), silver (Ag), zinc oxide (ZnO) and titanium dioxide (TiO_2). Widely used as a fuel additive in diesel engines to reduce particulate emissions (Park et al., 2008), CeO_2NPs are also used as glass polishers and in heat-resistant coatings (EPA, 2009). Exploited for their antibacterial properties (Jeong et al., 2005, Lee et al., 2005, Shrivastava et al., 2007), AgNPs are found in over two hundred consumer products, including deodorants

fabrics (Luoma, 2008), and are especially effective against *E.coli* (McQuillan et al., 2012). ZnONPs are found in paints, cosmetics, animal feeds and fertilisers and, much like TiO$_2$NPs, are commonly employed in sunscreens where the particles protect against cell damage by blocking UV light (JRC-IRMM, 2011).

The physical/chemical properties of NPs are attributable to their small size (surface area and size distribution), chemical composition (purity, crystallinity, electronic properties), surface structure (surface reactivity, surface groups, inorganic or organic coatings), solubility, shape, and aggregation (Nel et al., 2006, Stone et al., 2010). The surface area-to-volume ratio is a function of particle size, with smaller particles having a large surface area per weight than larger particles (Bottero et al., 2010). This higher surface to volume ratio makes NPs potentially more reactive than large particles. Approximately 35-40% of the atoms are localized at the surface of 10nm nanoparticles compared to less than 20% for a particle larger than 30nm (Auffan et al., 2009).

Currently there are no safe guidelines regarding the release of metals NPs into surface water, including marine environments. The UK has released Environmental Quality Standards, whilst the US EPA has released Aquatic Life Criteria based on the biotic free ligand model, which concerns metal ions (Table 1). However one major difference between metal NPs and metal ions is charge. AgNPs, for example, have a slightly negative charge, whilst ionic silver exists as the Ag$^+$ ion (Liu and Hurt, 2010).

Toxin	US fresh water (µg/L)		US salt water (µg/L)		UK salt water (µg/L)	
	Acute	Chronic	Acute	Chronic	Acute	Chronic
Cadmium	2.00	0.25	40	8.80	–	2.50
Copper	2.33	1.45	4.80	3.10	–	5
Gold	–	–	–	–	–	–
Iron	–	1000	–	–	–	1000
Nickel	470	52	74	8.20	–	30
Silica	–	–	–	–	–	–
Silver	3.20	–	1.9	–	1	0.50
Titanium	–	–	–	–	–	–
Zinc	120	120	90	81	–	10

Table 1 - US EPA Aquatic Life Criteria Water Quality Standards and UK Environmental Quality Standards for certain metals in fresh and/or salt waters (Baker et al., 2013).

SILVER NANOPARTICLES (AgNPs)

Silver nanoparticles (AgNPs or nanosilver) have attracted increasing interest due to their unique physical, chemical and biological properties compared to their macro-scaled counterparts (Sharma et al., 2009). AgNPs have peculiar physico-chemical properties: high electrical and thermal conductivity, chemical stability, catalytic activity and non-linear optical behavior (Krutyakov et al., 2008).

AgNPs find use in many fields, and the major applications include their use as catalysts, in textile engineering, in electronics, in optics, and most importantly in the medical field as a bactericidal and fungicidal. Silver ions are used in the formulation of dental resin composites; in coatings of medical devices; as a bactericidal coating in water filters; as an antimicrobial agent in air sanitizer sprays, pillows, respirators, socks, wet wipes, detergents, soaps, shampoos, toothpastes, washing machines, and many other consumer products; as bone cement; and in many wound dressings to name a few.

AgNPs in a liquid form can be used such as a colloid (coating and spray) or contained within a shampoo (liquid) and can also appear embedded in a solid such as a polymer master batch or be suspended in a bar of soap (solid).

Nanotechnology has been rapidly growing with utilization in a wide range of commercial products throughout the world.

Because of their widespread applications, the scientific community and industry has paid special attention to the research topic of AgNPs.

AgNPs characterization

Currently, there are a range of analytical tools employed to characterize nanoparticles in environmental media; but, it is clear from the literature that nanoparticle characteristics readily change in complex environments (e.g., body or environment) due to their high surface reactivity, and these characteristics evolve over time (Maurer-Jones et al., 2013).

Each method has its own limitation in applicable size and concentration ranges. For chemists it is an enormous challenge to measure samples for environmentally relevant concentrations, because detection limits are generally not sufficiently low (Jiang et al., 2009, Tiede et al., 2009). Environmental samples also contain a high background of natural and unintentionally produced nanoparticles (Hassellov et al., 2008).

To analyze the aggregation state of NPs in aqueous solution it is possible to use dynamic light scattering (DLS) or microscopy-based techniques such as scanning and transmission electron microscopy (SEM/TEM).

The DLS measurements are based on the relationship between a particle's diffusion coefficient in solution and its size-dependent Brownian motion.

Electron microscopy represents an important technique for directing viewing NPs at their original domain sizes.

Primary particle size, shape and size distributions can be determined directly from SEM and TEM images using digital processing or by scoring images manually. In SEM the interaction of the electron beam with the particle surface are scanned over the sample. Due to high depth of field in SEM a three-dimensional appearance can be obtained.

The atomic spectrometry is commonly used for characterization, where the supernatants of nanoparticle suspensions are analyzed using mass (inductively coupled plasma mass spectrometry, ICP-MS), atomic emission, or atomic absorption of the atom of interest (Elzey and Grassian, 2010, Gondikas et al., 2012). These techniques are highly selective and sensitive, with picomolar detection limits. Another common method is to monitor the localized surface plasmon resonance of AgNPs, where a decrease or shift in extinction correlates with dissolving nanoparticles, though this technique requires high concentrations of AgNPs that are largely environmentally unrealistic.

These techniques, however, only provide a snapshot of the ion content, failing to provide real-time, *in situ* assessment of the Ag^+ concentration. Another disadvantage to these techniques is that they are limited in their ability to differentiate between free and complexed Ag^+.

Another technique developed in the last year to study the kinetic of AgNPs dissolution is the Ion-selective electrodes (ISEs). This method provide inexpensive, continuous, sensitive, and selective measurements of Ag^+ and additionally, ISEs have the ability to quantitatively differentiate between free and complexed Ag^+, permitting a more accurate assessment of species contributing to AgNPs toxicity (Maurer-Jones et al., 2013).

Environmental fate of silver nanoparticles

The physico-chemical characteristics of metal-based NPs are fundamental to determine their environmental fate in water and subsequently their bioavailability and toxicity. Evaluate the

processes that control the stability of NPs is a crucial point to understanding their fate and bioavailability (Darlington et al., 2009).

In aqueous solutions the aggregation behaviour can be understood largely in terms of particle stability, while the soils present a solid phase with which NPs may interact, as well as with the dissolved (porewater) phase.

AgNPs may enter the aquatic system in several ways: 1) silver leached out from nanosilver consumer products, and eventually end up in streams and rivers (Benn et al., 2008) 2) suspended AgNPs in air finally depositing on water; 3) runoff scouring AgNPs polluted soils or landfill sewage sludge could result in AgNPs migrating to surface water. The water environment could largely affect the mobility of AgNPs.

In acquatic systems the natural organic material could adsorb on nanoparticles, and act as stable agents to make AgNPs more mobile. Clearly, the types, the concentration and functional groups of natural organic material influence the stability of the nanoparticles in the media (Nason et al., 2012). The divalent cations (e.g. Ca^{2+} and Mg^{2+}), commonly present in waters, could easily induce the aggregation of AgNPs; colloidal clusters would probably deposit in the sediment, reducing the bioavailability for aquatic organisms and plants.

Silver nanoparticle are not highly stable and can easily be oxidized, a slow dissolution of Ag^+ would be expected in aquatic environments. In seawaters, as sodium chloride is the dominant salt, Ag^+ associates with Cl^- to a great extent, and the main species observed are $AgCl_2^-$, $AgCl_3^{2-}$ and $AgCl_4^{3-}$, making silver more mobile in seawater (Barriada et al., 2007).

For the silver nanoparticles its possible identify several type of environmental transformation processes: oxidation and dissolution, aggregation, sulfidation, chlorination, regeneration (Yu et al., 2013).

- *Oxidation and dissolution*

The oxidation of metallic silver is thermodynamically favored at room temperature. The large surface area of AgNPs facilitates exposure to O_2 and so they are much more reactive than bulk silver. Nanoparticles with a small diameter (about 3 nm) within pores of a silica host could be oxidized at room temperature, and a dense Ag_2O layer was formed to prevent further corrosion: this process can be accelerated with the high humidity. Parameters such as dissolved oxygen, pH, temperature and salinity can affect Ag^+ dissolution in water. Liu et al. (2011) found that by removing dissolved oxygen in AgNPs suspension, the release of Ag^+ was completely inhibited, and the dissolution of Ag^+ was greatly enhanced with decreasing pH in air-saturated water, showing that both protons and dissolved oxygen played key roles in controlling Ag^+ release.

- *Aggregation*

The dissolution, mobility and toxicity of AgNPs are highly modified by the size and dispersion of the same nanoparticles.

Nanoparticle clusters can precipitate in the sediment and become less mobile than the original particles. The disperse state is closely related with the surrounding environment, such as electrolyte composition, solution ionic strength and pH.

The aggregation of nanoparticle induced by sunlight was recently shown by Cheng et al. (2011). Evidence showed that UV in sunlight induced this destabilization, possibly due to the oscillating of electrons at resonant conditions. They also observed that the toxicity of AgNPs was reduced significantly after sunlight irradiation, indicating the aggregation state of AgNPs is an important parameter influencing their bioavailability and cytotoxicity.

- *Sulfidation*

Similar to oxidation, when reacted with H_2S, COS, SO_2 or other S containing solutions, surface sulfidation of AgNPs probably occurs. Several studies have showed that PVP stabilized silver nanowires and nanoparticles are rapidly corroded at ambient laboratory conditions (Elechiguerra et al. 2005; McMahon et al., 2005). Silver sulfide nanoparticles were also discovered in the final stage sewage sludge materials of a full-scale municipal wastewater treatment plant (Kim et al., 2010). Liu et al. (2011) have focused the study of the mechanism of the AgNPs oxysulfidation, showing how the complex Ag_2S is slightly soluble.

- *Chlorination*

The corrosion behavior for silver exposed to the atmosphere was studied, and it was revealed that AgCl, as well as Ag_2S, was a main constituent of the corrosion layer, showing the reaction of silver with chloride is common (Graedel, 1992). Particulate chloride and possibly HCl acted as the primary inducing agents, and gaseous H_2O_2 strongly promoted silver corrosion.

- *Regeneration*

Several studies have reported the regeneration associated transformation of new small AgNPs near the original particles under particular ambient conditions (Glover et al., 2011). The authors hypothesized that this process involves three steps, including the oxidation and dissolution of silver from original particles, Ag^+ diffusion away due to the concentration gradients, and AgNPs formation by chemical or photoreduction.

Environmental exposure of silver nanoparticles

The widespread application of AgNPs has inevitably increased human and ecosystem exposure. AgNPs may be released in the environment during the production, transport, erosion, washing or disposal of AgNPs containing products. To date there is concern about the potential risks of AgNPs in the environment.

A number of literature reports have appeared on the leaching and fate of AgNPs in the environment. Benn et al (2008) revealed that AgNPs and Ag^+ can easily leach into water by simply immersing commercial AgNPs containing socks into water with shaking.

Evidence of water contamination by nanomaterials has been recently described. Kaegi et al (2008) showed that TiO_2NPs were released from self-cleaning exterior facades into run-off water, and demonstrated the presence of sub-micronic Ti based particles in raw sewage and effluents.

The increasing presence of manufactured nanomaterials in consumer products, large quantities of nanoparticles could be released and eventually enter aquatic ecosystems, either wittingly or by chance (Blaser et al., 2008, Reijnders, 2009, Kaegi et al., 2010, Farkas et al., 2011), posing serious possible risks to the environment. Therefore, investigating the potential aquatic toxicity of nanomaterials has become an important issue.

NPs in the marine environment

To date, the vast majority of aquatic nanoparticle research has been performed on freshwater species, including *Daphnia magna* (waterflea), *Lymnaea stagnalis* (pond snail) and *Caenorhabditis elegans* (nematode). From these studies, results have highlighted a range of sub-lethal effects including reduced swimming (Asghari et al., 2012), reduced growth and reproduction (Zhao and Wang, 2011), bioaccumulation (Rosenkranz et al., 2009), digestive stress and reduced feeding (Croteau et al., 2011a, 2011b). Despite extensive research on freshwater species, little study has been directed towards marine organisms. Published data at this time are available for just eight phyla and, of these, many reports are limited to a single class, order or species. It is not yet clear how best to extrapolate freshwater data for marine organisms given that the properties of NPs will change according

to exposure media, as will the biological, behavioural and respiration characteristics of marine organisms (Baker et al., 2013).

The behavior and toxicity of NPs in sea water are likely very different than in freshwater, even at high dilutions. For example, the vast input of natural colloids and suspended particulate matter have been shown to exhibit modified behaviors in estuarine systems (Dunphy et al., 2006, Hyung et al., 2007, Ju-Nam and Lead, 2008, Xie et al., 2008). As previously described, in seawater the high level of sodium chloride can induce a major mobility of the AgNPs. The typical environmental transformation such as agglomeration, aggregation and precipitation affect the behavior of NPs (Stolpe and Hassellov, 2007).

Transformation and bioavailability are dependent on the local chemistry of the environment. Transformations include physical, chemical, photochemical and biological reactions within organisms or in the presence of organisms (Ju-Nam and Lead, 2008).

The extent to which NPs interact with both abiotic and biotic entities, which includes the possibility of becoming carriers of other environmental pollutants, should be the focus of risk assessments in the marine environment (Hartmann and Baun, 2010).

It's possible grouped in three main sources the NPs released into marine ecosystems:

- *personal care products* such as cosmetics and sunscreens. The lifetimes of the latter NPs and the inert coatings that they are functionalized with, consisting of magnesium, silica, alumina, zinc and zirconium, are largely unknown (Botta et al., 2011; Labille et al., 2010);

- *urban and industrial sewage*. Few information are available on how nanomaterials may interact with the sewage treatment process or if NPs can be removed from waste water sludge (Brar et al., 2010; Kim et al., 2010);

- *anti-fouling applications* in paints that are designed to prevent the attachment and growth of aquatic organisms on ships (Dineshram et al., 2009; Upadhyayula and Gaghamshetty, 2010).

ECOTOXICOLOGY

Anthropogenic pollution is an old phenomenon, arising from the first industrial revolution, but it has achieved dramatic aspects only in recent years due to the extreme population growth, industrial development, and urban and assumed alarming proportions because the natural self-cleansing capacity of the environment are insufficient to eliminate all toxic substances that are released.

Since the 60s, the scientific community has posed a growing attention to different aspects related to anthropogenic pollution, including the chemistry and physics of trace contaminants, their toxicological effects at cellular, physiological and high order level - population, community and ecosystem- level. A new discipline was borne, ecotoxicology.

A proper ecotoxicological analysis should be aimed to:

- identify the sources of various pollutants and biological targets of contamination;

- characterize and quantify the amounts of pollutants present in environmental compartments, including the effective doses on biological targets;

- characterize pollutant toxicity on many different species, examining the direct or indirect mechanisms of action;

- evaluate the toxicity to organismal, population and biological community level.

Summarizing, the ecotoxicology is concerned with studying the mechanism of action of pollutants, to measure the biological damage of one or more species and finally to assess

and quantify the degree of ecosystem disturbance. An integrated study of this type is therefore the best approach for a proper evaluation and control of environmental quality.

Methodological approach for NPs toxicity testing

The nanoparticles are a new class of synthetic pollutants and potential ecotoxicological effects need to be studied (Cattaneo et al., 2009). Testing NPs in standardized laboratory tests can require adaptations to the guidelines. One of the adaptations would be the spiking procedure, which is used for the introduction on nanoparticles in environmental test media.

In the ecotoxicological studies, the NPs properties should be evaluated preferably at the beginning and at the end of the study, to take in consideration the possible transformations of NPs in environmental media (Stone et al., 2010).

Developing standardized spiking procedures for NPs is needed to improve comparability between multiple water toxicity tests. Several of spiking methods have been proposed for preparing test media including the use of solvents, sonication, filtration and stirring for prolonged periods and pH manipulations (Tiede et al., 2009).

At the same time, it should be important to standardize the expression of the dose and the methods used for the quantification of nanoparticles in environmental media. Nowadays, these two dose metrics are used in human toxicology (Oberdorster, 2004), but they seem to be impracticable for the environmental ecotoxicological studies.

In order to enable relating potential ecotoxicity to the (nano) size of NPs, ecotoxicity data of NPs needs be compared with a non-nano material with similar composition. Testing a soluble salt of the metal of interest could unravel the contribution of the soluble silver fraction to nanoparticle toxicity.

AgNPs ecotoxicity and mechanisms

As previously describe the nanotechnology has been rapidly growing with utilization in a wide range of manufacturing products throughout the world.

Because of their widespread applications, the scientific community and industry has paid special attention to the research topic of AgNPs.

At the same time, there is still a lack of information concerning the increase of human, animal and ecological exposure to NPs including AgNPs and the potential risks related to their short and long-term toxicity.

Several authors suggest that the NPs can induce various environmental and health problems (Wood et al., 1993, Hussain et al., 2005, Panyala et al., 2008).

It's possible to identify four major ways in which nanoparticles may interact with an organism: 1) adsorption to the surface (cell, organ or body), 2) cellular internalisation, 3) dissolution of ions from the NP and 4) mechanistic nano-effects (Baker et al., 2013).

The possible risks of AgNPs in mammalian and non-mammalian cells have been studied *in vivo* and *in vitro*.

Generally, in *in vitro* tests, the mechanism of AgNPs-mediated cytotoxicity is mainly based on the induction of reactive oxygen species (ROS). Particularly, exposure to AgNPs causes reduction in GSH, elevated ROS levels, lipid peroxidation and increased expression of ROS responsive genes, it also leads to DNA damage, apoptosis and necrosis. The cytotoxicity and genotoxicity of AgNPs are size-, concentration- and exposure time-dependent (Hussain et al., 2005, Carlson et al., 2008).

An important question is the real impact of AgNPs to human health and animals. Ahamed et al (2008) have indicated that AgNPs induce reproductive failure, developmental malformations and morphological deformities in a number of non-mammalian animal

models. Common causes of AgNPs-induced toxicity include oxidative stress, DNA damage and apoptosis.

Generally, few papers on the *in vivo* toxicology of AgNPs were found, so further investigation is needed to evaluate the real effects of AgNPs on the organisms.

To date, insufficient assessments are available for regulators to adequately control potential hazard associated with the environment. Potential release and dissolution of AgNPs poses a risk for organisms living in soil, sediments and water.

The few information and data regarding the potential impacts of NPs on the marine environment pose serious questions about the risks of exposure for wildlife and humans.

Unfortunately, most of the currently available ecotoxicological data regarding nanoparticles are limited to species used in ecotoxicity standard experiments or freshwater species (Blaise et al., 2008, Asghari et al., 2012).

Zhu et al. (2010) suggested that chronic or long-term exposure may more clearly reveal NP toxicity.

Recent studies on invertebrates and fish have provided data that suggest that harmful effects are possible, but few studies refer specifically to marine wildlife.

Therefore, it's particular importance employ classic and new endpoints on model marine organisms exposed to nanoparticles.

EFFECTS ON MARIN ORGANISMS

In general, three primary biological targets can be identified in the marine environment:

- *pelagic species* ranging from phytoplankton to fish and mammals, including deep sea species exposed during vertical migration of the particles;

- *benthic species* that are exposed to NPs deposited in sediment biofilms;

- *filter feeders*, such as bivalve mollusks, which can be exposed to high ENP concentrations present in surface waters released by terrestrial and atmospheric sources or existing aggregates (Matranga and Corsi, 2012).

More specifically, the marine organisms can be divided into 32 phyla of the animal kingdom, in addition to having representatives in the Archaea, Bacteria, Chromista, Fungi, Plantae and Protozoan kingdoms (Appeltans et al., 2012). Despite their diversity and abundance, most studies have examined effects on a few representative species including Pseudomonas spp. (bacteria), Thalassiosira spp. (diatoms) and Mytilus spp. (mussels).

In a recent review Baker et al. (2013) have try to summarize the potential uptake methods and effects of nanoparticles on various marine organisms (Table 2).

Organism	Uptake method and effects
Bacteria	Gram-positive bacteria more susceptible than Gram-negative. NPs may affect the composition, but not function, of biofilms, but accumulation may make them available to predatory grazers.
Algae	NPs may adsorb to cell surfaces. Ions dissolved from NPs can interfere with nutrient uptake and therefore limit growth. Negative charge on diatom casings may attract dissolved cations.
Arthropods	NP adsorption to nauplii limits movement; Nauplii may feed on NPs. Zn^{2+} dissolution a driver of sub-lethal toxicity.
Annelids	Ingestion of sedimented NPs by deposit feeders, and of floating NPs by filter feeders. Sub-lethal effects seen even with insoluble NPs.
Bivalves	NPs trapped by EPS; 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 NPs.
Gastropods	Adsorption to body may cause oxidative stress in situ. Not significant accumulators in mesocosm studies.
Echinoderms	Down-regulation of immune system in adults; ion dissolution

	may cause larval mortality in chronic exposures.
Stenohaline Fish	May lodge in gill mucus and dissolve. NPs accumulate in the gut following drinking; carbonate production may biotransform and release dissolved ions.
Euryhaline & Diadromous Fish	Ion dissolution interferes with change in gill function moving from full marine to fresh water. Encourages stress-related drinking, leading to uptake of NPs in the gut.
Mesocosms	Bivalves and biofilms suggested as major NPs accumulators in simple mesocosm studies, based upon mass:accumulation ratios.

Table 2 - Potential uptake methods and effects of NPs on various marine organisms (Baker et al., 2013)

Bivalve molluscs: a target groups for nanoparticles toxicity

Among the classic marine invertebrate organisms, the bivalves, have been recently investigated in terms of bioavailability and toxicity based on the importance of invertebrates as sensitive and relevant organisms. (Baun et al., 2008).

These organisms have highly developed processes for the cellular internalization of nano and micro-scale particles; they can filter large volumes of water (Canesi et al., 2012). Bivalve water pumping rates can vary from 90 cm^3/h (*Macoma nasuta*, 3.5 cm) to over 9.7 L/h (*Mytilus californianus*, 9.2 cm) (Meyhofer, 1985) when filter feeding, leaving them highly vulnerable to ingestion of NPs from the water column. Bivalves normally process microalgae, bacteria, sediments, particulates, natural nanoparticles and can potentially accumulating different chemicals in their tissues, raising the question of their uptake efficiency and selection of nano-size particles (Ward and Shumway, 2004).

The rate and ingestion dynamics of nanoparticles have been investigated in *Mytilus edulis* and *Crassostrea virginica*, indicating that aggregates significantly enhance the uptake of 100

nm particles (Ward and Kach, 2009). To date, few studies have been published on tissue localization, internal distribution and uptake of nanomaterials.

Several authors have reported stronger negative effects in gills and digestive glands, induced by different species of nanoparticles (e.g. glass wool, AuNPs, Fullerene), in the blue mussel *M.edulis* and *Crassostrea gigas* (Koehler et al., 2008, Tedesco et al., 2008, 2010, Ringwood et al., 2010).

Canesi et al. (2008, 2010a, 2010b) using the *Mytilus galloprovincialis* as a model species, reported a number of alterations in hemocytes immune parameters, oxidative stress, and lysosomal stability in the digestive glands where carbon black (NCB), C60-fullerene, TiO_2 and SiO_2 aggregated/agglomerates were observed.

In exposures with radio-labelled AgNPs (<40 nm, 0.7 mg/L), 60% of the uptake accumulated in the soft tissues with maximum concentration in the digestive organs, whilst some 7% was found in the mussel's extrapallial fluid (Zuykov et al., 2011). When a mussel closes, it retains some water within the shell to keep the organism moist, and this extrapallial fluid can contain a number of different compounds, including amino acids, carbohydrates, peptides and other (in)organics (Zuykov et al., 2011). Over 90% of the Ag recovered in the fluid in this study was retained by a 3 kDa filter, suggesting Ag may be complexed by the high molecular weight organics found in the fluid.

Comparisons between Me(O)NPs and their ionic counterparts shows form affects different organs. Over 48 h, AgNPs (20-30 nm, citrate-capped, 0.2 mg/L) have increased protein levels and caused greater oxidative damage in the hepatopancreas of *Crassostrea virginica*, whilst $AgNO_3$ caused greater stress in the gills (McCarthy et al., 2013). This suggests capture of AgNPs and transport to the hepatopancreas, where they cause damage *in situ*.

Comparing CuONPs (31 ± 10 nm) and ionic Cu at 10 mg/L, *M.galloprovincialis* exposures also highlight the effect of form on type of oxidative stress caused (Gomes et al., 2012). CuONPs were responsible for lower SOD activity than ionic Cu, whilst significant increases in both glutathione peroxidase (GPX) and CAT activity only occurred after seven days with both forms, but with contrasting results after fifteen days. Both caused lipid peroxidation, but whilst MT presence increased linearly with CuONPs, it was only noted after day fifteen with ionic exposure. Twice the concentration of CuONPs was seen in the gut compared to the gills, suggesting some selective particle sorting. NP aggregation was highlighted as the main cause of toxicity. At 10 mg/L this dose is also very close to the UK regulatory limit (5 mg/L)(Baker et al., 2013).

A fuller understanding of the long term consequences of these particles in the marine environment is warranted and, importantly, also a need to relate the effects and release of these NPs to biomarkers in ecologically relevant species that can be used to inform on potential impacts on marine systems.

As previously described, the widespread use of mollusks in the environmental studies suggest that Mytilus spp. – abundant in coastal marine and estuarine environments - can represent a model organisms for characterizing the potential impact of NPs and correlated effects in the marine environments.

Test organism Mytilus galloprovincialis

The *Mytilus galloprovincialis*, named Mediterranean mussel, is a species of bivalve, a marine mollusc in the family *Mytilidae*. It is considered an invasive species in many parts of the world.

Mussel have been long recognized as valuable indicators of pollution, and extensive background information is nowavailable on their biological responses to a wide range of both inorganic and organic chemicals. (Moore, 2006, Dagnino et al., 2007).

In this organism, food particles trapped by the gill are moved 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 intra-cellular digestion and nutrient accumulation for gametogenesis (Gosling, 1992).

In a recent review, Canesi et al. (2012) encourage the use of this organism as a sentinel species for investigating the effects and mechanisms of action underlying the potential toxicity of nanoparticles.

AIM AND OUTLINE OF THE THESIS

An in depth study about the bioavailability and the possible effects of silver nanoparticles in the environment is yet necessary.

This study has been conducted within the framework of NanoFATE (Nanoparticle Fate Assessment and Toxicity in the Environment), a FP7-EU-funded integrated research project that aims to fill knowledge and methodological gaps of environmental risks posed by ENPs.

The aim of this doctoral thesis was to investigate effects of silver in the form of nanoparticles (NPs) in the marine environment using the marine filter feeder organism *Mytilus galloprovincialis* Lam as a bioindicator. Questions addressed are: to what level are silver NPs toxic to mussels. What is the contribution, if any, of the nano scaled form to toxicity? What is the mode of action of AgENP and what differences with the ionic form? How can AgENP toxicity be projected to population level?

The plan for conducting to answer these questions on 3 layers.

First of all, it will be observed effects at the organismic level, i.e. the ecotoxicological characterization of ENPs by means of acute (mortality at 96 h), subchronic (survival probability in aerial conditions) and chronic (ability to bissogenesi) tests. The toxicity data will be associated to the actual AgENP dose in the water column. From these results we expect to identify the full range of toxicity endpoints (NOEC-LOEC-EC_x-EC_{50}).

From this level, then, two research lines will be developed. The first will be focused on mechanistic effects through a combination of molecular and biochemical techniques. In particular, the hypothesis of a Reactive Oxygen Species (ROS) based mode of action (MOA) for AgENP will be asessed. This research activity will include real-time quantitive PCR gene expression assays for determination of oxidative stress responding sequence mRNA levels (CAT, SOD, GPX, GST); heat shock proteins (HSP27, HSP70, HSP90) and metallothioneins (MT10 and MT20). Furthermore enzymatic activity of CAT, SOD, GPX measured by in gel assay; lipid peroxidation markers including lipofuscin accumulation and protein carbonyl levels will considered to strenghten the ROS MOA hypothesis.

The other research line will be based on the determination of ecophysiological parameters and the application of the Dynamic Energetic Budget (DEB) model. This will allow to link the effects at organism level to population. Long-term changes in the energy parameters/physiological responses of bivalves exposed to Ag-ENPs will be observed. This will include the evaluation of clearance rate, respiration rate, hearth beat rate (HBR), rate of excretion as ammonia nitrogen, pseudofecal and fecal ejection, assimilation efficiency.