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Nanostructured titanium dioxide: Fate in the aquatic environment and effects on the blue mussel *Mytilus edulis*

Siw Anette Busk

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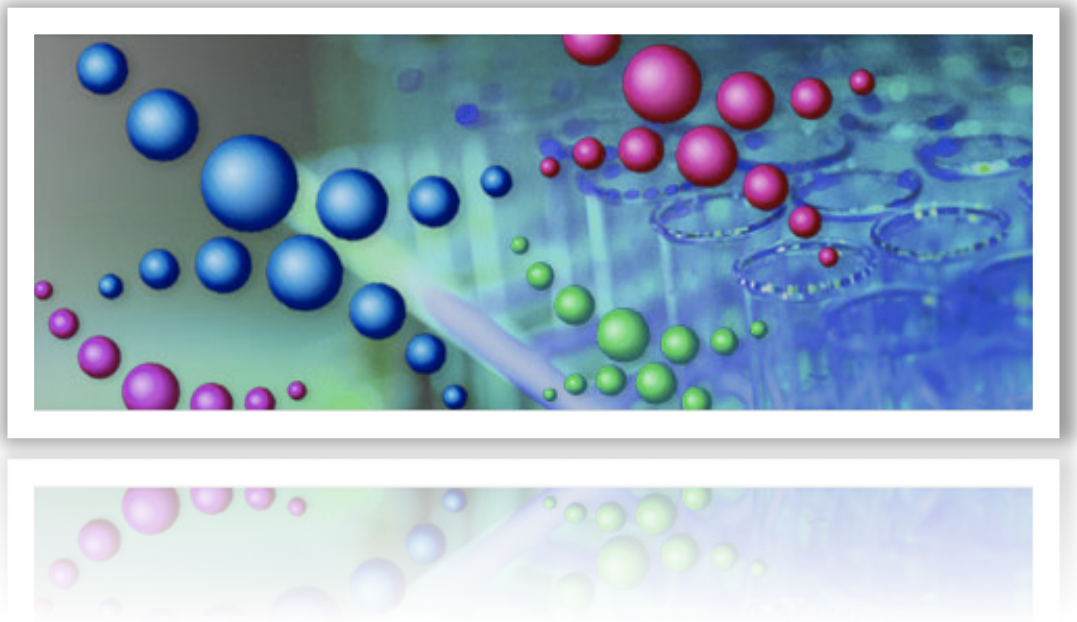

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IRIS
International Research Institute of Stavanger

Abstract

The rapid development of nanotechnology and the corresponding increase in the use of nanoparticles in commercial products have led to concerns about their health risks and environmental impact. As the aquatic systems act as a sink for many pollutants, nanoscale particles are likely to enter the aquatic environment and pose a threat to aquatic organisms. In particular, filter-feeding organisms, such as the blue mussel *Mytilus edulis*, may represent a unique target group for nanoparticle toxicology.

The present study reviews the key aspects concerning fate and behavior of nanoparticles in the aquatic systems, the availability for uptake by biota and toxic effects in aquatic invertebrates, with a particular focus on nanostructured titanium dioxide (nano-TiO_2). The experimental part of this study, explores the hypothesis that nano-TiO_2 can cause sub-lethal impacts on *Mytilus edulis* exposed through the water column. The behaviour of nano-TiO_2 in the aquatic system is explored and the possible effects of *in vivo* exposure to nano-TiO_2 on mussel haemocytes (blood cells) are investigated. Mussels were exposed to different concentrations of nano-TiO_2 suspensions for six days and a lysosomal biomarker was evaluated in the haemocytes.

The results show a great tendency of nano-TiO_2 to aggregate when dispersed in water, in particular in seawater, which will affect the subsequent fate within the aquatic environment. Significant lysosomal membrane destabilisation is found in mussel haemocytes exposed to 5 and 25 mg L^{-1} nano-TiO_2, as evaluated by the neutral red retention time (NRRT) assay. Overall, the obtained data demonstrates that nano-TiO_2 can induce sub-lethal effects in the filter-feeding organism.

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Siw Anette Busk, Stavanger 2011

Contents

List of Abbreviations

CNT	-	Carbon nanotube
EC_{50}	-	Effect concentration, 50%
ERA	-	Environmental risk assessment
GST	-	Glutathione S-transferase
ISO	-	International Organization for Standardization
K_{ow}	-	Octanol-water partition coefficient
LC_{50}	-	Lethal concentration, 50%
LMS	-	Lysosomal membrane stability
LOEC	-	Lowest observable effect concentration
LPO	-	Lipid peroxidation
Nano-SiO_2	-	Silicon dioxide nanoparticles
Nano-TiO_2	-	Titanium dioxide nanoparticles
NOEC	-	No observable effect concentration
NTU	-	Nephelometric turbidity units
PAH	-	Polycyclic aromatic hydrocarbons
PEC	-	Predicted environmental concentration
PNEC	-	Predicted no effect concentration
ROS	-	Reactive oxygen species
SOP	-	Standard operating procedure
TEM	-	Transmission electron microscopy

1. Introduction

Along with promises and possibilities, every new technology brings with it a concern regaring it's potential to cause harmful effects on human health and wildlife – nanotechnology is no exception.

1.1 Nanotechnology

The concept of nanotechnology and the possibility of manipulating matter at the atomic level were first brought into light in a lecture, "There's Plenty of Room at the Bottom", held by the physicist Richard Feynman in 1959. Without realizing it at the time, Feynman planted the seeds of a new era in technology that, 52 years later, is expected to bring benefits in almost all industries and areas of society.

Today, nanotechnology is a fast-growing, interdisciplinary field of science, combining engineering with biology, chemistry, physics, and medicine and erases the traditional boundaries between them (Ray et al., 2009). Nanotechnology gives the ability to observe and control individual atoms and molecules, and deals with structures in the size range from approximately 1 to 100 nanometers, known as the nanoscale. One nanometer (nm) is a billionth of a meter, or 10^{-9} meters. By comparison, the diameter of an average human hair is approximately 100,000 nm and a red blood cell is about 7,000 nm in diameter (Alberts, 2004).

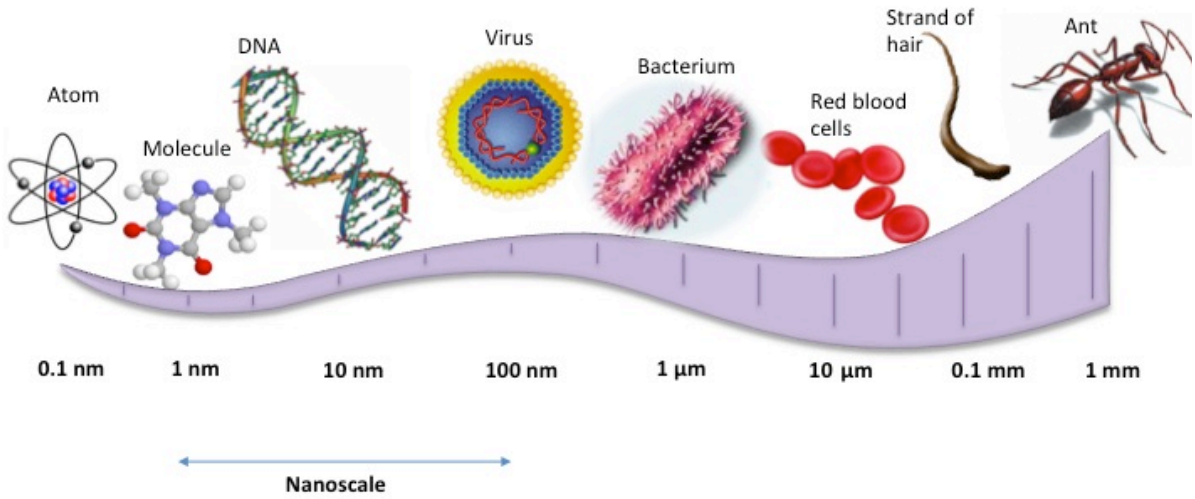


Figure 1: Visual examples of the size and the scale of nanotechnology.

By taking advantage of unique phenomena that naturally occur within this size range, scientist are making everyday material act in unimaginable ways. At the nanoscale, properties such as melting point, color, electrical conductivity, magnetic permeability, and chemical reactivity change as a function of the size of the particle (Boverhof and David, 2010). Thus, when a material is created with dimensions within the nanoscale, its properties change significantly from larger forms of that same material. For instance, particles of gold lose their golden color and appear red or purple at the nanoscale. Hence, nanotechnology opens a new dimension of materials, were the size can be controlled and every size behaves a little different from all other sizes.

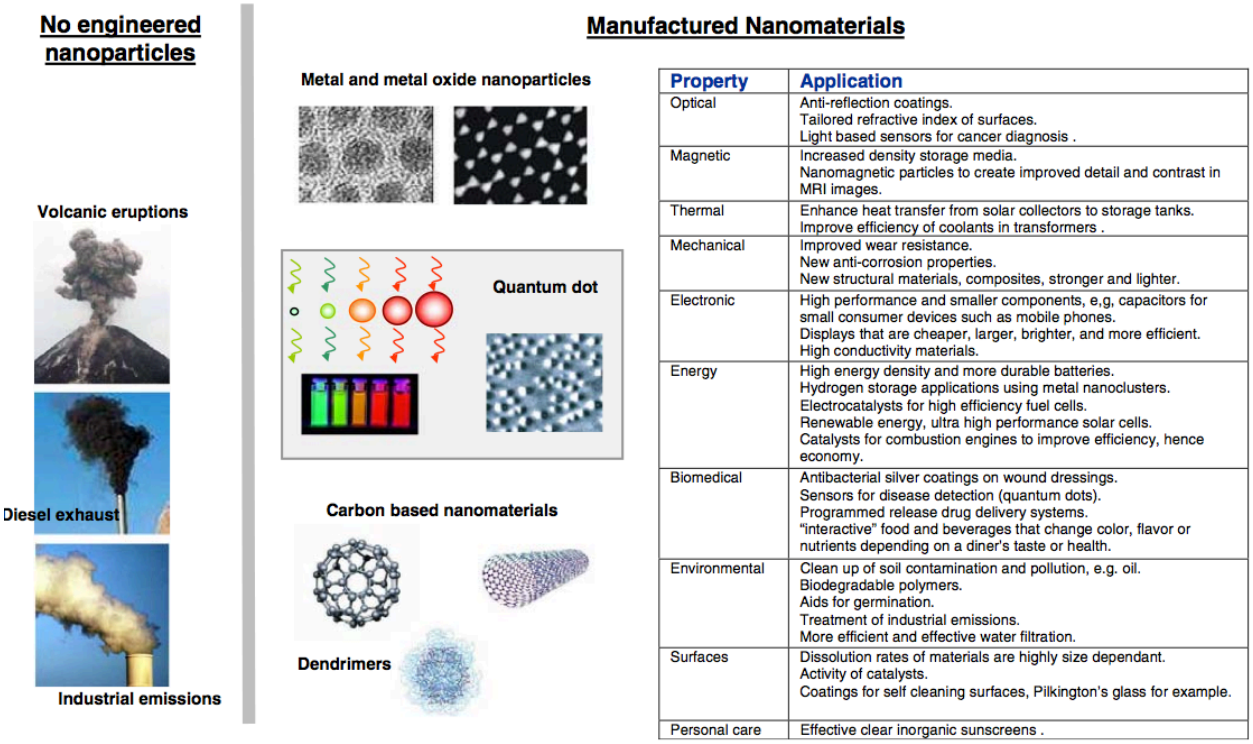
One of the principal factors that causes nanomaterials to differ from larger materials is the increased relative surface area (Ostiguy et al., 2006, Batley and McLaughlin, 2010). When a particle gets smaller, the surface-to-volume ratio gradually increases, leading to an increasing proportion of atoms on the surface of the particles compared to inside. A particle with a size of 30 nm has 5% of its atoms on its surface, whereas a particle of 3 nm has as much as 50% of its atoms on the surface (The Royal Society, 2004). Hence, a given mass of nanoparticulate material will have more atoms available on the particle surface compared to the same mass of material made up of larger particles. As chemical reactions occur on the surfaces, nanomaterials can interact with the environment more efficiently than their larger counterparts (Krug and Wick, 2011), thereby making them more chemically reactive, and potentially alter their strength and electrical properties (Ostiguy et al., 2006, Batley and McLaughlin, 2010). Another factor that shapes the nanoscale behavior is the predominance of quantum effects that takes place as the electrons are confined by the dimensions of the nanostructure. As the size is reduced to a few tens of nanometers, the quantum effects starts to dominate the properties of matter, affecting the optical, electrical and magnetic behavior of materials (Ostiguy et al., 2006). As for the gold nanoparticles mentioned above, the motion of the electrons is confined, and this restricted movement makes the gold particles react differently with light compared to larger sized gold particles, and consequently, their color changes (Ashby et al., 2009).

1.1.1 Natural and anthropogenic nanomaterials

As novel as nanomaterials seem, nanoscale materials have always existed from both natural and anthropogenic sources (Klaine et al., 2008). Aquatic colloids, the fine fraction of desert sand, oil fumes, fumes originating from volcanic activity or from forest fires, and certain

atmospheric dusts, all represent nanoparticles produced by nature (Ostiguy et al., 2006). In addition, a majority of biological processes occur at the nanoscale range and can be considered as nature's own nanotechnology. For instance, the DNA molecule fall within the nanosize range with a diameter of approximately 2.5 nm (Alberts, 2004), the ribosome acts as a nanosized factory combining amino acids in a specific order to create proteins (Ashby et al., 2009), and hemoglobin, the oxygen-transporting protein found in red blood cells, is 5.5 nm in diameter. Other nanomaterials are unintentionally released into nature from anthropogenic sources, which is byproducts of human activity, such as car exhaust, industrial emissions and welding fumes (Nowack and Bucheli, 2007, Ostiguy et al., 2006). Nanotechnology products, however, are deliberately manufactured to take advantage of the novel properties that become evident at the nanoscale.

Nanomaterials can be classified in a number of ways. One way of categorizing nanomaterials is based on their chemical composition (Handy et al., 2008b, Buzea et al., 2007). This classification separates nanomaterials into broad categories such as carbon-based structures including CNTs and fullerene C_{60}; metal-containing nanoparticles including metal oxides, such as titanium dioxide (TiO_2); or semiconductor nanocrystals, also known as quantum dots.



Property	Application
Optical	Anti-reflection coatings. Tailored refractive index of surfaces. Light based sensors for cancer diagnosis .
Magnetic	Increased density storage media. Nanomagnetic particles to create improved detail and contrast in MRI images.
Thermal	Enhance heat transfer from solar collectors to storage tanks. Improve efficiency of coolants in transformers .
Mechanical	Improved wear resistance. New anti-corrosion properties. New structural materials, composites, stronger and lighter.
Electronic	High performance and smaller components, e,g, capacitors for small consumer devices such as mobile phones. Displays that are cheaper, larger, brighter, and more efficient. High conductivity materials.
Energy	High energy density and more durable batteries. Hydrogen storage applications using metal nanoclusters. Electrocatalysts for high efficiency fuel cells. Renewable energy, ultra high performance solar cells. Catalysts for combustion engines to improve efficiency, hence economy.
Biomedical	Antibacterial silver coatings on wound dressings. Sensors for disease detection (quantum dots). Programmed release drug delivery systems. "interactive" food and beverages that change color, flavor or nutrients depending on a diner's taste or health.
Environmental	Clean up of soil contamination and pollution, e.g. oil. Biodegradable polymers. Aids for germination. Treatment of industrial emissions. More efficient and effective water filtration.
Surfaces	Dissolution rates of materials are highly size dependant. Activity of catalysts. Coatings for self cleaning surfaces, Pilkington's glass for example.
Personal care	Effective clear inorganic sunscreens .

Figure 2: Natural and engineered nanoscale substances (Farré et al., 2009)

Classification based on dimensionality is another way of categorizing nanomaterials (Buzea et al., 2007, Krug and Wick, 2011). According to the International Organization for Standardization (ISO), there are two main types of nanomaterials – nanoobjects and nanostructured materials. Nanostructured materials comprise a broad class of materials that contain nanosized structures, without necessarily having an overall nanoscaled size. These include nanoporous materials (which have nanosized pores within particles that may or may not be of nanoscale dimensions), nanocrystalline materials (which have nanosized crystalline grains within particles that may or may not be of nanoscale dimensions), and complex fluids containing nanosized objects. Nanoobjects are classified based on the number of dimensions confined to the nanoscale range, separating them into nanoplates, nanofibers, and nanoparticles as shown in Figure 3.

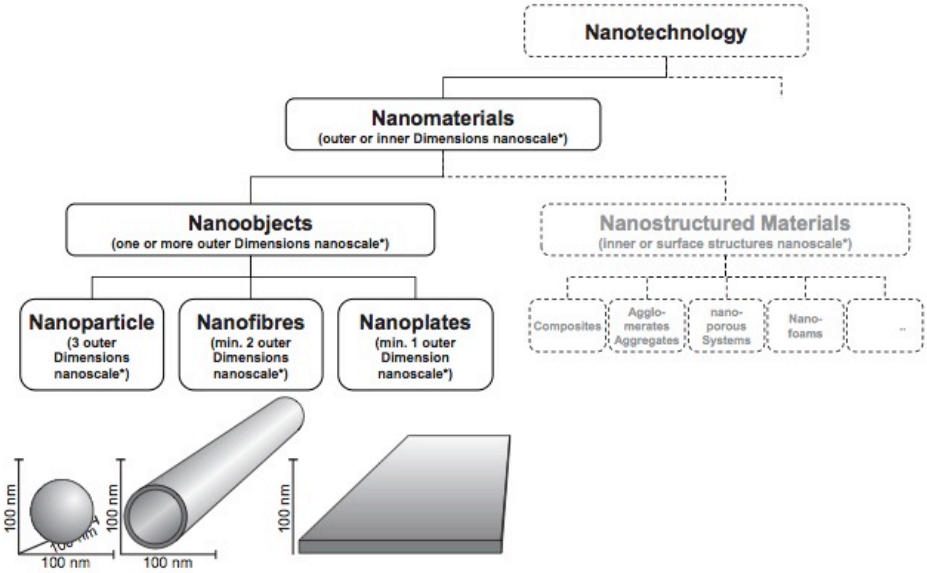


Figure 3: The ISO definition of nanoobjects. The shape of nanoobjects reflects the number of dimensions confined to the nanoscale (Krug and Wick, 2011).

Nanoplates, including nanofilms, nanolayers, and nanocoatings, are stretched out in two dimensions whereas one dimension is confined to the nanoscale. An example of a nanoplate is graphene, which consists of sheets of graphite and has interesting electronic properties (Geim and Novoselov, 2007). Nanofibers have three dimensions within the nanoscale, and includes nanotubes, nanorods and nanowires. Carbon nanotubes (CNTs) have been given a lot of attention, and these nanofibers with hollow interior is widely explored for their use in medical applications (Bianco and Prato, 2003, Bianco et al., 2005). Nanoparticles have all their

dimensions measured within the nanoscale, and are often synthesized to form spheres (Ashby et al., 2009, Krug and Wick, 2011).

1.1.2 Applications and benefits

The world of nanotechnology has led to a host of new material application. In commercial products, nanoscale materials can be added to consumer products, such as the frames of tennis rackets and motorcycle helmets, to make the material stronger, more durable, and lighter. Nanoscale additives to fabrics help them resist wrinkling, staining, and bacterial growth. Nanoscale thin films on eyeglasses, computer and camera displays, windows, and other surfaces can make them water-repellent, antireflective, self-cleaning, resistant to ultraviolet or infrared light, anti-fog, antimicrobial, or scratch-resistant (U.S. National Nanotechnology Initiative). In medicine, the use of nanotechnology offers some exciting possibilities. Nanotechnology allows researchers to build nanosized devices that, when conjugated with antibodies, can target tumor cells with high specificity and affinity. This enables drug-delivery systems, in which small drug molecules can be encapsulated in micelles formed by nanomaterials that transport them to the desired location, thereby lowering the total drug consumption and side effects (Jain, 2010). Researchers have also developed an imaging technique to measure the amount of an antibody-nanoparticle complex that accumulates specifically in plaque, thereby serve as an early diagnosis of atherosclerosis (Wickline et al., 2006). In environmental science, research is underway to use nanomaterial to detect and clean up organic solvents that are polluting ground water (Zhang, 2003, Long et al., 2006). Furthermore, nanotechnology could potentially have a great impact on clean energy production, such as more efficient solar cells and environmentally friendly batteries (Tian et al., 2007).

1.2 Nanotoxicology

Despite obvious benefits of the power of nanomaterials, there are open questions concerning whether, and to what extent, the novel properties seen at the nanoscale may pose a threat to the environment and to human beings (Buzea et al., 2007). As the production of nanomaterials increases, so does the possibility for human exposure and unintentional release into the environment. Workers can be exposed to nanoparticles released in the workplace during research, design, and development; consumers can be exposed during usage of these commercial nanotechnology products; and leakage or discharge of nanomaterials into the

environment causes exposure to ecosystems (Figure 4). The small size of nanoparticles may facilitate their entry into living cells (Ray et al., 2009). This, together their enhance reactivity that allow them to interact more efficiently with biological systems, raises concern about their potential to induce harmful biological effects (Tsuji et al., 2006).

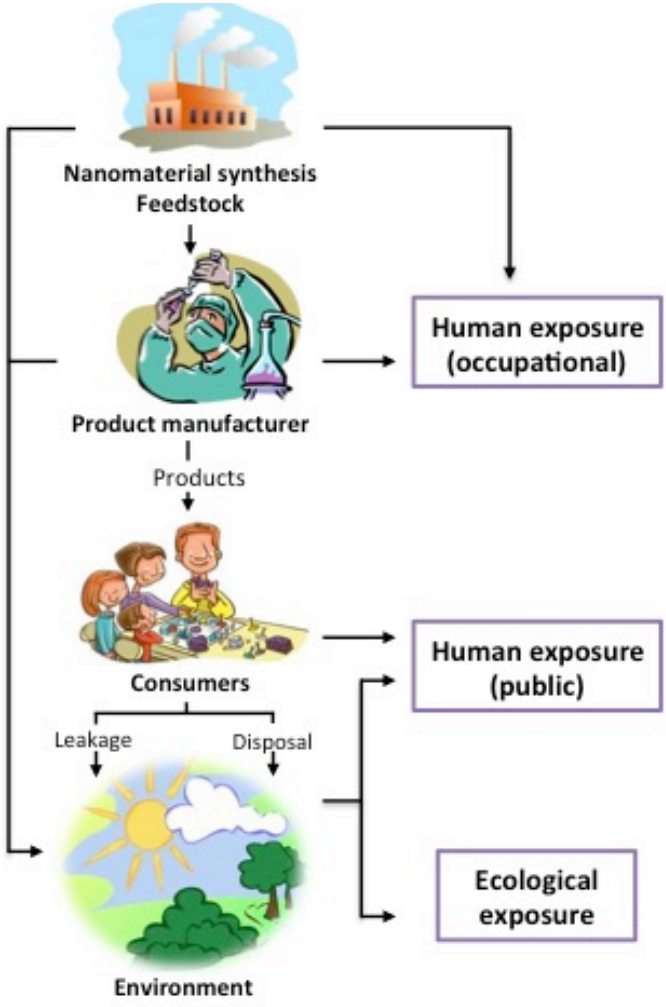


Figure 4: Possible release and exposure routes for nanomaterials. Based on figure by Tsuji et al (2006).

The term *nanoecotoxicology* can be defined as the study of harmful effects of nanomaterials upon ecosystems (Baun et al., 2008a). During recent years, several studies have been performed demonstrating that nanomaterials can affect biological systems at the cellular, subcellular and protein levels (Colvin, 2003, Oberdörster et al., 2005). The majority of studies conducted on nanoecotoxicology have focused on terrestrial vertebrates and the effects of nanoparticles by inhalation, with comparatively less attention paid to exposure of organisms living in other environmental compartments (Scown et al., 2010). Recently, however, studies

are emerging exploring the exposure effects of nanoparticles on aquatic organisms, investigating potential routes of uptake, translocation, fate, and effects, as well as how the surrounding exposure medium affect the fate of the nanoparticles. The investigation into the effects on the aquatic environment is of high interest, particularly since the aquatic systems act as a sink for many pollutants, receiving runoff and wastewater from domestic and industrial sources (Batley and McLaughlin, 2010).

Data from ecotoxicity tests in invertebrates, fish and algae have indicated low hazard potential of nanoparticles on aquatic species (Lovern and Klaper, 2006, Lovern et al., 2007, Oberdörster et al., 2006, Smith et al., 2007, Warheit et al., 2007a). However, studies on the sub-lethal effects have revealed oxidative damage in brain of largemouth bass exposed to the carbon-based fullerene C_{60} (Oberdörster, 2004). Increased lipid peroxidation in brain and gills, as well as the expression of cytochrome P450 isoenzymes (crucial for a number of catalytic biotransformation reactions) in the liver of fathead minnow exposed to fullerene C_{60} (Zhu et al., 2006a). Lipid peroxidation have been found in the gills, brain and liver of rainbow trout and juvenile carp exposed to nano-TiO_2 (Federici et al., 2007, Hao et al., 2009), indicating that these tissues suffered from oxidative stress. Quantum dots and nanosized gold have shown to induce oxidative stress and DNA damage in gills and digestive gland of mussels (Gagne et al., 2008, Tedesco et al., 2008).

While toxicity mechanisms have not yet been completely elucidated, available ecotoxicology data on the sub-lethal impact of nanoparticles in aquatic invertebrates suggests that oxidative stress, genotoxicity, and effects on the immune system are key features of their toxicity (Handy and Shaw, 2007). Most studies assessing the effects of nanoparticles in aquatic invertebrates have focused on freshwater species, in particular the crustacean *Daphnia magna* (*D. magna*) (Scown et al., 2010). Less attention has been given to species from estuarine and marine environments, such as the blue mussel *Mytilus edulis,* which is of focus in this study.

1.2.1 Ecological risk assessment

Particles in the nanometer size range do occur both in nature and as a result of existing industrial processes (section 1.1.1) and their effects have been studied over the years (Nel et al., 2006). However, the extraordinary properties of engineered nanomaterials, may need a new investigative approach to assess their potential hazard (Farré et al., 2009)

Ecological risk assessment (ERA) is a process that evaluates the potential for adverse ecological effects that can occur as a result of exposure to stressors (Chapman, 2002). The process involves identification of the hazard and then a structured approach to determine the probability of exposure to the hazard and the associated consequences. ERA is divided into a scientifically oriented risk analysis to estimate the magnitude and probability of effects, and a technically oriented risk management to implement solutions to the problem and determine acceptability of risks (van der Oost et al., 2003). The entire risk assessment process comprises several steps, as illustrated in Figure 5.

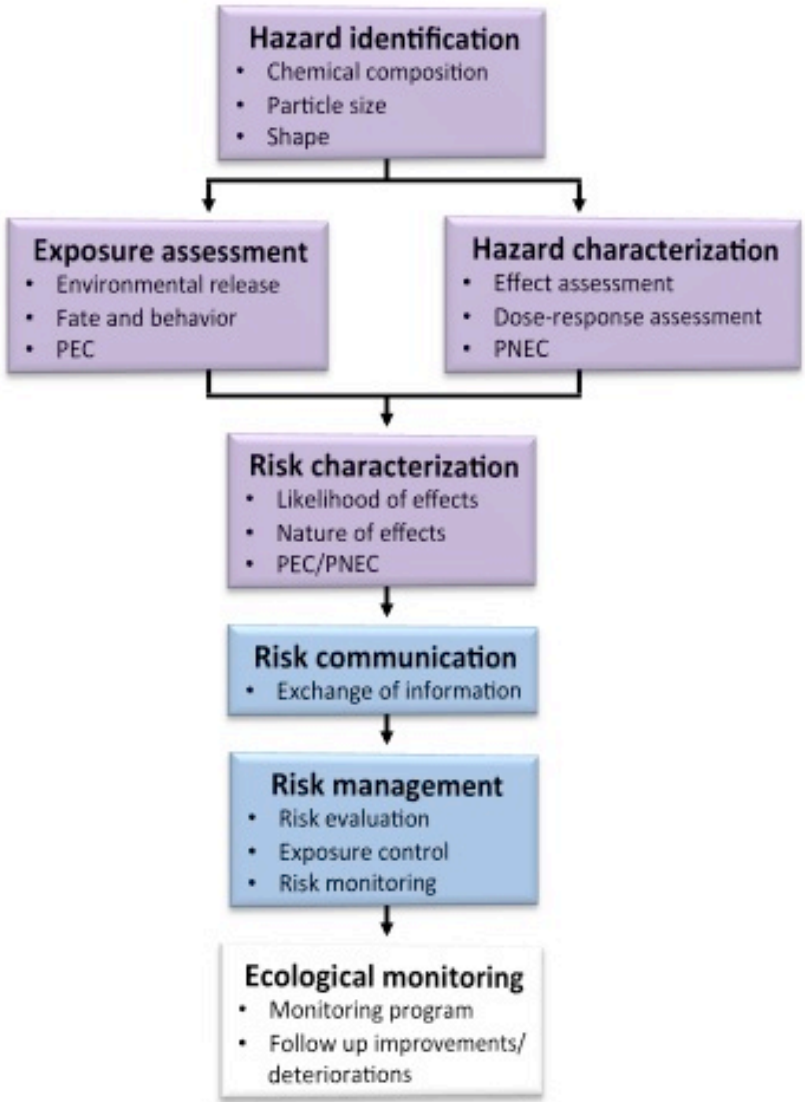


Figure 5: Ecological risk assessment (ERA) framework. The risk analysis (1. part of ERA) is marked purple, whereas risk management (2. part of ERA) is shown in blue.

The first step is the hazard identification that involves the identification of the type and nature of adverse effect that may be caused by the chemicals. The properties of nanomaterials depends on several factors, such as chemical composition, size, and shape (Colvin, 2003), creating a huge group of substances, all with the potential to be hazardous in their very own way. For instance, *in vitro* studies using nanosized gold, have shown that very small (1.4 nm) nanoparticles can cause cell death through induction of oxidative stress and mitochondrial damage (Pan et al., 2009), whereas slightly larger (3.7 nm) gold nanoparticles can penetrate the nucleus of the cells without being toxic (Gu et al., 2009). This makes the hazard identification quit complex when it comes to nanomaterials.

The exposure assessment is the estimation of concentrations to which the environment may be exposed – predicted environmental concentrations (PECs). This assessment should include the entire life cycle of the nanoparticles, as release and exposure may occur from synthesis to disposal (Tsuji et al., 2006, Colvin, 2003). The fate and behaviour of the nanoparticles in different environmental compartments after their release should also be included as this is likely to have a great impact on their concentration and form (Johnston et al., 2010, Moore, 2006, Nowack and Bucheli, 2007).

The hazard characterization, or effect assessment, is the estimation of the relationship between level of exposure and severity of an effect, and estimation of a predicted no effect concentration (PNEC). The risk characterization brings all the information from the first three steps together to estimate the risk based on exposure compared to effects. Risk arises from the likelihood of harm to occur; it is a combination of the *hazard* and the probability of *exposure* to it (Krug and Wick, 2011). The ratio between the PEC and PNEC values gives a simple indication on whether or not there is a reason of concern. The second part of ERA involves evaluation of the risks to decide if there is need for further testing or risk reduction.

Thus, to assess the potential risks of nanoparticles, both the toxicokinetic and toxicodynamic behavior should be considered. The toxicokinetic behaviour, including their entry into the environment, their movement and dispersion in different environmental compartments, the process of uptake by biota, and fate within the organism, will decide whether (and to what degree) nanoparticles reach the site of toxic action (exposure). Their toxicodynamic behaviour includes all harmful effects in the living organism, including biochemical or physiological changes that adversely affect the organism's reproduction, growth, or mortality rates (hazard).

1.3 Objectives

This thesis presents the beginning of the process of establishing nanoecotoxicology as a new discipline at IRIS Biomiljø. The goal of this project is therefore to explore the basics of nanoecotoxicology regarding the possible impact of nanoparticles on the aquatic environment.

The main objective of this thesis is to complete a literature survey on the impact of nanoparticles on the aquatic systems with focus on the following questions:

a. How do nanoparticles behave in the aquatic environment?
b. How do nanoparticles interact with aquatic invertebrates, in particular the blue mussel?
c. Do nanoparticles induce toxic responses in aquatic invertebrates?

The literature assessment has a main focus on nano-TiO_2, and reflects about 2/3 of the work of this thesis. Nano-TiO_2 was chosen as test material after recommendation from collaborating partners in Los Angeles who already has started working on this branch of ecotoxicology. Nano-TiO_2 is inexpensive and can therefore be provided easily and they are considered to be of low toxicity (Tran et al., 2000, Hext et al., 2005). Nano-TiO_2 is used in a number of applications (Nowack and Bucheli, 2007, Kaida et al., 2004) and they are therefore likely to reach the aquatic environment in significant amounts.

Experimental work was conducted to further investigate the questions numbered a and c. Here, the behaviour of nano-TiO_2 in water was examined and two exposure studies was set up to investigate the possible effects of nano-TiO_2 on the haemocytes (blood cells) of the marine mussel *Mytilus edulis*. Mussels are tolerant to a wide range of environmental conditions, they are suspension-feeders pumping large volumes of water, and they are able to concentrate many chemicals within their tissues, which makes them suitable for laboratory testing (Widdows and Donkin, 1992). Additionally, mussels have been used in previous studies on nanoecotoxicity. For instance, Canesi et al (2010) evaluated several biomarkers after exposure to the carbon-based fullerene C_{60}, nano-TiO_2 and nanosized carbon black; Tedesco et al (2008) studied the biological impact of gold nanoparticles on the mussel's gills, mantle, and digestive gland; whereas Ward and Kach (2009) examined the effect of nanoparticle aggregation on the ingestion of nanosized polystyrene.

1.4 Outline

The review (**Chapter 2**) is structured to highlight the fate of nanoparticles in the aquatic system and their interactions with the invertebrates in this environmental compartment, with a focus on the test organism and type of nanoparticles chosen for the experimental part. **Section 2.1** briefly brings in some aspects of the blue mussel's anatomy and physiology, which is important to keep in mind in order to predict and understand the interactions between nanoparticles and living organisms. **Section 2.2** gives a short introduction to the testing material nanostructured TiO_2. **Section 2.3** presents an overview on the fate of nanoparticles in the aquatic environment, with a focus on the tendency of nanoparticles to aggregate after entering the aquatic systems. This behaviour is of huge importance both in relation to their interaction with biota as well as their behaviour during laboratory experimentation, and has therefore gained a lot of attention in this thesis. **Section 2.4** discusses the interaction between nanoparticles and aquatic invertebrates, in which the potential mechanisms of uptake by the blue mussel is of focus. **Section 2.5** is the last part of the review, and focuses on the published literature regarding the organism's response to nanoparticles. This section includes the lethal responses to nano-TiO_2, as well as biochemical, physiological, and indirect effects of nanoparticles.

Chapter 3 describes the experimental procedures followed for the examination of nano-TiO_2 behaviour in water, characterisation of nanoparticles in transmission electron microscopy, turbidity testing of nano-TiO_2 seawater solutions and the generation of a standard curve to determine nanoparticle concentration in seawater. Two exposure studies have been conducted followed by specific analysis to evaluate the mussels' response to the presence of nano-TiO_2 in different concentration levels. The results from the experimental part of this thesis are presented in **chapter 4. Chapter 5** discusses the obtained results and describes the hypothetical and the planned continuation studies, and is followed by a short conclusion in **Chapter 6.**

2. Background

2.1 The blue mussel

The phylum *Mollusca* is one the largest and most diverse group of animals, comprising more than 50 000 described species. Molluscs are soft-bodied organisms enclosed by a hard protective shell, and within the shell a fold of tissue called the mantle covers the internal organs of the animals. Mussels belong to one of the major classes of molluscs, namely *Bivalvia*, that includes animals with two shell valves. In *M. edulis*, the two shell valves are similar in size and have a roughly triangular shape, hinged together at the anterior of the shell. The shell acts as a skeleton for the attachment of muscles and protect against predators. Two muscles, the anterior and the posterior adductors, control the opening and the closing of the shell valves. The mantle, which consists of connective tissue with haemolymph vessels, nerves and muscles, encloses the animal within the shell. The mantle also contains most of the gonad and is the main site for storage of nutrient reserves. Cilia on the inner surface of the mantle direct particles onto the gills and draw away heavier material towards the inhalant opening where it can be discharged (Gosling, 2003, Bayne et al., 1976a). A schematic drawing of the blue mussels anatomy is shown in Figure 7 and Figure 7.

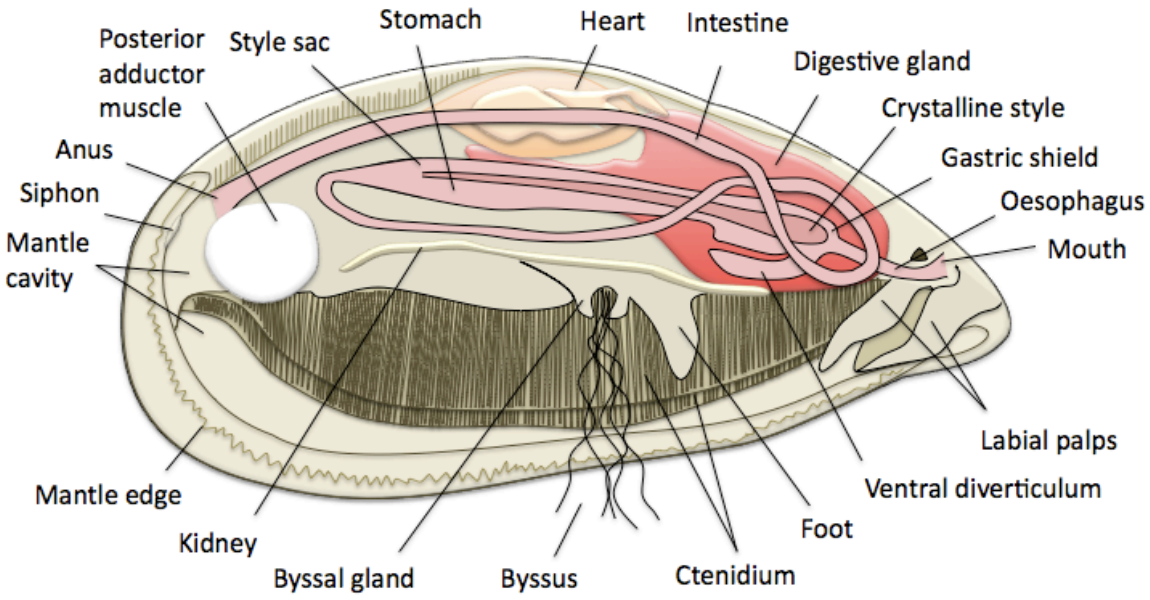


Figure 6: The anatomy of the blue mussel *Mytilus edulis* showing the organs seen from the right side. Figure is redrawn from SOP: "Collection and preparation of histology samples" (IRIS Biomiljø, 2009a).

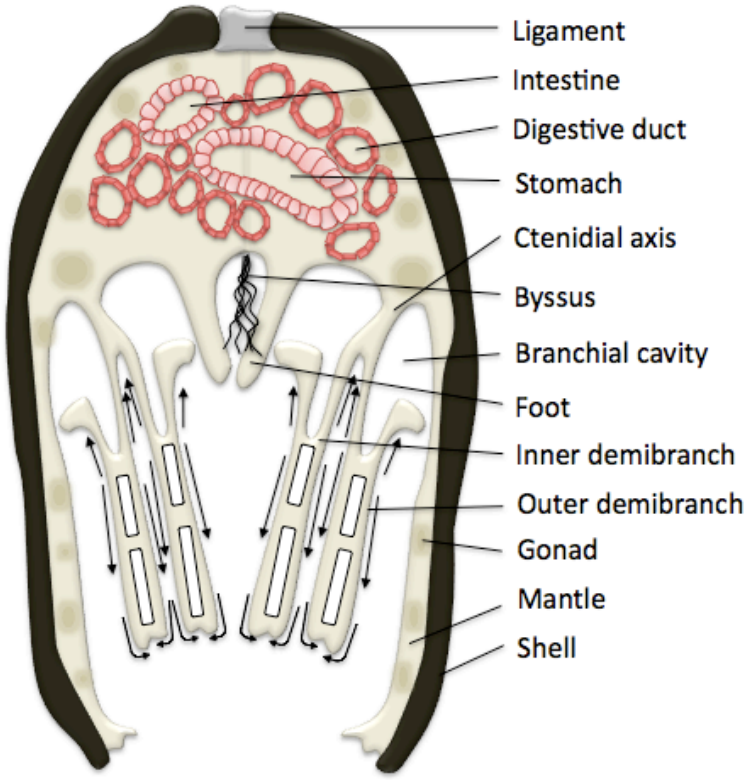


Figure 7: Transverse section of the mussel showing the form of the gills. The arrows present the direction of the main ciliary currents. Figure is redrawn from Bayne et al (1976a)

2.1.1 Feeding and digestion

The blue mussel, *M. edulis*, uses mucociliary mechanisms on the gills and labial palps to filter and ingest particles suspended in the ambient water, such as bacteria, phytoplankton, detritus, microzooplankton and dissolved organic matter (Widdows and Donkin, 1992, Gosling, 2003). This method of nutrient acquisition is known as filter feeding. Water flows into the mantle cavity through the inhalant siphon, is transported through the gill filaments where particles are captured, and exits through the exhalant siphon. The amount of particles captured depends on the volume of water transported across the gills (pumping rate) and the efficiency with which the particles are retained on the gills (Bayne et al., 1976a). After capture, the particles are transported towards the ventral ciliated particle grooves on the gill filaments, incorporated into mucus strings and further transported along ciliated grooves to the labial palps for particle sorting (Beninger and StJean, 1997). The particles are either directed towards the mouth for ingestion or rejected as pseudofaeces (Beninger et al., 1992, Foster-Smith, 1978). Ingested material is subjected to extracellular digestion throughout the gut, and selected particles are transported from the stomach to the tubules of the digestive gland for a more

comprehensive intracellular digestion (Bayne et al., 1976a). Subsequently, the material is directed to the intestine where absorption can occur throughout its length before elimination as faeces (Reid, 1968). The intestine terminates in an anus, and faecal pellets are swept away through the exhalant opening of the mantle.

2.1.2 Circulation

The blood, or haemolymph, flows from the gills into the heart, which contracts to drive the fluid into the anterior aorta, which divides into many arteries. Among these are the pallial arteries supplying the mantle and the pallial muscles, and the gastro-intestinal arteries that supply the stomach, intestine, shell muscles and foot with haemolymph. The arteries break up into a network of vessels in all tissues (Gosling, 2003). The venous system collects haemolymph from three main sinuses from which the haemolymph is carried to the kidneys for purification, passes through the gills and finally back to the heart (Bayne et al., 1976b).

The haemolymph contains cells known as haemocytes that float within colourless plasma. Three morphological types of haemocytes have been described in mussels by Moore and Lowe (1977), namely granulocytes, lymphocytes and macrophages. The granulocytes are recognized by a large number of spherical granules within the cytoplasm, the lymphocytes are small spherical cells with a large nucleus and little cytoplasm, whereas the phagocytic macrophages are characterized by an irregular appearance and a large vacuolated cytoplasm. These cells are not restricted to the haemolymph system but move freely out of the sinuses into the mantle cavity, the gut lumen and other surrounding tissues (Bayne et al., 1976b). The haemocytes play a number of important roles in the mussel physiology, including gas exchange, digestion, nutrient transport, elimination of wastes, regeneration and repair, and internal defence (Bayne et al., 1976b, Gosling, 2003).

2.1.3 Defence mechanism

The haemocytes are important in the internal defence system in mussels. As a first response against foreign substances, there is a measurable increase in the number of circulating haemocytes, which then recognise and react to the substances by phagocytosis or encapsulation (Moore and Lowe, 1977). The haemocytes adhere to the foreign material before it is taken into the cell by endocytosis, and is enclosed within a primary phagosome that subsequently fuses with lysosomes to form phagolysosomes (Gosling, 2003). The

phagocytised material is destroyed by haemocytes either through the release of lysosomale enzymes into the haemolymph (Pipe, 1990), or by the release of ROS, such as superoxide, hydrogen peroxide, and oxygen and hydrogen oxides (Pipe, 1992).

2.2 Titanium dioxide nanoparticles

Bulk materials based on TiO_2 have been massively produced and widely used as a white pigment for many years. More recently, nanosized versions of this metal oxide has been manufactured and introduced in a number of commercial products. Today, nano-TiO_2 is used in a wide range of products designed for direct human use and consumption, such as in cosmetics, sunscreens, toothpaste, and as a food additive (Nowack and Bucheli, 2007, Kaida et al., 2004). Other applications are their use in environmental remediation to decontaminate soil and water (Long et al., 2006, Zhu et al., 2005), and in the pharmaceutical industry as drug delivery vehicles (Contado and Pagnoni, 2008).

Photocatalysis using nano-TiO_2 has recently become very important. This nanostructured material becomes photoactivated under near UV light and reactive oxygen species (ROS) are formed on the surface of the crystal (Fadeel and Garcia-Bennett, 2010). This photocatalytic property of nano-TiO_2 have been successfully used in environmental technology for the treatment of wastewater, the removal of organic compounds (benzothiophene) from diesel fuel and the degradation of air pollutants (Ju-Nam and Lead, 2008).

Titanium dioxide exists in three main crystallographic structures, namely anatase, rutile and brookite. Each of these forms presents different properties and therefore different applications (Fadeel and Garcia-Bennett, 2010). The toxic impact of nano-TiO_2 also appears to depend on their phase composition. For instance, Sayes et al (2006) demonstrated that anatase TiO_2 was 100 times more toxic than an equivalent sample of rutile TiO_2. The reason for these differences was suggested to be due to the fact that anatase TiO_2 generates ROS more effectively (Mo and Ching, 1995). Thus, the ability of nano-TiO_2 to transfer energy to nearby oxygen molecules, that is of huge advantage in environmental remediation, may adversely and unintentionally affect living organisms (Ju-Nam and Lead, 2008).

2.3 Fate of nanoparticles in the aquatic environment

The fate of the nanoparticles within the aquatic environment is of huge importance both in relation to their interaction with biota as well as their behaviour during laboratory experimentation. The pattern of distribution of nanoparticles in the aquatic environment will depend on their transport, partitioning, transformation and degradation after entering the aqueous environmental compartment.

2.3.1 Nanoparticles entering the aquatic system

As more products containing nanomaterials are developed, there is a greater potential for environmental exposure. Nanomaterials released in the environment are likely to be transported to the aquatic systems by wind and rainwater runoff, where it can accumulate and pose a threat towards aquatic organisms (Colvin, 2003, Moore, 2006). Nanoparticles can be released as a consequence of human activity in the following ways

- Disposal of wastes
- Unintended release
- Deliberate application in remediation

The aquatic systems can receive nanomaterials from various sources, including atmospheric deposition, leaching from soil and through direct inputs, such as wastewater discharges (Scown et al., 2010). Some nanomaterials are produced in large amount and are therefore likely to enter the environment from manufacturing effluents or from spillage during manufacture and processing (Oberdörster et al., 2005, Ray et al., 2009). Nanomaterials are incorporated in an increasing number of commercial products including computer boards, automobile tires, and clothing, and might be unintentionally released during use and disposal. For instance, washing off of consumer products containing nanoscale materials, such as cosmetics and sunscreens, allows the nanomaterials to enter the environment on a continual basis (Oberdörster et al., 2005), whereas environmental remediation is an application that will deliberately inject nanomaterials to soils and groundwater in significant levels (Tratnyek and Johnson, 2006).

Methods for detecting and characterizing nanoparticles in the aquatic environment are still in their early stages and the environmental concentrations of most nanoparticles remain

unknown (Battin et al., 2009). Estimates for levels of nano-TiO_2 in the aquatic environment based on modeling approaches reveal significant levels of these nanosized particles in natural waters. Mueller and Nowack (2008) estimated the PEC value of nano-TiO_2 in surface waters in Switzerland, and suggested a concentration level up to 16 $\mu g\ L^{-1}$ for high emission scenarios.

2.3.2 Persistence

Little is known about the relative stability of nanomaterials in the environment (EPA, 2007, Kadar et al., 2010). Persistence will depend on whether the material decomposes and on whether the particles are modified in the environment (The Royal Society, 2004). Some nanomaterials can be affected by environmental factors such as light, oxidants or microorganisms, leading to their degradation. Biological and abiotic degradation can result in breakdown of the nanoparticles, or give byproducts with altered physical structure or surface characteristics. Other nanomaterials can be persistent in the aquatic environment; they are non-degradable, or the degradation occurs very slowly, and will therefore have a longer residence time in nature (Boxall et al., 2007). Persistence of nanoparticle-derived contaminants can lead to accumulation the environment, magnification in the food chain and long-term effects on the ecosystems.

2.3.3 Behaviour in the water – Aggregation

Waterborne nanoparticles generally settle more slowly than larger particles of the same material (EPA, 2007), which makes them capable of movement over considerable distances as a consequence of the mass movements of water. However, the distance travelled can be greatly reduced due to the phenomenon of aggregation. Data on the behavior of manufactured nanoparticles in aquatic systems is limited. However, available information, together with the extensive literature on natural colloids in aquatic systems can provide a useful basis for prediction of nanoparticle fate (Jarvie and King, 2010, Ju-Nam and Lead, 2008, Klaine et al., 2008). Their fate is likely to be governed by aggregation, in which the nanoparticles attach to each other to form larger clusters (Figure 8), and by interactions with natural substances in the system (Batley and McLaughlin, 2010, Lead and Wilkinson, 2006, Moore, 2006).

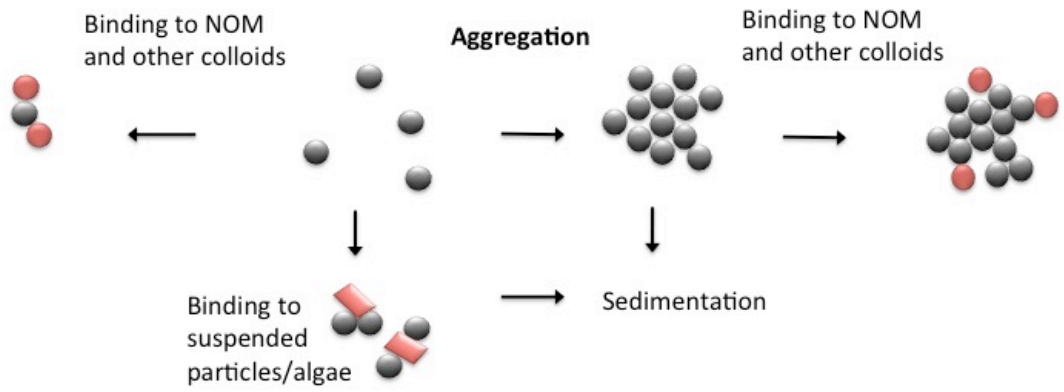


Figure 8: After dispersing nanoparticles in solution, they can remain as dispersed or form larger clusters followed by possible interactions with natural organic material (NOM) within the system. Based on figure by Batley and McLaughlin (2010)

Natural aquatic colloid is defined as material with at least one dimension smaller than 1 µm (Lead and Wilkinson, 2006) and, clearly, this material also contains natural occurring nanoparticles, defined as material smaller than 100 nm. There is a variety of aquatic colloids, including viruses and bacteria, natural organic matter (NOM), such as humic acids, proteins, and polysaccharide exudates from microbes, and inorganic matter such as oxides of manganese, iron, aluminium and silicon (Klaine et al., 2008). Colloidal fate and behaviour is dominated by aggregation; they interact among themselves and with other larger substances in the environment to form aggregates that can be lost through sedimentation (Gustafsson and Gschwend, 1997, Lead and Wilkinson, 2006).

When entering the aquatic systems, engineered nanoparticles will become components of these colloids (Scown et al., 2010) and the colloidal behaviour of the NPs – whether they remain dispersed within the water phase, or interact with each other or with natural colloidal matter, and subsequently sediment out – has a great impact on their environmental pathways, their interactions with biota, and their ecotoxicity (Jiang et al., 2009, Jarvie and King, 2010, Lead and Wilkinson, 2006).

Stability of nanoparticles and the DLVO theory

Several researchers have investigated the stability of nanoparticles when dispersed in water. A stable dispersion of nanoparticles in an aquatic medium is called a colloidal system, which is defined as a system containing particles of colloidal size (1 nm – 1 μm) in one phase (e.g. solid) distributed in a phase of a different state (e.g. liquid) (IUPAC, 1997). The term "stable" in this context describes a liquid where the particles may collide, but do not stick together to form aggregates (Handy et al., 2008b). Several factors influence the rate of aggregation, such as size and surface properties of the particles themselves, their interactions with each other, and factors relating to the surrounding media.

The DLVO theory, named after Derjaguin and Landau, Verwey and Overbeek, describes the forces between charged surfaces interacting through a liquid medium, and can be used to describe the stability of the nanoparticles in the aquatic environment (Chen and Elimelech, 2007, Jiang et al., 2009, Liu et al., 2009, Handy et al., 2008b). Colloids carry an electrical charge and the stability of colloidal suspensions is determined by the by the sum of attractive and repulsive forces that exist between the individual particles. These forces are governed by surface charges of the colloidal material (Scown et al., 2010). The attraction between particles is due to van der Waals forces. The repulsion between particles is caused by a double layer of charge that surrounds the particles in a liquid, the so-called electrostatic diffuse double layer (Jiang et al., 2009). Two important properties of the electrical double layer are the zeta potential and the thickness of this double layer (Morrison and Ross, 2002). The zeta potential is the potential difference between the bulk and the slipping plane situated some distance from the particle surface (Figure 9).

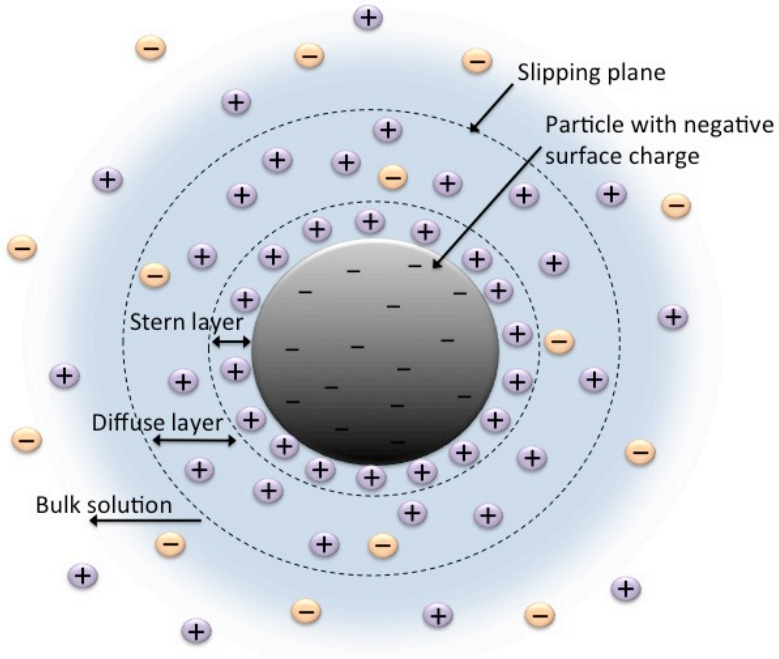


Figure 9: Stern-Gouy-Chapman model of the electrical diffuse double layer. Increasing the repulsive force, by increasing the surface charge or the thickness of the electrical double layer, results in electrostatic stabilization. Redrawn from Technical Note by Malvern Instruments (2006).

According to the DLVO theory, two particles are prevented from approaching one another and adhering together due to the repulsive forces (the electrostatic double layer) between two particles. So, if the surface charge (measured as zeta potential) is high enough, the colloids will remain discrete, and are stabilized in suspension (Scown et al., 2010). However, reducing or eliminating the charge allows the nanoparticles to come into contact, adhere strongly together and form aggregates (Derjaguin and Landau, 1941, Verwey and Overbeek, 1948).

Ionic strength, pH and electrostatic stabilization

As the stability of nanoparticles is controlled by interactions between the nanoparticles, factors influencing these inter-particulate forces will obviously also affect their behaviour. When increasing the ionic strength (by adding a salt) of the aqueous medium holding the nanoparticles, opposite charges will attract each other. Some of these salt ions will accumulate in the electrostatic double layer, and thereby reduce the zeta potential (surface charge is not necessarily altered) (Gustafsson and Gschwend, 1997, Handy et al., 2008b). This leads to a reduction in the thickness of the electrostatic double layer, allowing two particles to come more closely and to be affected by the attractive forces that exist between the particles. Their collisions may now lead to an attachment and subsequent aggregation. Jiang et al (2009) demonstrated this effect of ionic strength on the aggregation behaviour of

nano-TiO_2. At ionic strengths up to 0.005 M NaCl, the electrostatic repulsive forces were greater than the attractive forces trying to bind the nanoparticles together, characterised by a stable nano-TiO_2 dispersion. Increasing the NaCl concentration to 0.1 M, however, resulted in reduced zeta potential (compression of the electrical double layer) and a highly aggregated state of the nanoparticles. Similar results were found by Domingos et al (2009), also showing an increased aggregation and deposition of nano-TiO_2 with increasing ionic strength. Moreover, the carbon-based fullerene C_{60} was found by Brant et al (2005) to stay stable over a two-month period in the absence of electrolyte, however, already at an ionic strength of 0.001 M the fullerenes showed a strong tendency to aggregate (Brant et al., 2005).

The pH of the medium also affects the stability of nanoparticles in water. The surface charge of metal oxides dispersed in water, including TiO_2, is controlled by surface ionization (Morrison and Ross, 2002). They have a positive charge at low pH and a negative charge at high pH values. The pH value, at which a particle has no net charge, is called the isoelectric point, and for nano-TiO_2 this point of zero charge has been found to be around pH 6 (Jiang et al., 2009, Kosmulski, 2002). Thus, when pH is far from the isoelectric point, the absolute value of zeta potential increases, resulting in increased electrostatic repulsion. When aggregation is suppressed in this way, it is called electrostatic stabilisation. On the contrary, when the pH approaches the isoelectric point, the repulsive force is weakened due to the reduced zeta potential, and aggregating takes place. The tendency to form aggregates at pH values near zero point of charge has also been reported for several nanoparticles, including nano-TiO_2 (Domingos et al., 2009, Jiang et al., 2009), fullerene C_{60} (Brant et al., 2007) and nanosized iron oxide (Baalousha et al., 2008).

Natural organic matter and steric stabilization

The presence NOM can also affect the aggregation behaviour of nanoparticles. Binding of NOM to the surface of nanoparticles has shown to increase the stabilization of nanoparticles in water, including nano-TiO_2 (Domingos et al., 2009, Yang et al., 2009). The increased stability is likely to be caused by steric stabilization mechanisms, in which the NOM adsorbed onto the nanoparticles physically prevent two particles from approaching each other (Chen and Elimelech, 2007) as visualized in Figure 10. However, Yang et al (2009) presumed that the reduced aggregation was caused by electrostatic stabilisation, as adsorption of humic acids were found to increase the surface charge (measured as zeta potential) of the nanoparticles.

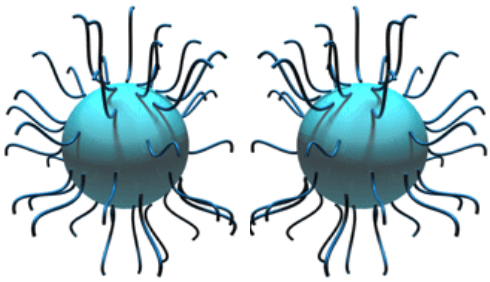


Figure 10: Binding of natural organic matter (NOM) can lead to steric stabilization in which the particles are physically prevented from approaching one another. Based on Christian et al (2008).

The affect of NOM on the aggregation behaviour is complex, and appears to depend on the concentration of NOM as well as the presence of salts. Chen and Elimelech (2007) demonstrated that the addition of HA increased the stability of fullerene C_{60} in the presence of NaCl and $MgCl_2$, and at low concentrations of $CaCl_2$. However, enhanced aggregation was observed at higher concentrations of $CaCl_2$, which was presumed by the authors to be caused by bridging mechanisms between Ca^{2+}, humic acids and fullerene C_{60} (Chen and Elimelech, 2007).

2.4 Interactions between nanoparticles and aquatic invertebrates

If present in the water in a bioavailable form, the xenobiotics, or foreign substances, can interact with organisms in several ways, potentially leading to toxic responses. After entering an organism, the xenobiotic may reach four different "locations" within the organism as shown in the simplified model in Figure 11. This section focuses on the toxicokinetic part of Figure 11 that deals with the fate of nanoparticles within the living organism. Many questions regarding the fate of nanoparticles within biota remain to be elucidated, and we are far from reaching any general consensus view on absorption, distribution, metabolism, or excretion of nanoparticles in marine invertebrates (Klaine et al., 2008, Handy et al., 2008b).

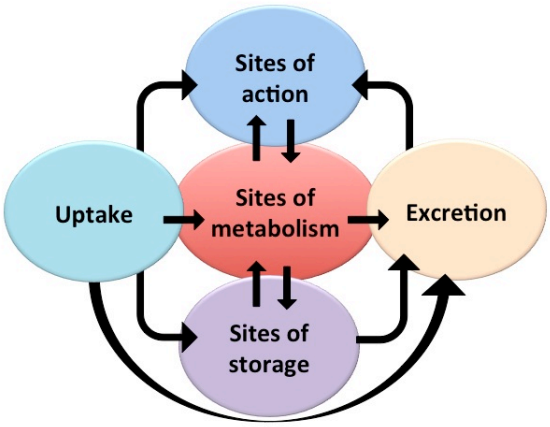


Figure 11: General model showing the fate of xenobiotics in living organism. Modified from Walker et al (2006).

2.4.1 Uptake across the respiratory epithelium

The movement of many dissolved aquatic pollutants into aquatic animals occurs as a consequence of passive diffusion across epithelial boundaries of the gills and body wall (Walker et al., 2006). This type of passive transport is considered an important route of entry of nanoparticles as well (Moore, 2006). Due to the filter-feeding nature of mussels, large quantities of water are continuously filtered through their gills in search for food. The gills, or ctenidia, of the blue mussel are made up by a mesh of filaments with the outer surface exposed to the water and the inside well supplied with haemolymph and circulating blood cells. The construction of the gills gives them a large surface area, through which diffusion of nanoparticles can occur. The small size of single nanoparticles facilitate this form of transport across biological barriers (Alberts, 2004) and passive diffusion across the epithelium of the mussels gills is therefore likely to take place. This pathway of uptake was demonstrated by

Koehler et al (2008) in which nanoparticles of silicon dioxide smaller than 5 nm diffused into the gill tissue of *M. edulis*.

2.4.2 Ingestion and subsequent cellular uptake

Despite the observations made by Koehler et al (2008), several authors suggest that the major route of entry into the aquatic invertebrate is via capture and ingestion (Roberts et al., 2007, Ward and Kach, 2009, Gagne et al., 2008, Baun et al., 2008b). In *M. edulis*, the process of ingestion and subsequent cellular internalisation involves several steps of critical particle selection that the nanoparticles have to overcome in order to enter the marine invertebrate. The potential fate of nanoparticles within the blue mussel is represented Figure 12.

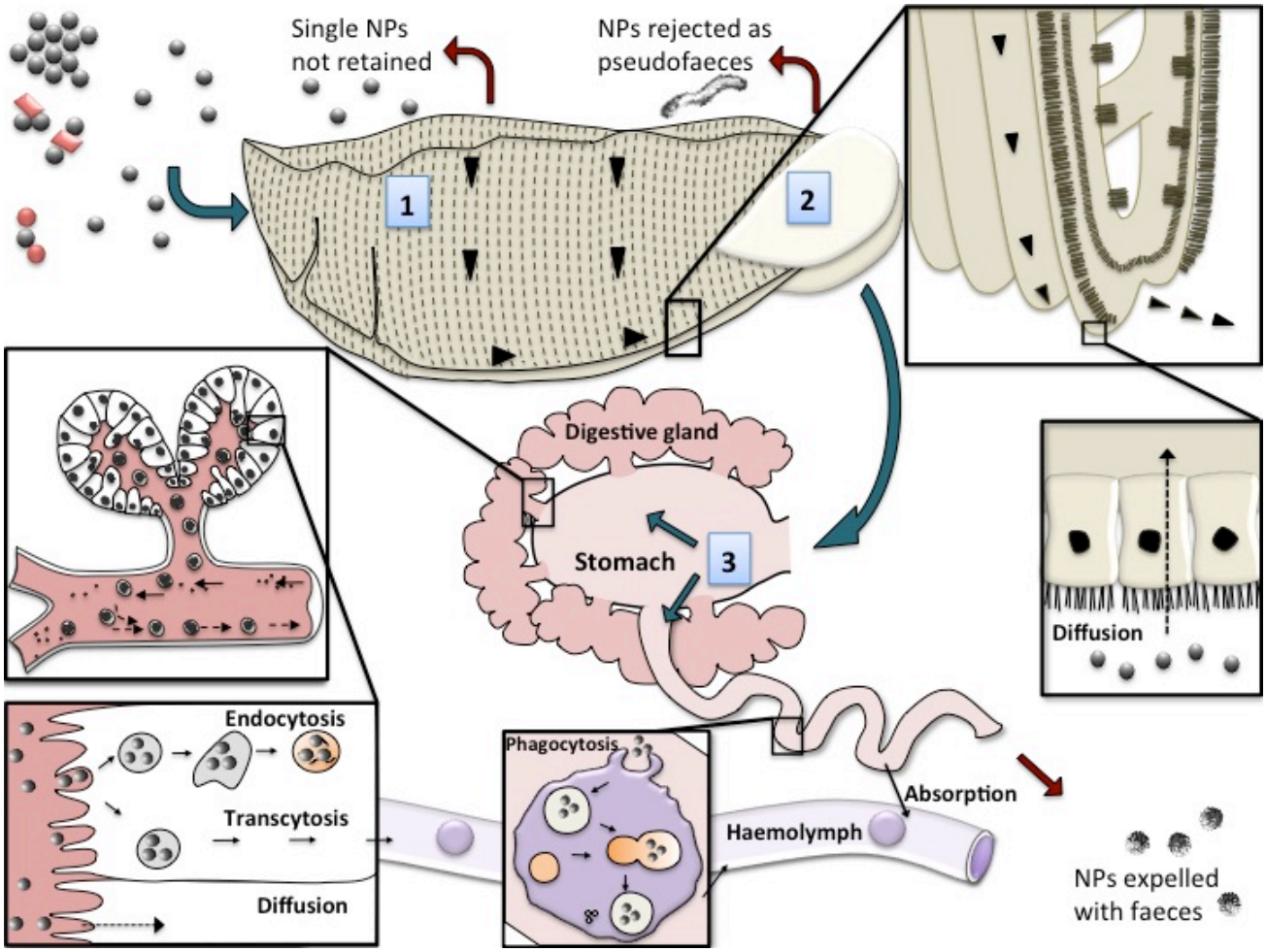


Figure 12: Schematic drawing showing the potential fate of nanoparticles within the blue mussel. Nanoparticles (NPs) enter the bivalve through an opening in the mantle tissue, as single NPs, NP aggregates, or bound to other particles. 1: Particles are removed from the incoming water; single NPs probably too small to be retained. 2: Selection by the labial palps resulting in ingestion of NPs or rejection as pseudofaeces. 3: Selection of particles in the stomach. Transport of NPs to the digestive gland for intracellular digestion can occur, followed by a potential distribution within the body. Non-digested NPs are expelled with faeces but may be engulfed (phagocytosis) by circulating haemocytes within the intestine.

The gills and preferential particle capture

The efficiency at which the mussel removes particles from the water current, known as the retention efficiency (Bayne et al., 1976a), depends upon the diameter of the particles (Langdon and Newell, 1990, Riisgård, 1988). *M. edulis* is able to retain particles of 1 μm in diameter with an efficiency of 50% (Jørgensen, 1990) and this efficiency improves with increasing particle size, reaching 100% for particles larger than 3-4 μm (Shumway et al., 1985, Riisgård, 1988). In contrast to uptake via passive diffusion, the process of aggregation may therefore promote uptake of nanoparticles via ingestion. In a study by Canesi et al (2010), fullerene C_{60} with a primary size of 0.7 nm, formed aggregates in the size range from 35 nm to 4000 nm after one hour in seawater, whereas 22 nm sized nano-TiO_2 formed masses of 150 nm and 1600 nm. Due to the growth in size, aggregated nanoparticles are therefore likely to be captured onto the gills with an efficiency of more than 50%.

The labial palps and pre-ingestive selection

After capture, all particles are transported along the ventral particle groove toward the labial palps (Beninger et al., 1995). According to Ward and Kach (2009), some aggregated nanoparticles may be broken down by the action of cilia on the gills and labial palps after capture, potentially leading to ingestion of smaller aggregates as well as single nanoparticles. When the particle abundance is low, all particles are further transported towards the mouth for ingestion (Beninger et al., 1995). However, as the particle availability increases above a concentration at which the digestive capacity of the mussel is saturated, the ration of ingested particles remains constant and surplus material is rejected as pseudofaeces (Foster-Smith, 1978, Beninger et al., 1992). It is generally believed that a pre-ingestive particle selection takes place in this process of rejection on the labial palps, and that the bivalves are capable of selecting nutritive particles, such as algae, while rejecting particles of poor nutritive value in their pseudofaeces (Hawkins et al., 1996, Hawkins et al., 1998, Kiørboe and Møhlenberg, 1981, Kiørboe et al., 1980, Prins et al., 1991). Rejection of nanoparticles at this point is therefore a possibility.

The stomach and post-ingestive selection

From the mouth, the now mucus-bound particles are transported through the narrow oesophagus into the stomach by the movement of cilia. Such movement mechanism is also found throughout the alimentary tract. The stomach is an oval-shaped flattened sac and lies totally embedded in the digestive gland that opens into the stomach by several ducts (Gosling,

2003). The crystalline style is rotating against a thickened part of the stomach wall, known as the gastric shield, which releases enzymes and breaks apart large particles and particle aggregates (Bayne et al., 1976a). Due to the dissolution of the style in this process, as well as acid secretion from the digestive gland, the pH of the stomach contents can fall to pH 5.5 (Owen, 1974). Several researchers have demonstrated the ability of aquatic organisms to ingest nanoparticles, including nano-TiO_2 (Galloway et al., 2010, Ramsden et al., 2009, Canesi et al., 2010, Zhu et al., 2010a). Within the stomach, ingested nanoparticles meet an environment that is totally different from that of the seawater, which might alter their subsequent behaviour and fate, potentially resulting in smaller aggregates or even single nanoparticles. While all the mixing and extracellular digestion take place, the stomach contents come under the influence of complex ciliated tracts and folds that cover certain parts of the stomach and act as sorting areas (Bayne et al., 1976a, Brillant and MacDonald, 2000). In this post-ingestive selection, some particles are channelled into the intestine for elimination without being absorbed into the cell tissue, as has been reported for nano-TiO_2 and CNTs in sediment dwelling organisms (Galloway et al., 2010, Petersen et al., 2008). In theory, however, the rejected particles are not necessarily lost. Haemocytes can migrate into the gut to ingest particles of nutritional value that are too large to enter the digestive gland (Reid, 1968). Thus, the haemocytes also play an important role in intracellular digestion and transport of food particles, and potentially take up the nanoparticles rejected as waste material from the stomach.

Digestive gland and intracellular digestion

Translocation of nanoparticles from the stomach into the digestive gland, the major site of intracellular digestion (Bayne et al., 1976a), have been reported (Ward and Kach, 2009, Koehler et al., 2008, Ringwood et al., 2009). The primary ducts of the digestive gland branch into smaller secondary ducts that end in digestive tubules. Using *M. edulis* as test organism, Ward and Kach (2009), observed a longer gut retention time for polystyrene nanoparticles (100 nm) in comparison of 10-µm polystyrene particles, and the authors insinuated that this prolonged gut retention time was due to transport of nanoparticles into the digestive gland. At the cellular level, Koehler et al (2008) reported passive diffusion of SiO_2 nanoparticles smaller than 5 nm into the digestive cells of *M. edulis*, whereas nanoparticles in the size range of 5-25 nm were taken up by the endocytic pathway. Uptake by endocytosis, where the cell membrane encloses the nanoparticles, results in deposition within enclosed vesicles in the cytoplasm, and subsequently fusion with lysosomes for degradation or interactions with other

intracellular organelles. Koehler and colleagues observed that nanoparticles smaller than 10 nm were able to diffuse into mitochondria and those smaller than 7 nm even found their way into the nucleus of the digestive cells (Koehler et al., 2008). Using oyster as study organism, Ringwood et al (2009) also demonstrated active uptake of nanoparticles and observed accumulation of fullerene C_{60} aggregates within the lysosomes of digestive cells, indicating intracellular digestion.

2.4.3 Bioaccumulation

Only a few studies have investigated the potential bioaccumulation of nanoparticles that can have great impact on the ecosystems (Franke et al., 1994). Bioaccumulation, or bioconcentration, refers to the accumulation of substances in the organism and occurs when the rate of uptake exceeds the rate of elimination (excretion and metabolism). Zhu et al (2010a) investigated the uptake and depuration of nano-TiO_2 in the crustacean *D. magna*, and found significant amount of aggregated nanoparticles accumulated within the gut of the organisms. The size of the aggregates ranged from a few hundred nanometres to several microns, and the authors implied that the large size was inhibiting the nanoparticles from being excreted (Zhu et al., 2010a). Furthermore, in a study by Rosenkranz et al (2009), the elimination of nanosized (20 nm) polystyrene by *D. magna* was slow in comparison to micrometre sized (1 µm) particles of the same material. Both particle sizes were found to accumulate within the organism's gut. After 4 h, a decrease of more than 90% was observed for the 1 µm sized particles, whereas a 40% decrease was found for the nanoscale particles. Furthermore, in a study on CNTs conducted by Petersen et al (2009), *D. magna* was unable to excrete the ingested nanotubes during a depuration period of 24 h, however, an addition of algae resulted in the release of a significant fraction of the accumulated CNTs. In a study by Petersen et al (2008) the bioaccumulation of CNTs was compared to that of polycyclic aromatic hydrocarbons (PAHs) using a sediment-burrowing oligochaete *Lumbriculus variegatus* as test organism. These PAHs are environmental contaminants of similar fuse-ring composition known to accumulate in fat-rich tissue. The bioaccumulation factors for CNTs were much lower than those for the PAHs, and it was concluded that CNTs, unlike PAHs, do not readily absorb into the tissue and are rapidly excreted (Petersen et al., 2008).

2.4.4 Transfer along food chain

Accumulation of nanoparticles in aquatic invertebrates calls for concern on the potential food chain transfer of nanoparticles and the associated ecological consequences (Petersen et al., 2008, Handy et al., 2008b). Zhu et al (2010b) investigated the trophic transfer and biomagnification of nano-TiO_2 in a simplified food chain showing that nano-TiO_2 can be transferred from *D. magna* to its predator, *Danio rerio* (zebrafish). The concentration of nano-TiO_2 did not increase from the one level in the food chain to the next (biomagnification factor <1), and, hence, no biomagnification was observed. According to Zhu and colleagues, the lack of magnification during food chain transfer might be due to the non-lipophilic nature of nano-TiO_2. For aquatic organisms, there is a close relationship between the hydrophobicity of the compound, often indicated by the octanol-water partition coefficient (K_{ow}), and its propensity to in the build up within aquatic organism (Walker et al., 2006). Lipophilic compounds tend to seek lipophilic environments (biota) rather than the surrounding water and can be retained in tissue for a long time (Walker et al., 2006), allowing biomagnification to take place. Highly lipophilic nanoparticles, such as fullerene C_{60} with a K_{ow} value of 6.67 (Jafvert and Kulkarni, 2008), have been found to localize into fat-rich compartments *in vitro* (Oberdörster, 2004), and may therefore show a greater tendency to biomagnify than nano-TiO_2 and other hydrophilic nanoparticles.

2.5 Biological effects

The ecosystem responses to a pollutant can be observed at several levels of biological organization as visualized in Figure 13. This "molecules-to-ecosystem" approach allows linkage to be made between different levels of organization, from molecules to biochemistry to physiology to populations (Walker et al., 2006). This means that any effect seen at the higher levels of organization, such as reduction in a population or changes in ecosystem function, can be linked to responses occurring at the lower levels of organisation.

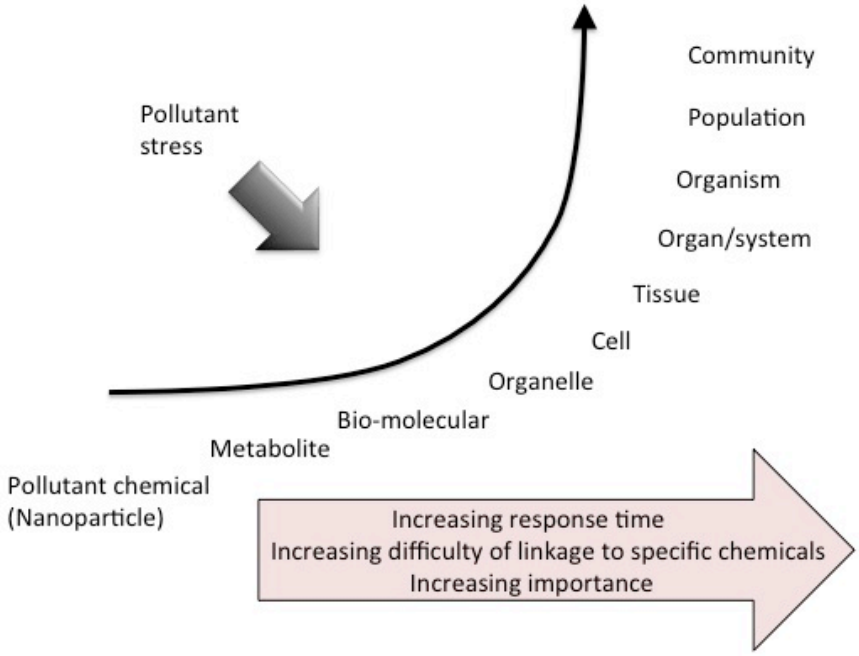


Figure 13: Nanoparticles may trigger a sequential order of responses through the hierarchical levels of biological organization. Modified from (Bayne et al., 1985).

This is the underlying basis for the biomarker strategy. Biomarkers are broadly defined as a change in a biological response at the individual level or below that can be related to exposure to or toxic effects of environmental chemicals (Peakall, 1994). Thus, biochemical, physiological, morphological, and behavioural responses can be regarded as biomarkers, providing "early warning signals" before the effects develop and become evident at higher levels of organization. Responses at the higher levels, such as changes in individual number or changes in the ecosystem function, are too general to be considered as biomarkers and are often referred to as bioindicators.

2.5.1 Lethal responses of nano-TiO_2

Lovern and Klaper (2006) reported a relatively high acute toxicity of nano-TiO_2 (30 nm) towards *D. magna* (LC_{50}=5.5 mg L^{-1}) when pre-treating the nanoparticles with the organic solvent tetrahydrofuran, whereas nano-TiO_2 (100-500 nm) prepared by sonication at the highest concentration of 500 mg L^{-1} led to only 9% mortality. This discrepancy might be due to the size of the particles, in which the small nano-TiO_2 achieved by filtration in THF, promotes their entry into the organisms and allows distribution within the body. However, it has been suggested that the THF is inherently toxic and is responsible for the low lethal concentration level (Johnston et al., 2010, Klaine et al., 2008). Heinlaan et al (2008) compared the effects of nanoscale TiO_2 with non-nanoscale TiO_2 using crustaceans and bacteria, and found no toxic effects even at a concentration of 20 g L^{-1}. Moreover, Zhu et al (2010a) and Wiench et al (2009) reported a low acute toxicity (48 h) of TiO_2 on *D. magna*, with LC_{50} values greater than 100 mg L^{-1}. According to these studies, nano-TiO_2 appears to exert low acute toxicity to the aquatic wildlife. However, by extending the exposure time to 72 h and to 21 days, Zhu et al (2010a) reported a drastic decrease in the LC_{50} value to 2.02 and 2.62 mg L^{-1}, respectively. Studies on the chronic exposure to nanoparticles are very limited, but the obvious differences in lethal concentrations reported by Zhu and colleagues suggest that exposure duration should be considered as a potentially important factor in nanotoxicity.

2.5.2 Biochemical effects of nanoparticles

Biochemical biomarkers can provide an indication of the sub-lethal impacts of a stressor, the biochemical mechanism that can be affected by a stressor, and an early warning of population-level impacts (Klaper et al., 2009).

Protective responses

Xenobiotics can cause a variety of biochemical effects in an organism, both protective and non-protective. When the concentration of a xenobiotic exceeds a certain level in the cell, it can trigger responses designed to protect the organism against potential toxic effects. One protective response is the induction of enzymes to metabolize and control the levels of free pollutants. Nano-TiO_2 has been found to increase the activity of GST in marine mussel (*Mytilus galloprovincialis*) and in water flea (*D. magna*), at a concentration level of 1 mg L^{-1} and 500 mg L^{-1}, respectively (Canesi et al., 2010, Klaper et al., 2009). GST is an enzyme in

the second stage (phase II) of biotransformation (Walker et al., 2006), and is a commonly used biochemical biomarkers in toxicity studies providing an early warning of the response of an organism to pollutants (Klaper et al., 2009). Other nanoparticles, including fullerene C_{60} and nanosized carbon black, have also been found to induce GST in aquatic invertebrates (Canesi et al., 2010, Klaper et al., 2009).

Oxyradical production and oxidative responses

A diverse range of nanoparticles has shown to induce production of ROS, and is thought to be one of the primarily mechanisms of nanoparticle-induced toxicity (Nel et al., 2006, Handy and Shaw, 2007, Oberdörster et al., 2005). ROS are ions or small molecules, including oxygen ions, peroxides and inorganic and organic free radicals that are very reactive due to the presence of unpaired valence shell electrons. In aerobic cells, ROS are formed as natural by-products of cellular metabolism, and have important functions in host defence mechanisms and cell signalling (Han et al., 2001). The cells have antioxidant systems designed to mechanisms to keep the concentration of ROS at non-toxic levels and to protect them against damage by ROS. These defense systems include antioxidant enzymes, such as superoxide dismutases, catalases, and glutathione peroxidases, and low molecular weight non-enzymatic antioxidants such as glutathione, ascorbic acid (vitamin C), retinol (vitamin A), α-tocopherol (vitamin E), and carotenoids (Livingstone and Pipe, 1992).

Some nanoparticles, including TiO_2, are redox active and can generate ROS directly on their surfaces (Farré et al., 2009). The generated ROS can interact with components within the cell, an interaction that tends to trigger further radical formation (Klaine et al., 2008). Another potential way for nanoparticles to indirectly cause elevated ROS levels is through the activation of the immune system, in which reactive oxygen species are generated to destroy the invading nanoparticles (Livingstone and Pipe, 1992). If the cellular concentration of ROS becomes greater than the cells capacity for ROS neutralization, these reactive species can react with components within the cell, possibly leading to oxidative stress, inflammation, and damage to proteins, membranes, and DNA (Viarengo et al., 1991, Johnston et al., 2009).

Alterations in the production of antioxidant defenses indicate that the delicate balance between antioxidant defenses and production of ROS is being disturbed, and can potentially serve as biochemical biomarker of nanoparticle exposure (Klaper et al., 2009). Still, changes in these antioxidants are observed with exposure to a wide range of xenobiotics (Livingstone

and Pipe, 1992), and, hence, the specificity of these biomarkers is low. The activity of catalase was elevated in digestive gland, but not in gills, of *M. galloprovincialis* exposed to nano-TiO_2, nano-SiO_2, fullerene C_{60}, and carbon black nanoparticles in a study by Canesi et al (2010). Similarly, the activity of catalase increased in the digestive gland of *M. edulis* exposed to gold nanoparticles but remained unchanged in the gills (Tedesco et al., 2008), suggesting that the digestive gland might be more susceptible to exposure of these nanoparticles. Elevated activity of catalase was also reported in *D. magna* upon exposure to nano-TiO_2 and fullerene C_{60} (Klaper et al., 2009). In fish, Hao et al (2009) reported significant changes in the activity of the antioxidant enzymes catalase, superoxide dismutases and peroxidase in the liver, gill and brain tissues of juvenile carp after exposure to nano-TiO_2, indicating that the antioxidant defence systems of these tissues were being stressed. The greatest impact on enzyme activities were observed in the liver, indicating that the liver might be the most susceptible organ to nano-TiO_2 exposure (Hao et al., 2009). Furthermore, depletion of GSH was observed in the liver of fish exposed to nano-TiO_2 (Federici et al., 2007, Hao et al., 2009), suggesting that the liver was using up antioxidant defenses to prevent oxidative stress, whereas an increase in GSH was observed in the gills (Federici et al., 2007). On the contrary, Oberdörster (2004) reported depletion of GSH in the gills of fish (largemouth bass) exposed to fullerene C_{60}.

In addition to the changes in the antioxidant levels, increase in oxidative products may also provide as evidence of oxidative stress (Sayeed et al., 2003). ROS can cause damage to the cell membranes by oxidizing double bonds on fatty acid tails of membrane phospholipids (Klaine et al., 2008). This process, called lipid peroxidation (LPO), increases membrane permeability and fluidity, which makes the cells more susceptible to osmotic stress and potentially block nutrient uptake (Cabiscol et al., 2000). Nanoparticles of TiO_2 have been found to increase LPO in various tissues of fish. Hao et al (2009) reported increase in LPO in liver, gill, and brain tissues of juvenile carp exposed to nano-TiO_2. Here, the greatest elevation was found in the liver tissue, indicating that the liver might be the most susceptible organ to nano-TiO_2 exposure (Hao et al., 2009). Federici et al (2007) also observed increase in the LPO levels in various tissues of rainbow trout exposed to nano-TiO_2, but observed no increase in LPO in the fish liver. The observed depletion of total GSH that was found in the liver tissue implies that the liver was using up antioxidant defences to prevent oxidative stress, and, hence, zero increase in LPO in this organ. In freshwater mussel (*Elliption complanata*), LPO increased in the gills, but not the digestive gland, after exposure to cadmium-telluride quantum dots (Gagne et al., 2008). Also, Ringwood et al (2009) failed to

find increase in LPO in the digestive gland of oyster upon exposure to fullerene C_{60}. Furthermore, significant increase in lysosomal lipofuscin, which is the end product of lipid peroxidation (Moore, 1988), was observed in marine mussel (*M. galloprovincialis*) exposed to nano-TiO_2, carbon black nanoparticles, nano-SiO_2 and C_{60} (Canesi et al., 2010), indicating that the bivalves suffered from oxidative stress. Among the nanoparticles tested, nano-TiO_2 was the strongest inducer of this biomarker with a strong increase at 1 mg L^{-1} that did not increase further at higher concentrations.

Genotoxicity

DNA damage has been reported in aquatic invertebrates after exposure to nano-TiO_2 and quantum dots (Galloway et al., 2010, Gagne et al., 2008). However, the mechanism of this genotoxicity is unclear. Damage of the genetic material can be a result of direct binding of the nanoparticles to the DNA molecule, leading to adverse impact on stability and function of the molecule. Fullerene C_{60} has been found to bind both single-stranded and double-stranded DNA *in vitro*, which might have an negative impact on the self-repairing process of the molecule (Zhao et al., 2005). The nanoparticles can cause DNA damage indirectly through the production of ROS as these free radicals both can react with components of the DNA, including purine and pyrimidine bases as well as the deoxyribose backbone (Wang et al., 2007, Gurr et al., 2005).

Lysosomal membrane destabilisation

Lysosomal membrane stability is a sensitive biomarker of bivalve cellular stress (Lowe et al., 1995a). A common method to measure lysosomal stability is based on substrate permeability, and bears a quantitative relationship to the magnitude of stress imposed (Livingstone and Pipe, 1992). A membrane Mg^{2+}-ATPase dependent proton pump maintains an acid environment in the lysosomes (Ohkuma et al., 1982, Poole and Ohkuma, 1981). Dysfunction of the pump would lead to a marked increase in the pH in the lysosomes and allow free passage of the lysosomal contents including neutral red into the cytosol. Failure or dysfunction of the proton pump may be a direct consequence of contaminant action or alternatively the result of a reduction in ATP synthesis following contaminant damage to mitochondria (Lowe et al., 1995a).

Lysosomal membrane destabilization has been reported in haemocytes of *M. edulis* after exposure to nano-iron (Kadar et al., 2010) and nano-gold (Tedesco et al., 2008), in the

haemocytes and digestive cells of *M. galloprovincialis* exposed to nano-TiO_2, fullerene C_{60}, nano-SiO_2 and nanosized carbon black (Canesi et al., 2010), in the coelomocytes (blood cells) of the marine lugworm *A. marina* exposed to nano-TiO_2 (Galloway et al., 2010), and in marine oysters *Crassostrea virginica* exposed to fullerene C_{60} (Ringwood et al., 2009). Destabilisation of the lysosomal membrane can potentially lead to enhanced protein catabolism and cellular atrophy (Moore, 1988). This can have implications for impacted mussels in terms of reduced growth and reproductive potential (Lowe et al., 1995a).

2.5.3 Physiologic effects of nanoparticles

Interactions between nanoparticles and aquatic invertebrates may disturb the normal physiology of the organisms, such as changes in behaviour, reproduction, or nutrition acquisition and uptake. Lovern et al (2007) investigated the effects of nano-TiO_2 and fullerene C_{60} on *D. magna* by measuring several behavioural endpoints. Significant changes in the behaviour were observed after exposure to fullerene C_{60} (0.26 mg L^{-1}), including repeated collisions with the glass beakers, swimming in circles at the water surface, and altered hopping frequency, feeding behaviour, and heart rate. On the contrary, no effects were observed on behavior or heart rate upon exposure to 2 mg L^{-1} TiO_2. As highlighted by the authors, the altered swimming behaviour could potentially affect predation risk and thereby lead to an increase in mortality, with potential negative impact on the population level.

Effects on the reproduction machinery, with possibly impacts at the population and ecosystem level, have been reported. At chronic exposure regimes (21 days), Zhu et al (2010a) and Wiench et al (2009) observed reproductive defects in *D. magna* exposed to nano-TiO_2, with EC_{50} values of 0.46 mg L^{-1} and 26.6 mg L^{-1}, respectively. Furthermore, Zhu et al (2010a) demonstrated a negative impact of nano-TiO_2 on the organism's feeding behaviour, with a significant reduction in the food ingestion that might cause a reduction in growth and reproduction dynamics (Hanazato, 2001). Alterations in feeding behavior were also observed in *A. marina* upon exposure to nano-TiO_2 in a study by Galloway et al (2010) but they failed to find the same response in the lugworms after exposure to CNTs. Oberdörster et al (2006) reported significant delay in molting and significantly reduced offspring production in *D. magna* exposed to 2.5 and 5 mg L^{-1} fullerene C_{60} for 21 days, respectively. Taken together, these findings indicate that nanoparticle exposure may exert negative impact on population of aquatic organisms and on food web dynamics in aquatic systems.

2.5.4 Interactive effects

Nanoparticles can also exert an indirect effect by interacting with other xenobiotics present in the environment, and thereby affect the bioavailability of these contaminants to aquatic organisms (Baun et al., 2008b, Cheng et al., 2004). A few studies have investigated the potential carrier effect of nanoparticles and their influence on bioaccumulation of other contaminants in the aquatic environment. Knauer et al (2007) showed that the strong sorption to nanosized carbon black decreased the bioavailability of the pesticide diuron to algae, whereas the presence of nano-TiO_2 have been found to increase the bioaccumulation of cadmium and arsenate in fish (Zhang et al., 2007, Sun et al., 2007, Sun et al., 2009). Baun et al (2008b) investigated the influence of fullerene C_{60} aggregates on aquatic toxicity and bioaccumulation of four different xenobiotics, using *D. magna* as test organism. They found that the toxicity was altered for only the two contaminants with significant sorption to the C_{60} aggregates, where the toxicity of pentachlorophenol was reduced with 25% (antagonistic effect), whereas a more than 10 times increase in the toxicity was reported for phenantrene (additive effect). Furthermore, it was also shown that uptake of phenantrene was faster in the presence of fullerene C_{60} aggregates, and that a 1.7 times higher steady-state concentration was reach in *D. magna* in the presence of C_{60}. Still, no bioaccumulation was found due to rapid excretion (Baun et al., 2008b). In the case for pentachlorophenol in the study by Baun et al (2008b), the authors suggested that sorption to C_{60} aggregates may inhibit the compound from entering the cell and to reach its toxic site of action, which is the mitochondria. Thus, the bioavailability of pentachlorophenol will be reduced, as fewer pentachlorophenol molecules are able to exert their toxic effect.

3. Materials and methods

3.1 Behaviour of nanoparticles in seawater

Powdered nanoscale P25 Titanium dioxide (CAS no. 13463-67-7) with a particle size of 21 nm, a specific surface area of 35-65 $m^2 g^{-1}$ and a phase composition of 80% anatase and 20% rutile was purchased from Sigma-Aldrich (Oslo, Norway).

To determine the possible effects of natural seawater on the solubility of nano-TiO_2, the aggregation and deposition characteristics of nano-TiO_2 in seawater was visually compared to the behaviour in fresh water.

Suspensions of nano-TiO_2 with six different concentrations (0.1, 0.5, 1.0, 1.5, 2.0, 2.5 g L^{-1}) were prepared in seawater or distilled water. To break up particle aggregates, the nano-TiO_2 suspensions were incubated in an ultrasonic bath (Branson sonicator 3210) for 40 minutes, in which ultrasound energy is applied to speed the process of dissolution by breaking intermolecular interactions (Branson Operator's Manual). All the handling of nanoparticles was done under local exhaust ventilation. All suspensions were visually inspected at the end of each incubation period.

3.2 Transmission electron microscopy

The morphology of nano-TiO_2 was characterized using a transmission electron microscope (TEM, JEOL 200CX) utilizing an acceleration voltage of 200 kV. In electron microscopy, a beam of electrons is accelerated towards a specimen under high vacuum, leading to interactions between the electrons and the sample as the beam hits it. These interactions and the generated signals are the basis of the various imaging techniques that can be utilized in electron microscopy. In TEM, the image is formed by electrons that transverse the sample. The image is then magnified and focused onto an imaging device, such as on a layer of photographic film (Ashby et al., 2009). Prior to TEM analysis, powdered nano-TiO_2 was suspended in ethanol, dropped onto 400-mesh copper grid coated with carbon and allowed to dry in room temperature over night. After drying, the samples were systematically inspected in the transmission electron microscope using 50,000 and 100,000 x magnification.

Additional samples for TEM imaging were made to look at the effect of sonication on the nanoparticles. Nano-TiO_2 solutions were prepared by dispersing 5 mg L^{-1} nanopowder in seawater or freshwater; each solution was then separated in two beakers, in which one was incubated for 40 min in ultrasonic bath. Two drops of the solutions were placed onto 400-mesh copper grid followed by drying over night. As the process of sonication breaks up particle aggregates, it was hypothesized that a greater proportion of single nanoparticles could be observed in the samples exposed to ultrasound.

3.3 Concentration of nano-TiO_2 in seawater

The presence of dispersed particles causes a liquid to become cloudy. This cloudiness, or turbidity, is an expression of the optical property that causes light to be scattered and absorbed by particles and molecules rather than transmitted in straight lines through a water sample (EPA, 1999). The turbidity, often given in Nephelometric Turbidity Units (NTU), of the sample can be determined by using a nephelometer (Figure 14). An intense beam of light is directed up through the bottom of a glass cell containing the test sample. The light scatter by the suspended particles is detected by a sensitive photomultiplicator tube at an angle of 90° from the incident light. The amount of light reaching the photomultiplier tube is proportional to the turbidity in the sample. The photomultiplier tube converts light energy into an electrical signal, which is amplified and displayed on the instrument meter. A light shield is used to cover the sample cell during the measurement to exclude external light.

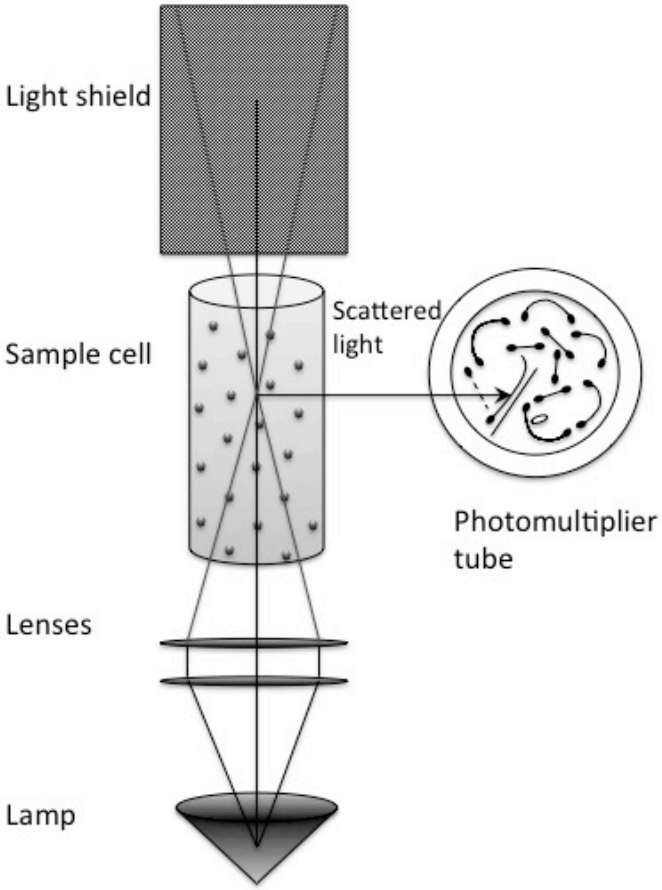


Figure 14: Schematic drawing of the principle of the nephelometric operation. Based on figure in Instrument Manual, Hach Company (1989)

To test whether this methodology could provide as a method for determination of nano-TiO_2 concentration in seawater, the relationship between the concentration of nano-TiO_2 in seawater and the NTU value of the sample was evaluated by making a standard curve. Several samples of known nano-TiO_2 concentrations between 0.02 – 50 mg L^{-1} were prepared and transferred to a glass cell for turbidity testing using a Hach 2100A Turbidimeter. All samples were measured in duplicates. One test cell was used for all measurements to avoid variations between the test tubes that could influence the amount of scattered light. To obtain defined, reproducible results, the turbidity meter was calibrated and adjusted using reference standards of known NTU values. A standard curve was then made in Excel to see if there was a correlation between the turbidity and nano-TiO_2 concentration.

3.4 Acute toxicity test

A preliminary experiment was conducted to give an indication of the mussels' response to a high dose of TiO_2 nanoparticles. The exposure solution was prepared by dispersing nano-TiO_2 in seawater to a concentration level of 100 mg L^{-1}, followed by incubation in ultrasonic bath for 90 min. Mussels were transferred into a 600 ml beaker containing 500 ml seawater for acclimation (3 h) prior to the experiment. The seawater was gently removed and mussels (five mussels per treatment) were exposed to the nano-TiO_2 seawater solution for 21 h (on ice). A parallel control group of mussels was kept in clean seawater for 21 h. Animals were not fed during the experiment.

3.5 Chronic toxicity test

A stock suspension of 100 mg L^{-1} nano-TiO_2 was prepared by dispersing the nanopowder in seawater with incubation in ultrasonic bath for 90 min; a further 20 min of sonication was conducted immediately before water replacement each day. The exposure solutions were prepared immediately prior to use by diluting the stock solution in seawater to the concentration levels of 1, 5, or 25 mg L^{-1}. Mussels (10 for each condition, tagged with an individual number) were exposed to nano-TiO_2 for six days. A parallel control group of mussels was kept in clean seawater. The beakers were oxygenated and placed in a cool box to keep a cold environment. Nano-TiO_2 seawater solutions were changed daily, and the turbidity of the nano-TiO_2 seawater solutions was measured right before and after each water replacement in order to monitor the exposure concentrations. Animals were not fed during the experiment.

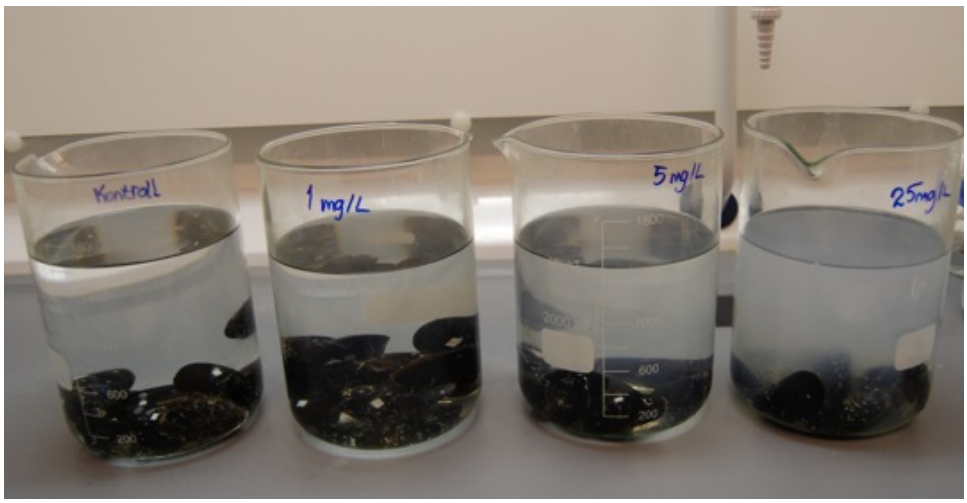


Figure 15: Mussels in beakers of different nano-TiO_2 concentrations

3.6 Haemolymph sampling

The mussel valves were prised apart with a solid scalpel and 0.1 ml haemolymph was withdrawn from the posterior conductor muscle of mussels, using a 1 ml syringe with a 0.6 x 25 mm needle. Having obtained the haemolymph sample, the needle was discarded to reduce shearing forces that can harm the cells during the subsequent expulsion of the haemolymph into an Eppendorf tube prefilled with 0.1 ml filtered seawater and held on ice. The contents were gently mixed.

3.7 Neutral red retention time

In vivo lysosomal membrane stability (LMS) in the haemocytes of control and exposed mussels was evaluated by Neutral red retention time assay (NRRT). The release of the neutral red dye into the cytosol following exposure to a stressor may be due to damage to the lysosomal membrane and, possibly impairment of the proton pump that maintains a low intralysosomal pH (Lowe et al., 1995a). Destabilization of the lysosomal membrane is used as a reliably indicator of invertebrate health (Lowe et al., 1995b), and has been shown to be an appropriate biomarker for the impacts of nanoparticles in aquatic invertebrates (Canesi et al., 2010, Galloway et al., 2010, Kadar et al., 2010, Ringwood et al., 2009, Tedesco et al., 2008).

The procedure was carried out according to the Standard Operating Procedure (SOP) for "Neutral Red Retention Time in *M. edulis*, blue mussel" (IRIS Biomiljø, 2009b), based on the method described by Lowe et al (1995a), with slight modifications. In brief, a stock solution of neutral red was made by dissolving 20 mg of neutral red dye in 1 ml of Dimethyl sulfoxide (DMSO). A working solution was then prepared by adding 5 µl of the stock solution to 995 µl of filtered seawater.

A 30-µl aliquot of the cell suspension was dispensed onto a microscope slide and put on a rack in a lightproof humidity chamber for 15 min to allow the cells to attach. At this point, a new number was given to each mussel in order to perform the analysis anonymously. The excess solution was then carefully tapped off to leave a monolayer of cells on the microscope slides, and 30 µl of Neutral Red working solution was added to the area with the attached cells. The slides were sealed with a 22x22 mm cover slip. After 15 min of incubation in the chamber, the slides were systematically examined in a light microscopy using x 40 magnification. Following a further 15 min incubation, the slides were examined again and

thereafter at 30 min intervals until reaching the endpoint of the assay, defined as the time at which 50% of the cells showed sign of lysosomal leaking (the cytosol becoming red and the cells rounded). Following each inspection in the microscope, the slides were returned to the lightproof humidity chamber to prevent the haemolymph samples from drying out and photo-activation of the neutral red probe.

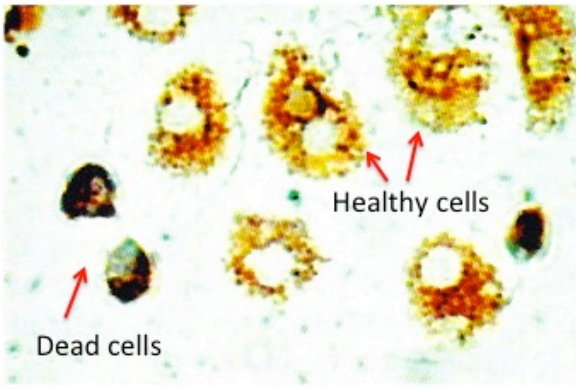


Figure 16: Neutral red retention time assay (NRRT) assay. Dye loss is evident in some of the cells and the cell morphology is changing from irregular to round (IRIS Biomiljø, 2009b).

3.8 Collection of histology samples

For histological examination of the gills, gonads and digestive gland, dissection was carried out according to the SOP for "Collection and preparation of histology samples" (IRIS Biomiljø, 2009a) with small modifications. To make a Baker's calcium buffer to store the histology samples, 25 g NaCl and 10 g $CaCl_2$ was dissolved in 100 mL formaldehyde, and diluted with distilled water up to 1 L. A solid scalpel was inserted into the ventral byssal cavity, and the posterior adductor muscle was cut to open the shell. A piece of the gills, digestive gland and gonads were dissected, placed in a histocassette and immediately transferred to the Baker's calcium solution and stored at 4°C.

Figure 17: Collection of histological samples.

3.9 Data management and statistical analysis

3.9.1 Turbidity and concentration

The mean NTU value of all 25 samples of known concentration (between 0.02 and 50 mg L^{-1}) was calculated. Regression analysis was performed with the use of StatPlus® software from AnalystSoft to evaluate the relationship between concentrations and NTU values.

3.9.2 Neutral Red Retention Time

Statistical tests were performed with the use of JMP® software from the SAS institute. Parametric tests were preceded by tests for the normal distribution and homogeneity of variance. Appropriate transformations (log) of the sample data were used, when necessary, in order to allow the use of the parametric statistical method. Dunnett's test for multiple comparisons of means was used to test for differences between exposed groups and control group.

4. Results

The goal of this work was to investigate the possible impact of nanoparticles on the aquatic environment. A literature survey regarding fate of nanoparticles in the aquatic systems and their interactions with aquatic invertebrates was the main objective in attaining this goal. Additionally, basic experiments were conducted to explore the hypothesis that nano-TiO_2 can cause sub-lethal impact on bivalve mollusc *M. edulis* exposed through the water column. The fate of nanoparticles in water has a great impact on their interactions with biota. Therefore, the aggregation behaviour of nano-TiO_2 in seawater versus freshwater was investigated (4.1) and the nanoparticles were visualized in TEM to reveal their size and aggregation state (4.2). Turbidity testing was done to investigate whether this method could be used to quantify nano-TiO_2 in seawater (4.3). Mussels were exposed to nano-TiO_2 seawater solutions for 21 h (4.4) or six days (4.5) and NRRT assay was performed to evaluate the cytotoxic impact on the haemocytes. Concentrations of nanoparticles in exposure solutions were measured on certain time intervals to determine whether the exposure concentrations were constant.

4.1 Solubility of nano-TiO_2 in water

To determine the possible effects of natural seawater on the solubility of nano-TiO_2, the aggregation and deposition characteristics of nano-TiO_2 in seawater was visually compared to the behaviour in fresh water. This experiment was performed to define the preparation of nano-TiO_2 solutions in the following experiments.

Addition of nano-TiO_2 2.5 g L^{-1} to freshwater and to seawater resulted in their immediate precipitation to the bottom as a milk-white mass. By carefully mixing the beakers, nano-TiO_2 appeared to dissolve to some extent in freshwater resulting in a more cloudy solution. This was not observed for nano-TiO_2 in seawater. After incubation in ultrasonic bath for 40 min, obvious differences could be seen for between the two solutions as shown in Table 1.

Table 1: Solubility of nano-TiO_2 in water

[Nano-TiO_2], g L^{-1}	Type of water	Time in ultra-sonic bath, min	Observations
2.5	Freshwater	40	Dissolved
2.5	Seawater	40	Not dissolved (Deposits at the bottom)
2.0	Seawater	40	Not dissolved (Deposits at the bottom)
1.5	Seawater	40	Not dissolved (Deposits at the bottom)
0.5	Seawater	40	Not dissolved (Deposits at the bottom)
0.1	Seawater	40	Not dissolved (Some deposits at the bottom)
0.1	Seawater	90	Dissolved

The procedure was completed three times. For the nano-TiO_2 freshwater solution, the nanoparticles were totally dissolved after 40 min of exposure to ultrasound energy. In seawater, the same treatment resulted in an almost clear solution with large amounts of milk-white deposits on the bottom. Several concentrations of nano-TiO_2 in seawater were tested and a concentration dependent solubility was observed. Decreasing the concentration of nano-TiO_2 to 0.1 g L^{-1}, as well as increasing the time of incubation to 90 min, resulted in an apparently stable nano-TiO_2 suspension without any observable nanoparticle precipitates. Therefore, stock suspensions for the subsequent experiments were produced by suspending 100 mg L^{-1} powder in seawater followed by 90 min of sonication.

4.2 Transmission electron microscopy

The size and morphology of nano-TiO_2 were investigated with TEM.

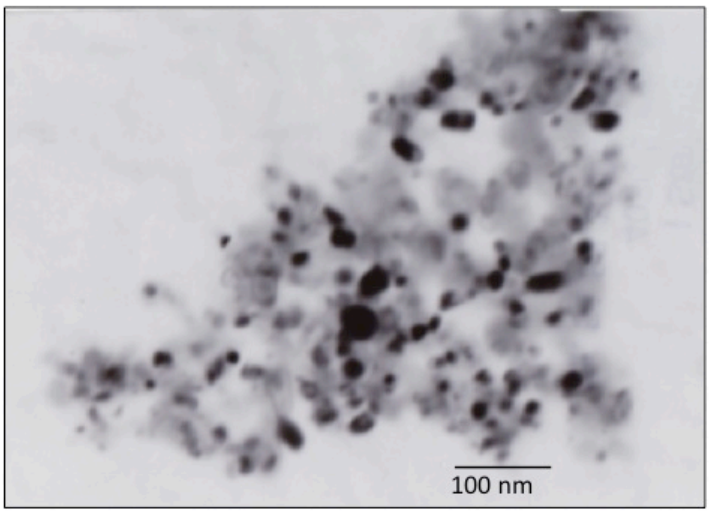


Figure 18: TEM image of nano-TiO_2 suspended in ethanol (100,000x magnification)

Observed by TEM, nanoparticles of titanium dioxide (suspended in ethanol) were spread out on the grid in large clusters with sizes from a few hundreds nanometres to several microns in diameter. Each cluster (Figure 18) appears to be comprised of single particles arranged in thin sheets on top of each other. By focussing on one of these layers, single nanoparticles could by seen within the clusters, with a diameter of about 21 nm that is the announced TiO_2 particle size.

Preparation of nano-TiO_2 in seawater (with and without sonication) resulted in a thick layer of salt ions on the grid, and, thus, no nanoparticles could be seen in TEM. TEM analysis of nanoparticles prepared in freshwater, with and without ultrasonic incubation, revealed an image very similar to that of nano-TiO_2 in ethanol, and, despite diverse pre-treatment, no observable differences in the nanoparticle dispersion state in could be detected. The TEM image with ethanol as solvent is shown (Figure 18) due to better quality.

4.3 Determination of nano-TiO_2 concentrations in seawater

The turbidity of several samples of known nano-TiO_2 concentrations between 0.02–50 mg L^{-1} was measured using a nephelometer, in order to determine the relationship between nano-TiO_2 in seawater and the turbidity of the sample. The procedure was done twice, and the average NTU value was used to create a standard curve.

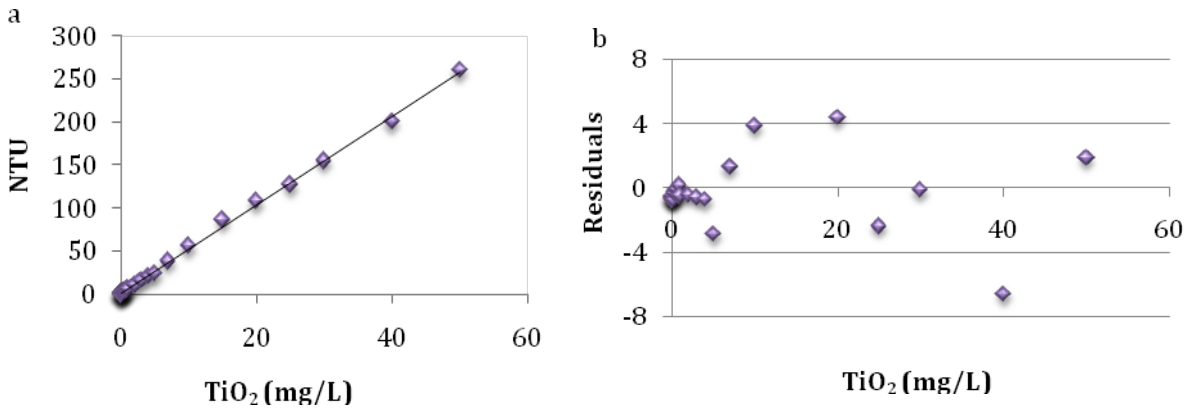


Figure 19: a: Relation between sample turbidity and concentration of nano-TiO_2. Regression line is shown (R^2=0.9986). b: Residuals from the regression line shown in a, plotted against nano-TiO_2 concentration.

Nephelometric analysis of seawater samples with known concentrations of nano-TiO_2 show an increase in turbidity with increasing concentration. Figure 19a shows the sample data together with the least square regression line. The equation of the regression line is:

$$Y = 5.15\ X + 0.64 \qquad \text{(Equation 1)}$$

A strong correlation ($R^2 = 0.9986$) was calculated between the two variables, indicating that the variability in turbidity can be explained by the variation in sample concentration of nano-TiO_2. According to the slope of the regression line, there is an estimated increase in the turbidity of 5.15 NTU for every increase of one unit (mg L^{-1}) of nano-TiO_2 in seawater. The scatter of data points around the fitted line is even and symmetric but show an increase in variability of the residuals as the sample concentration increases above 4 mg L^{-1}, as shown in Figure 19b, indicating a higher specificity in the lower concentration levels. This standard curve was utilized to monitor the concentration in the following experimentation.

4.4 Short-term exposure

An acute (short-term) toxicity test was performed to determine whether a high concentration of nano-TiO_2 could induce detectable responses in the mussels. In general, acute toxicity studies are used to assess the toxicity and to measure the concentration that will affect the test organism. In this preliminary experiment, mussels (five individuals) were exposed to nano-TiO_2 at a concentration of 100 mg L^{-1} for 21 h. A parallel control group of mussels was kept in clean seawater for 21 h. To evaluate the mussel's response, haemolymph was extracted and the lysosomal membrane stability in the haemocytes was evaluated by the NRRT assay. The ability of cells to take up and retain the neutral red dye has been used in several studies as a measure of cytotoxicity (IRIS Biomiljø, 2009b). The NRRT assay is a well-established method at IRIS Biomiljø and is used as a reliable indicator of invertebrate health.

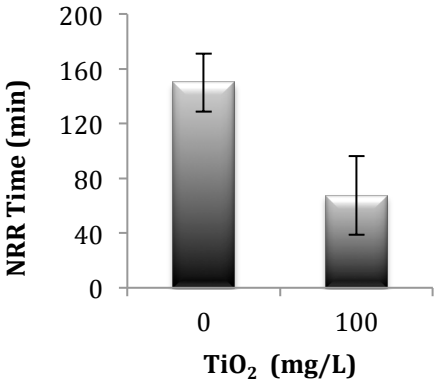


Figure 20: Effects on lysosomal membrane stability of mussel haemocytes after 21-h exposure to 100 mg L^{-1} nano-TiO_2, evaluated by the NRRT assay.

The haemocytes incubated with neutral red dye were inspected in a light microscope until reaching the endpoint of the assay, defined as the time at which 50% of the cells showed sign of lysosomal leaking. At this point, the cytosol becomes red and the cells rounded. The average time until reaching the endpoint for the exposed group was compared to that of the control group. Exposure to 100 mg L^{-1} nano-TiO_2 for 21 h led to a reduction in the ability of the lysosomes to retain neutral red dye, resulting in a NRRT that was 45% of that of the control group (Figure 20). Nano-TiO_2 reduces LMS in the mussel haemocytes when exposed to high concentrations under acute toxicity testing regimes. One individual died during exposure and was therefore not included in the NRRT assay.

4.5 Long-term exposure

A more comprehensive exposure study was performed to evaluate whether the same responses (as found in the acute toxicity test) could be detected at lower concentrations of nano-TiO_2 when the organisms were exposed over a longer period of time. Mussels were exposed to different concentrations (1, 5, or 25 mg L^{-1}) of nano-TiO_2 for six days. A parallel control group of mussels was kept in clean seawater. Each condition comprised 10 individuals, giving a total of 40 mussels in this exposure experiment. Exposure solutions were changed daily.

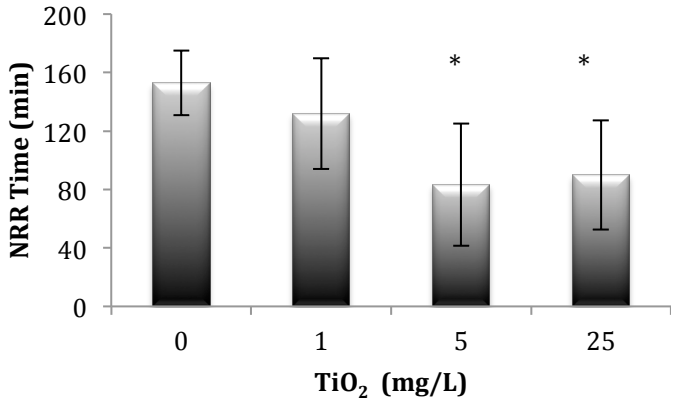


Figure 21: Effects on lysosomal membrane stability of mussel haemocytes after six days of exposure to 1, 5, or 25 mg L^{-1} nano-TiO_2, evaluated by the NRRT assay. Asterisks (*) indicates significant differences at $P<0.05$ from the control group.

Haemolymph was extracted from all mussels, and the LMS of haemocytes was evaluated using the NRRT assay. All mussels were tagged with an individual number in order to perform the NRRT assay anonymously. A significant impact of nano-TiO_2 exposure on lysosomal membrane stability in the haemocytes was observed at the two highest concentrations tested. The neutral red retention times of haemocytes exposed to 5 and 25 mg L^{-1} TiO_2 nanoparticles were reduced to 49% and 59% of control values (Figure 21). One individual died after 5 days of exposure to 5 mg L^{-1} nano-TiO_2.

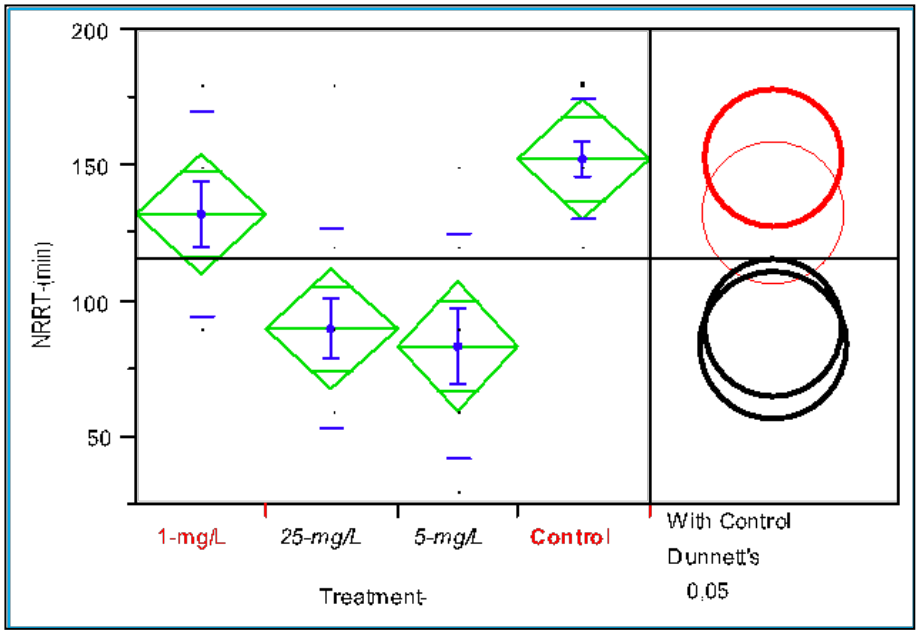


Figure 22: Statistical analysis of data using Dunnett's test. The 5 mg L^{-1} and 25 mg L^{-1} treatment groups are significantly different from the control group ($P < 0.05$).

Dunnett's test for multiple comparisons of means was used to test for differences between exposed groups and control group. The statistical data is shown in Figure 22. The results show that the 5 mg L^{-1} and 25 mg L^{-1} treatment groups are significantly different from the control group, indicated by no overlaps between the circles representing them (black circles) and the control group (red bold circle). Although not significant, both Figure 21 and the statistical analysis show a tendency for LMS reduction for the group exposed to 1 mg L^{-1} as well (86% of control values).

4.6 Variations in exposure concentrations

During the exposure period of six days (4.4), the concentration of nano-TiO_2 was measured using the turbidity method evaluated in section 4.3. This was done in order to quantify the concentration of nanoparticles the organisms were experiencing. It was preferred to keep the concentrations at the same level throughout the period of exposure.

Observable alterations in exposure solution were seen during the experimental period. At 24 h from water replacement, all solutions appeared clear to the eye and aggregates of nanoparticles could be observed. At the highest concentrations tested, aggregates were observed along the byssal threads of the mussels, and also lining the beakers and plastic tubes

of the oxygenation system (Figure 23). Also, precipitates of nanoparticles were observed on the bottom of the beakers in all exposure conditions before each water replacement.

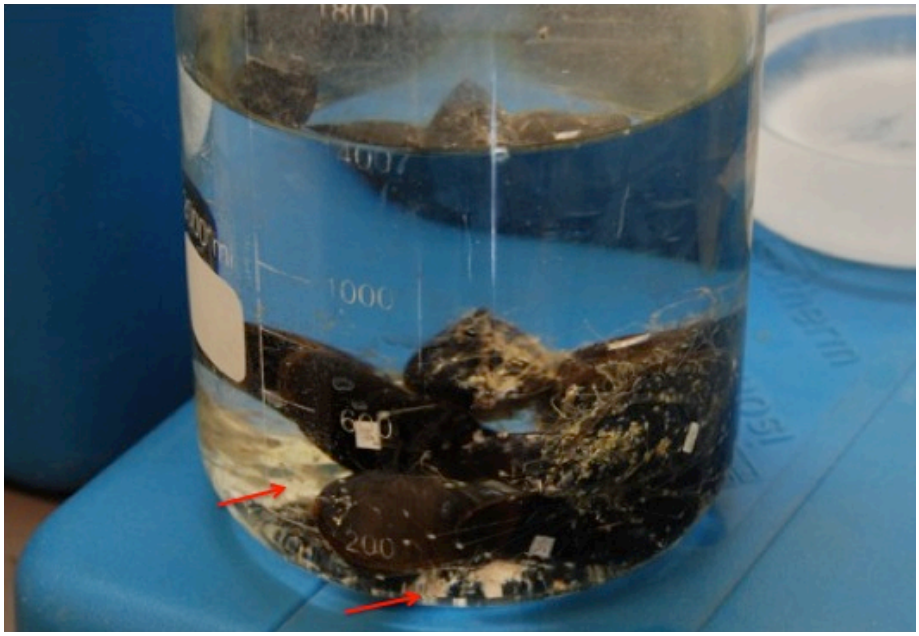


Figure 23: Photo taken at 24 h from replacement of exposure solution (25 mg L^{-1}). Aggregates of nano-TiO_2 can be observed at the bottom (red arrows).

The visual observations indicated an alteration in nano-TiO_2 concentration in the water phase that was confirmed by measuring the turbidity of exposure solutions. The turbidity could then be related back to nano-TiO_2 concentration by using the generated standard curve.

Turbidity testing of water samples before and after each water replacement reveals large gap between nominal (1, 5 or 25 mg L^{-1}) and actual concentrations at 24 h from water replacement. Figure 24a, b, and c shows the variations in the exposure concentrations during the experimental period of six days. A line is drawn between the data points to visualize the change in concentration. However, it should be noted that the kinetics of concentration decrease is unknown. Still, there is a clear variation in the concentrations that the mussels experience during the experimental period.

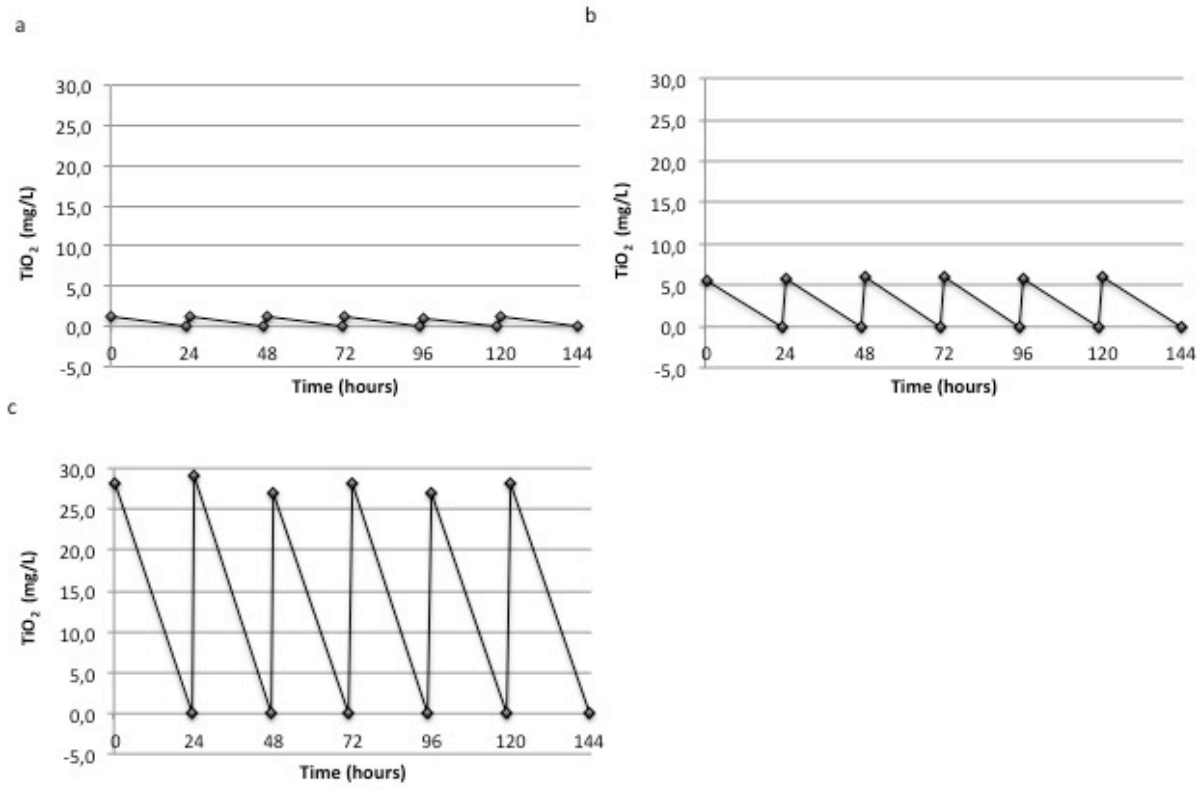


Figure 24: Variations in nano-TiO_2 exposure concentration during the experimental period (six days). The lines between the data points show an assumed constant decrease in nano-TiO_2 concentration but the kinetics are unknown.

5. Discussion

The goal of this work was to explore the basics in the field of nanoecotoxicology. To achieve this goal, available literature has been reviewed (chapter 2) and experiments have been conducted in the laboratory based on the collected information. The following discussion focuses on the observations and data obtained during the experimental part of this work.

The behaviour of nanoparticles in the aquatic systems has great impact on their subsequent fate within this environmental compartment. The nanoparticles can remain freely suspended or flocculate into larger clusters in the process of aggregation. The tendency to aggregate when suspended in water has been reported by several authors (section 2.3.3), and has shown to be favoured by increased ionic strength (Brant et al., 2005, Domingos et al., 2009, Jiang et al., 2009). Observations made in the present study support these earlier findings and reveal obvious differences in the behaviour of nano-TiO_2 in freshwater versus seawater. In this study, the addition of nano-TiO_2 to water resulted in their immediate precipitation to the bottom of the beakers; however, gently mixing resulted in dissolution of nano-TiO_2, to some extent, in freshwater. This was not observed for the nano-TiO_2 seawater solution. The observations indicate that the solubility of nano-TiO_2 is dependent on the ionic strength in the water. Despite the fact that the concentrations used in this experiment is higher than the concentrations predicted to be found in nature (section 2.3.1), the tendency shown here can be predictive for the nanoparticle behaviour in natural waters.

After entering the aquatic systems, the nanoparticles can be transported over long distances as a consequence of large mass movements of water. The increased tendency of nanoparticles to aggregate with increased ionic strength will have implications for their distribution in the environment. As for pollutants in general, the distance travelled depends on factors such as the stability and physical state of the nanoparticles, and is likely to be greatest where stable compounds are in solution (Walker et al., 2006). If released into rivers, the lack of salt ions combined with a turbulent water flow is likely to keep the nanoparticles stable in suspension, resulting in their distribution and transportation over considerable distances depending on the speed of flow of the river. However, based on the literature and the observations made in the present study, the nanoparticle behaviour is likely to change drastically as the polluted freshwater reaches the sea. As the nanoparticles meet the salty ocean water, the flow of the water slows down and the presence of salt ions can screen the surface charge of the particles

allowing them to come into contact and form aggregates (Brant et al., 2005, Domingos et al., 2009, Jiang et al., 2009). The subsequent transport of micrometre-sized aggregates is dominated by sedimentation (Handy et al., 2008a), and the nanoparticles are therefore likely to settle on the bottom once they are brought into the estuaries. In this way, aggregation will remove the nanoparticles from the water and, hence, limit their transport within the water phase and probably avoid their interactions with pelagic species.

The aggregation and subsequent precipitation ensures unequal nanoparticle distribution in the aquatic environment. If released into rivers on a continual basis, their precipitation as they enter water with higher ionic strength potentially leads to their build-up in the estuarine sediments. This, together with their potential persistent nature, may result in high concentration levels in these areas over time. As a dominant member of estuarine communities (Widdows and Donkin, 1992), the blue mussel will therefore be exposed to aggregated nanoparticles in significant concentrations. Previously published data indicate that aggregation of nanoparticles will increase their bioavailability to filter-feeding organisms. Aggregation leaves only minor part of the total of nanoparticles in the size ranges relevant for passive diffusion, which is a prominent route of uptake of waterborne chemicals by aquatic organisms (Walker et al., 2006). However, the increase in size allows the nanoparticles to be captured and ingested by organisms that actively collect particles from the water column in search for food (Canesi et al., 2010, Ward and Kach, 2009, Gagne et al., 2008, Baun et al., 2008b). Thus, the phenomenon of aggregation and subsequent sedimentation does not necessarily reduce the risk of ecotoxicity; it simply changes the target organisms.

Moreover, the tendency to aggregate has obvious implications for aquatic ecotoxicity experiments by making it difficult to achieve stable dispersions, especially when conducting studies on marine organisms (Boxall et al., 2007). The main approaches to achieve stable dispersions include the use of organic solvents, prolonged stirring in water, and incubation in ultrasonic bath. The use of solvents in the preparation of suspension for ecotoxicological experiments has been criticized as it is not necessarily environmentally relevant and because these solvents themselves can be inherently toxic (Johnston et al., 2010). In the present study, the dispersion procedure by sonication, in which ultrasound energy is applied to break up the particle aggregates, was adopted to provide a more environmentally relevant condition. Still, it should be noted that even this type of pre-treatment might enhance toxicity with respect to non-treated nanoparticles (Zhu et al., 2006b, Oberdörster et al., 2006). The aggregates of

nano-TiO_2 could be seen as large clusters of particles in transmission electron microscopy. Samples for TEM analysis were prepared in seawater and freshwater with and without sonication, and in ethanol. The salts, not surprisingly, covered the samples prepared with seawater and no nanoparticles could be seen. Despite an expected difference in the nanoparticles' aggregation state due to diverse preparation methods, the other three samples appeared similar in the microscope. Less aggregation among the nanoparticles was expected for the samples pre-treated to break up particle aggregates (sonication and ethanol) in comparison to those suspended in freshwater without sonication. Their aggregation state can, however, have been altered during the preparation period before TEM analysis, in which the samples were allowed to dry over night. Furthermore, only two drops from each solution were used to prepare the sample for TEM analysis, and are not necessarily representative for the entire solution (if not totally homogeneous). If several TEM samples had been made for each solution (with different pre-treatment) it might have been possible to observe differences in the nano-TiO_2 state due to different pre-treatments.

Aggregation behaviour of nanoparticles in water also makes it difficult to quantify the actual amount of nanoparticle that the test organisms experiences (Christian et al., 2008). The need for a methodology to measure the concentration of nano-TiO_2 in seawater was met by discovering a linear relationship between turbidity and nano-TiO_2 concentration. Thus, by measuring the turbidity using a nephelometer, it was possible to control the exposure concentration in a simple and rapid way. During the experimental period of six days, there was a significant variation in the nanoparticle concentration in the water phase. At 24 h from solution exchange (1, 5, and 25 mg L^{-1}), the concentration of nano-TiO_2 in the water phase dropped to zero. However, it should be noted that the nephelometric method has a low sensitivity with a measured detection limit of about 20 µg L^{-1} of nano-TiO_2 in seawater (Appendix 1). It is therefore unknown whether the concentration is stabilized at 20 µg L^{-1} or if it falls to zero. The drop in concentration is most likely due to aggregation of the nanoparticles and their subsequent sedimentation, a theory supported by observable white aggregates in the bottom of the beakers. Thus, the behaviour of nanoparticles in seawater leads to a large gap between the nominal dosing concentrations and the actual exposure concentrations. This makes it difficult to relate observed toxic effects to exact concentration levels. One possible way to avoid this problem is by using a continuous flow, rather than the semi-static system that was used in this study. A continuous flow could have provided a more realistic exposure situation and allowed the concentration of nanoparticles to be constant over

the exposure period with exposure solution provided on a continual basis. A semi-static system was chosen here due to all uncertainties related to the behaviour of the nanoparticles. It was unknown whether the nano-TiO_2 would aggregate within the tubes of a continuous flow set up.

It is questionable whether actual discharges are sufficient in volume to cause severe environmental impact (Battin et al., 2009, Moore, 2006). Estimates for levels of nano-TiO_2 in the aquatic environment based on modeling approaches have suggested a concentration of 0.7–16 μg L^{-1} in freshwater (Mueller and Nowack, 2008). It should be noted that this predicted concentration range is much lower than the exposure concentrations used in the present study. However, if the nanoparticles behave as predicted above, they are liable to fall to the bottom with estuarine deposits leading to higher concentrations in the estuaries compared to that in rivers and in the open sea. Thus, the concentrations used in this study do, perhaps, represent a realistic scenario for highly exposed areas.

A short-term exposure (21 h) to 100 mg L^{-1} nano-TiO_2 was conducted to determine the acute response to a high dose of nano-TiO_2 and to examine whether destabilisation of lysosomal membrane was induced at this high concentration. Previous studies suggest that nano-TiO_2 has a low acute toxicity toward aquatic invertebrates. Using the freshwater water flea *D. magna* as test organism and sonication to form stable suspensions, Zhu et al (2010a) and Lovern and Klaper (2006) reported $LC_{50,\ 48h}$ values greater than 100 and 500 mg L^{-1}, respectively. In line with these results, only one of five individuals died after exposure to 100 mg L^{-1} nano-TiO_2 for 21 h in the current work.

The sub-lethal impact of nano-TiO_2 on the mussels was investigated by the use of the NRRT assay. The NRRT assay is based on the principle that lysosomes in the haemocytes take up and retain the cytotoxic dye neutral red. Lysosomal membranes' damage caused by the impact of test substances induce the leaking of lysosomal components, thereby leading to a decrease in the retention times (Lowe et al., 1995a). Destabilization of the lysosomal membrane is used as a reliably indicator of invertebrate health (Lowe et al., 1995b), and has been shown to be an appropriate biomarker for the impacts of nanoparticles in aquatic invertebrates (section 3.9.2). In this preliminary study, only five mussels were included in each group (exposed and control group). Furthermore, as one individual died during exposure, only four individuals were included in the biomarker (NRRT) assay. The obtained data show a clear reduction in the

ability of the lysosomes to retain the neutral red dye after exposure to 100 mg L^{-1} nano-TiO_2, indicating that early changes in the health status of the mussels are induced by the nanoparticles. The average retention time in the exposed group was 45% of the retention time found in the control group. A small number of mussels were included in this preliminary exposure set-up, and the obtained data is therefore not necessarily representative for a larger group of individuals. The NRRT normally decreases dramatically in spawning organisms, leading to false positive results. Therefore, these organisms should not be included in the test. The reproductive condition of the mussels can be revealed by macroscopic features such as the colour, texture, and thickness of the gonad can reveal the reproductive (Seed and Suchanek, 1992). However, spawning mussels can have been included due to inexperience in identifying them, and thus contributed to the strong reduction in LMS in the exposed group. A larger number of individuals would therefore make the data more reliable. Still, the main goal of conducting this "proof-of-principle" exposure was to evaluate whether LMS could provide as an indication of sub-lethal impacts of nano-TiO_2 in order to conduct a longer exposure study using lower, more realistic concentrations.

Since exposure duration has been suggested to be an important factor in nanotoxicity (discussed in section 2.5.1), it was of interest to conduct a longer-term exposure experiment using lower concentrations. Mussels were exposed to 1, 5, or 25 mg L^{-1} nano-TiO_2 for six days. These concentrations were chosen to correlate with those utilised in ecotoxicity tests utilising the same type of nanoparticles (Zhu et al., 2008, Hao et al., 2009, Canesi et al., 2010, Zhu et al., 2010a, Wiench et al., 2009), although some of these have utilized lower concentrations as well. After six days of exposure, nano-TiO_2 induced significant decrease in lysosomal membrane stability in circulating haemocytes at 5 and 25 mg L^{-1}, indicated by the NRRT assay. As this analytical method is based on subjective observations in the microscope, it should be noted that the assay was performed anonymously. Surprisingly, a stronger effect was observed in the group exposed to 5 mg L^{-1}, compared to the group exposed to a concentration level of 25 mg L^{-1} with mean retention times that was 49% and 59% of the control value, respectively. If the mussels take up nano-TiO_2 through capture and ingestion, the mussel's digestive capacity can become saturated, so that the ration of ingested nanoparticles remains constant and surplus nanoparticles are rejected as pseudofaeces. Thus, the uptake is not necessarily greater when exposed to 100 mg L^{-1} compared to 5 mg L^{-1}, and might explain why the greatest response was not found in the group exposed to the highest concentration. However, this does not explain why the greatest response was found in the

group exposed to 5 mg L^{-1}. Moreover, one individual in this group (5 mg L^{-1}) died after five days of exposure. The mussels (40 individuals) used in this exposure study were randomly distributed into the four groups; however, there might have been differences in their health conditions prior to the exposure contributing to the results. Statistical analysis was performed to see if there was significant difference in the size (Appendix 2) of the mussels between the groups, which could have affected the result (small mussels can be weaker compared to larger mussels), but was not considered significantly different (data not shown). Also, as discussed above, the presence of spawning musses can affect the results. At the lowest concentration tested, a slight reduction in the retention time (86% of control value) was observed, but was not considered statistically significant.

Based on these data the lowest observed effect concentration (LOEC) was determined to be 5 mg L^{-1} resulting in a no-observed effect concentration (NOEC) of 1 mg L^{-1}. Although the lowest observed effect concentration found in the present study is higher than the range of the predicted environmental levels of nano-TiO_2 (Mueller and Nowack, 2008), the obtained data suggest that the mussels haemocytes represent a significant target for *in vivo* exposure to TiO_2 nanoparticles. Conflicting results have been reported by Canesi et al (2010), who observed significant destabilisation of the lysosomal membrane in the marine mussel *M. galloprovincialis* haemocytes after exposure to 1 mg L^{-1} nano-TiO_2 for 24 h. This inconsistency is surprising as a longer period of exposure is expected to reduce the NOEC values (Zhu et al., 2010a). Nevertheless, minor differences in the experimental conditions may lead to variations in the results. The oxygenation of the beakers holding the mussels that was applied in the present study, might affect the behaviour of nanoparticles as well as improve the quality of the water, thereby leading to better environment for the test animals, which might have had a positive impact on their general physiological condition. Moreover, the NRRT assay was performed in slightly different ways, in which Canesi and co-workers examined the slides at closer intervals (every 15 min), leading to a more sensitive evaluation of the cytotoxic impact. It should also be noted that the endpoint of the NRRT assay is based on a visual evaluation of the cells in the microscope, and is therefore a factor that can lead to variations in the test results depending on the person performing the analysis.

The significant changes in lysosomal biomarkers in the circulating haemocytes found in the present study, implies that uptake of nano-TiO_2 have occurred. Both passive diffusion across the respiratory epithelium of the gills and active uptake through ingestion may lead to

exposure of the circulating haemocytes. However, as discussed above, the behaviour of nanoparticles in the water is likely to affect the way they interact with biota. Due to aggregation, uptake through capture and ingestion is the most likely portal of entry for the nanoparticles (Ward and Kach, 2009). Several authors show localisation of nanoparticles within the gut by TEM imaging as evidence of direct ingestion (Wiench et al., 2009, Galloway et al., 2010, Zhu et al., 2010a, Koehler et al., 2008). The fate of these nanoparticles after ingestion varies considerably among the studies. For instance, in the lugworm *Arenicola marina*, nano-TiO_2 accumulated in the lumen of the gut without evidence of uptake into digestive gland but did, however, induce destabilisation of the lysosomal membrane in the circulating cells (Galloway et al., 2010). Based on these observations, the authors implied that even if nanoparticles are not digested by the organism it is possible that a small fraction might be taken up leading to toxic effects elsewhere (Galloway et al., 2010). The nanoparticles rejected by the stomach, might have been absorbed from the intestine and entered the circulating system without entering the digestive gland for digestion. In *M. edulis*, nanoparticles of metal-oxide (SiO_2) entered the primary and secondary tubules of the digestive gland in a study by Koehler et al (2008). After entering the digestive system, the nanoparticles may translocate to the haemolymph, as reported for polystyrene microspheres in *M. edulis* (Browne et al., 2008) and eventually lead to distribution within the body, accumulation, or toxic effects. Regarding uptake of nano-TiO_2 by the blue mussel in this study, no conclusion can be drawn based on the effects on the haemocytes alone. Still, based on the literature and the observed behaviour of the nanoparticles in seawater, it can be assumed that nano-TiO_2 enter the mussels through direct ingestion.

5.1 Future work

In order to provide more constant exposure concentrations, future studies should use a flow-through system in the experimental set-up. Stock suspensions of nano-TiO_2 should be kept in ultra-sonic bath, and be delivered to the beakers holding the mussels on a continual and controlled basis together with fresh seawater to give the correct concentrations. Based on the observations on nano-TiO_2 behaviour in the present study, it would probably be necessary to make sure that aggregated and precipitated nanoparticles are cleared out of the systems to avoid a continually increase in the concentrations (on the bottom). Although not significant, a slight decrease in LMS was seen in the group exposed to 1 mg L^{-1} nano-TiO_2. Because of this, together with the fact that nano-TiO_2 has been reported to induce responses in aquatic invertebrates at concentrations as low as 0.2 mg L^{-1} (Canesi et al., 2010) and 0.1 mg L^{-1} (Zhu et al., 2010a), it would be interesting to use lower concentrations in both short- and long-term exposures.

In this work, histological samples of gills, gut, and digestive gland were taken from the mussels included in the exposure study of six days. In future experiments, it would be of great interest to examine these histological samples by TEM, which could reveal the fate of nano-TiO_2 within the mussel, should any uptake have occurred. This has previously been done following exposure to a metal-oxide nanoparticles (nano-SiO_2) in *M. edulis* (Koehler et al., 2008), in which the nanoparticles could be seen inside the digestive cells and the epithelial cells of the gills. Furthermore, analysing the histological samples in light microscope for pathological alterations should be considered as a main focus for future research as the material to be examined is already provided.

To further assess the effects of nano-TiO_2, it would be interesting to investigate whether nano-TiO_2 induces alterations in other biomarkers in *M. edulis*, such as enzymes related to protective detoxification mechanisms or evidence of a disturbed balance between antioxidant defenses and production of ROS.

In the current work, the mussels were not fed during the experimental period. It could be useful, in the future, to investigate whether feeding of the animals during the exposure period has an impact on the mussel's response to nano-TiO_2. The presence of algae can potentially promote uptake of nano-TiO_2 if the nanoparticles interact with the algal cells. On the contrary,

the mussels might selectively ingest algae nanoparticles when available, leading to reduced uptake of the nanoparticles.

Furthermore, as the crystallographic structure (anatase and rutile TiO_2) has been found to impact the toxic effect of nano-TiO_2 (section 2.2), it could be of interest to compare the effects of anatase TiO_2 and rutile TiO_2. The mixed phase composition of nano-TiO_2 that was chosen in the current study has been utilized in several toxicity studies (Warheit et al., 2007b, Zhu et al., 2010a, Long et al., 2007, Zhang et al., 2007). Also, experiments could be conducted in order to compare different sizes of nano-TiO_2, as different sizes within the nanoscale can behave differently.

In a longer time perspective it would be useful to expose other types of test organisms besides the blue mussel, and perhaps even use organisms from two different trophic levels in the ecosystem to investigate the potential for transfer along the food chain and biomagnification. Nonetheless, it would be exciting to introduce other types of nanoparticles as well, in order to compare the responses to different types of nanoparticles. This could allow for investigation on whether there is a common response pattern to all nanoparticles or if all nanoparticles induce a specific set of responses. If a certain pattern of biochemical responses could be detected in the organisms when exposed to nanoparticles in general, this set of biomarkers could be used to reveal exposure to nanoparticles in the field.

6. Conclusion

In this work, the basics of nanoecotoxicology has been explored by reviewing current literature concerning behaviour and fate in the aquatic systems, interactions between nanoparticles and biota, and the toxic impact on aquatic invertebrates, with a particular focus on nano-TiO_2. The behavior of nano-TiO_2 in freshwater and seawater was examined in the laboratory, and the possible effects of *in vivo* exposure to nano-TiO_2 on mussel were investigated.

The results show that nano-TiO_2 can induce sub-lethal effects in the filter-feeding organism under higher concentrations (5 and 25 mg L^{-1}). However, the lowest concentration at which adverse effect was observed in the present study is orders of magnitudes higher than the concentrations predicted to exist in nature. This implies that nano-TiO_2 pose little environmental risk. Still, the aggregation behaviour of nano-TiO_2, suggests an unequal distribution of nano-TiO_2 within the aquatic environment. As aggregation is favoured by higher ionic strength, there is a potential for their build up in the estuarine sediments, which will make the blue mussel a significant target for exposure to higher concentrations of nano-TiO_2. Before definitive conclusions can be drawn regarding the potential risks to aquatic organisms, further exposure studies should be performed, and the concentrations in highly exposed areas should be determined.

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Appendices

Appendix 1. Turbidity measurements of nano-TiO_2 samples

To determine a relationship between concentration of nano-TiO_2 in seawater and turbidity of the samples, several samples of known nano-TiO_2 concentration were measure using a turbidimeter. Raw data are shown below.

Concentration mg/L	NTU	NTU	Mean	% Deviation	NTU-Blank
0	0,43	0,42	0,43	2	
0,02	0,43	0,47	0,45	2	0,03
0,04	0,62	0,59	0,61	2	0,18
0,06	0,78	0,8	0,79	2	0,37
0,08	0,9	0,87	0,89	2	0,46
0,1	1	1,1	1,05	2	0,63
0	0,3	0,3	0,30	0,0000	0
0,1	0,95	0,9	0,93	0,0541	0,63
0,2	1,2	1,2	1,20	0,0000	0,90
0,3	2,2	2,25	2,23	0,0225	1,93
0,4	2,65	2,6	2,63	0,0190	2,33
0,5	3,4	3,4	3,40	0,0000	3,10
0,7	4,4	4,4	4,40	0,0000	4,10
0,9	5,75	5,7	5,73	0,0087	5,43
1	5,95	5,9	5,93	0,0084	5,63
0	2,5	2,5	2,5	0	0
1	7,5	7,5	7,5	0	5
2	13	13	13	0	10,5
3	18	18	18	0	15,5
4	23	23	23	0	20,5
5	26	26	26	0	23,5
0	2	2	2	0	0
7	40	40	40	0	38
10	58	58	58	0	56
15	88	88	88	0	86
0	0	0	0	0	0
20	108	108	108	0	108
25	127	127	127	0	127
30	155	155	155	0	155
40	200	200	200	0	200
50	260	260	260	0	260

Fill color pink	Standard 1.0
Fill color peach	Standard 10
Fill color yellow	Standard 100
Fill color green	Standard 1000

Appendix 2. Data on test organisms

Individual #	Treatment	Anonymous #	Size (cm)	NRRT (min)
20110313-1	Control	22	5,5	150
20110313-2	Control	12	5,5	150
20110313-3	Control	21	6,5	180
20110313-4	Control	10	6	150
20110313-5	Control	27	6	150
20110313-6	Control	11	6	120
20110313-7	Control	16	6	150
20110313-8	Control	39	7,5	180
20110313-9	Control	37	5,5	120
20110313-10	Control	33	5,5	180
20110313-11	1 mg/L	19	5,5	150
20110313-12	1 mg/L	13	6	150
20110313-13	1 mg/L	25	6	180
20110313-14	1 mg/L	31	5,5	90
20110313-15	1 mg/L	4	5	90
20110313-16	1 mg/L	23	5	150
20110313-17	1 mg/L	14	7,5	90
20110313-18	1 mg/L	34	5,5	90
20110313-19	1 mg/L	32	5,5	150
20110313-20	1 mg/L	6	6,5	180
20110313-21	5 mg/L	35	5,5	30
20110313-22	5 mg/L	18	4	120
20110313-24	5 mg/L	38	5,5	60
20110313-25	5 mg/L	15	6	30
20110313-26	5 mg/L	29	6	120
20110313-27	5 mg/L	24	4,5	60
20110313-28	5 mg/L	9	6	150
20110313-29	5 mg/L	40	6,5	90
20110313-30	5 mg/L	8	4,5	90
20110313-31	25 mg/L	20	5,5	90
20110313-32	25 mg/L	17	5,5	60
20110313-33	25 mg/L	30	6	90
20110313-34	25 mg/L	2	6	60
20110313-35	25 mg/L	5	6,5	90
20110313-36	25 mg/L	1	5	90
20110313-37	25 mg/L	36	5,5	120
20110313-38	25 mg/L	26	6	60
20110313-39	25 mg/L	28	5,5	180
20110313-40	25 mg/L	7	7	60

Fill colour orange	1 mg/L
Fill colour yellow	5 mg/L
Fill colour green	25 mg/L