The ecotoxicology of nanoparticles in *Daphnia magna*

by

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Declaration

I declare that all the work presented in this thesis was undertaken by me. I also declare that this thesis was written by me and that the work contained herein is my own responsibility. One publication resulted from this project and was written in collaboration with Prof. Vicki Stone, Prof. Teresa Fernandes, and Dr. Qasim Chaudhry.

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Abstract

Manufactured nanoparticles are increasingly being used in the production of consumer products and appliances. A release in the environment, either intended through remediation or unintended through a spill at production sites, through wastewater or product degradation, is most likely to occur. Due to their small size, nanoparticles have a far greater surface area to unit mass ratio than conventional substances, rendering them potentially more reactive. This project aims to obtain key data on the ecotoxicology of nanoparticles in the aquatic environment. Initially, data from acute and chronic toxicity tests were gathered by exposing the invertebrate *Daphnia magna* to nanoparticles of carbon black, cerium dioxide, silver and titanium dioxide. The endpoints were mortality, moulting frequency, growth and number of offspring. The results indicate that a gradient of toxicity can be drawn, with cerium dioxide being the least toxic, to silver being the most toxic. Also a size dependent increase of toxicity was observed, with exposures to nano sized particles being more toxic than micro sized particles. Uptake and fate of nano sized materials were studied by exposing *D. magna* to fluorescent polystyrene beads of 20 nm and 1000 nm sizes and the results were compared. Both particle sizes were readily taken up in the gut and relocated in storage droplets within the body of *D. magna*. A quantification of the results showed that the mass of 1000 nm sized particles taken up was higher at equal exposure concentrations than the 20 nm sized particle but the excretion rate was higher as well for the 1000 nm particles. However, when assessing uptake as surface area or particle number dose, uptake of 20 nm particles exceeds uptake of 1000 nm particles. To assess the effect of nanoparticles on oxidative stress, the total antioxidant capacity was measured as well as the glutathione concentration of exposed *D. magna*. A decrease in total glutathione in *D. magna* was detected due to exposure to nano sized carbon black, while measuring the total oxidant capacity proved to be impossible due to interferences with the method used.

The results show that, when negative effects are observed, these are more severe in exposures to nanoparticles than their micro sized counterparts and furthermore a clear route of uptake of nanoparticles in the body of *D. magna* can be observed.

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CONTENT

1. Introduction

1.1 Definition of nanomaterials and their uses

The word “nano” has its origin in the Greek language, meaning “dwarf” (Bergeron and Archambault, 2005) and is used today in the scientific community as a prefix for one billionth (Scientific Committee on Emerging and Newly-Identified Health Risks (SCENIHR), 2007). By this definition, a nanometre is $1X10^{-9}$ metre. A nanomaterial (NM) is a “material having one or more external dimensions in the nanoscale or which is nanostructured” (British Standards Institution (BSI), 2007), with nanoscale defined as a dimension of 100 nm or less (Scientific Committee on Emerging and Newly-Identified Health Risks (SCENIHR), 2007). This definition incorporates nanosurfaces, nanosheets and many others. The nanorod, nanofibre and the nanotube can be described as subsets of nanomaterials. Here two dimensions are in the nanoscale. Lastly, nanoparticles have all three dimensions in the nanoscale (Borm et al., 2006, British Standards Institution (BSI), 2007, Scientific Committee on Emerging and Newly-Identified Health Risks (SCENIHR), 2007).

Nanoparticles are not an invention of humanity as they are also widespread in nature. They are produced by volcanoes, as volcanic dust, by algae and natural burning processes such as wildfires (Department for Environment Food and Rural Affairs (DEFRA), 2005, Moore, 2006). Even evaporating sea water produces sea salt nanoparticles (European Commission Community Research, 2005). Other classes of natural occurring nanoparticles are colloids or humic substances (Muirhead and Lead, 2003, Redwood et al., 2005). Humic substances are heterogeneous organic materials with a high molecular weight that are major constituents of soils and aquatic environments. They play a role in the binding and bioavailability of trace metals and trace organic pollutants because of their large surface area and strength of binding (Redwood et al., 2005). Humic substances typically fall in the size range 1 nm to several hundred nm, depending on their source, concentration, solution conditions, extraction and analysis method (Redwood et al., 2005).

Although not from a natural source, nanomaterials can also appear as manmade pollution, for example as ultrafine particles (ultrafine = particle in air of aerodynamic diameter less than 100 nm (U.S. National Library of Medicine, 2009)) emitted by diesel fuelled cars, in smelting processes of metals, heating of polymers or frying (Moore, 2006, Reijnders, 2006). Combustion-derived NPs have been the centre of much research focusing on their role in adverse effects on respiratory and cardiovascular health including both morbidity and mortality (Donaldson et al., 2005).

According to the National Nanotechnology Initiative (National Nanotechnology Initiative, 2009), nanotechnology is defined as the understanding and control of matter at dimensions between approximately 1 and 100 nanometers. Although the term nanotechnology is modern, the usage of nanomaterials dates far back in history. One example is the Lycurgus cup that dates back to the 4th century AD (The British Museum, 2009). The opaque green cup turns to a glowing translucent red when light is shone through it due to colloidal gold and silver incorporated in the cup. The same phenomenon, light scattering by nanoparticles, is used in many cathedral and church windows, dating back to the 10th century (The Royal Society & The Royal Academy of Engineering, 2004).

Science became preoccupied with nanotechnology in the second half of the 20th century, according to several sources (Bergeron and Archambault, 2005, Hardman, 2006, Lauterwasser, 2005, Seaton and Donaldson, 2005), inspired by the talk “There’s plenty of room at the bottom” by the physicist Richard Feynman. The term nanotechnology itself was created by Norio Taniguchi of Tokyo University in 1974 (Lauterwasser, 2005). Other important milestones that enhanced the interest in nanotechnology were the invention of the scanning tunnelling microscope by Gerd Binnig and Heinrich Rohrer in 1982 (Binnig and Rohrer, 1982, Binnig et al., 1982), allowing imaging well in the nanoscale, the discovery of the buckyball (a C_{60} fullerene) in 1985 (Kroto et al., 1985), a new shape of carbon, and leading from that, the discovery of carbon nanotubes in 1991 (Iijima, 1991)(It has to be mentioned here, that the topic of who synthesized the first carbon nanotubes is still under discussion although it is generally agreed that Iijima was the first to synthesize and correctly characterize a

carbon nanotube (Monthioux and Kuznetsov, 2006)). Since then new discoveries and applications for nanomaterials are widespread.

Due to their small scale, there are physical and chemical differences between nanomaterials and their parent bulk material. For example, due to their small size, nanomaterials have a greater surface area per unit mass than their parent bulk material. This renders them more chemically reactive, since more of their surface is exposed to the environment, thus providing more sites for reactions per unit mass. In tandem with enhanced surface-area, effects that change the optical, electrical and magnetic behaviour of materials become more important at the nanoscale (Owen and Depledge, 2005).

Due to their large surface area, nanoscale materials have also an improved catalytic effect when compared to their parent material (Zhang et al., 1998). Quantum dots can be modified through changing of the particle size, to emit light at different wavelengths and can therefore be used to replace dyes in biomedical applications (Chan et al., 2002). Carbon nanotubes are reported to be ten times stronger than steel and 1.2 times stiffer than diamond (Donaldson et al., 2006).

Fullerenes and ultrafine (uf) carbon black, due to their high electron affinity, can produce, if oxygen is available, highly reactive oxygen radicals through electron transfer, like the superoxide anion (Koike and Kobayashi, 2006, Sayes et al., 2004, Wilson et al., 2002). Nevertheless, further studies suggest that C_{60} fullerenes, due to their thirty carbon π-bonds can also react with free radicals and so be an efficient free- radical scavenger (Gharbi et al., 2005).

An early estimate indicated that nanotechnology is anticipated to revolutionise product development and manufacture that could contribute up to one trillion US dollars to the worldwide economy by 2015 (Roco, 2001).

A number of nanomaterials have already found their way into consumer products, such as TiO_2 in paints (Tran et al., 2005), cosmetic formulations (Reijnders, 2006), self-clean glass (Bergeron and Archambault, 2005), and ZnO in sunscreen lotions (Reijnders, 2006). Many other applications are currently

being developed, such as for targeted drug delivery, gene therapy, stain resistant coatings, industrial lubricants, advanced tyres and semiconductors. There are also some more ambitious uses of nanomaterials, such as in bioremediation of polluted environments, which involves their deliberate release into the environment (Boxall et al., 2007, Zhang, 2003).

Some of the nanoparticles widely used, like nano sized carbon black or oxides of titanium, alumina, zirconium or fumed silica have been in mass production for over half a century for a series of applications, ranging from use in pigments and rubber products to cosmetics and as the basis for fine polishing powders used in the microelectronics industry (Borm et al., 2006).

An important application of nanoparticles is in the area of cosmetics. Two significant nanoparticles used for this purpose are TiO_2 and ZnO (Royal Commission on Environmental Pollution, 2008). Their main function is to provide UV protection without the whitening effect of non nanoscale substances. Nanoparticles of aluminium, fullerenes, silver and silicon, among many others, are also widely used today in the cosmetic industry (Miller et al., 2006).

Fullerenes have also been the focus of many scientific studies (Brant et al., 2005, Gharbi et al., 2005, Nakamura and Isobe, 2003, Samal and Geckeler, 2000, Thompson et al., 2001). Fullerenes are carbon based molecules with a different molecular configuration than diamond or carbon black. Their chemical composition resembles that of graphite but they are composed of pentagonal instead of hexagonal rings (Sayes et al., 2004), making it a three dimensional structure. This class of molecules, as already mentioned earlier, were discovered in 1985 and earned their discoverers, Kroto, Curl, and Smalley, the Nobel Prize of Chemistry in 1996. Fullerenes have a potentially broad range of applications, foremost in pharmaceuticals, as a drug delivery vehicle (Nakamura and Isobe, 2003) but also as lubricants (Whatmore, 2005) or semiconductors (Hood, 2004). Carbon nanotubes due to their stability and their excellent electrical conductivity have been used in the manufacture of reinforced composites, sensors, nanoelectronics and display devices (The Royal Society & The Royal Academy of Engineering, 2004).

TiO_2 (induced by UV light) and silver, as bulk materials possess antibacterial properties, which are enhanced when the materials are used at the nanoscale (Lee et al., 2003). This leads to an array of applications, from washing machines (Samsung, 2003), to socks that give anti-microbial protection, preventing bacteria and fungus that cause itchiness and odour (JR Nanotech, 2003). Further applications of silver nanoparticles will be discussed in chapter 3.

1.2 Toxicity of nanomaterials

The evidence from the toxicological studies carried out so far suggest that NPs can penetrate through cellular barriers (Geiser et al., 2005, Oberdörster et al., 2005, Tran et al., 2005), and so could reach different areas within a cell or organism when compared with larger particles. In *in vitro* tests, NPs have been demonstrated to increase production of highly damaging reactive oxygen species (Stone et al., 1998) that can lead to oxidative stress (Stone et al., 1998) and inflammation (Brown et al., 2001). This may result in the intensification of certain pre-existing ailments such as asthma and cardiovascular diseases (Brown et al., 2001).

The two main nanoparticles used in the cosmetic industry, ZnO and TiO_2, are thought not to be able to penetrate healthy skin deep enough to pose a danger to human health (Cross et al., 2007), Gamer et al., 2006). It was stated that the particles were limited to the outer stratum corneum, and were not able to penetrate to the epidermis or dermis of porcine skin (Gamer et al., 2006). Using *in vitro* human epidermal membrane it has been shown, that ZnO causes oxidative stress, cytotoxicity and DNA damage to human epidermal cells even at low concentrations (Sharma et al., 2009). Although the skin presents a good physical barrier to penetration of nanoparticles in humans with healthy skin, people with skin diseases like eczema could still be vulnerable to increased particle uptake and, as a consequence, to absorption and toxicity (Royal Commission on Environmental Pollution, 2008). Another possible gateway of uptake of nanoparticles is through hair follicles. A study showed that uptake of a dye in the hair follicles of porcine and human skin was greater when the dye was incorporated in particles near the nano size (320 nm) than the dye in non-

particle form (Lademann et al., 2007). Although the same report stated that massage was needed in order to reach a significantly higher uptake of the particulate dye compared to the non- particle form. Furthermore, the particle form of dye could still be detected within the hair follicles at 10 days after exposure, while the non-particle dye could only be detected at times of up to 4 days. The study states that by selecting the correct size of particles as drug carriers, an efficient selective drug delivery and storage in the hair follicles is possible.

It has been reported that elevated concentrations of air pollution ultrafine particles (primary particle diameter less than 100 nm) can increase morbidity and mortality arising from pulmonary and cardiovascular causes, with both long term and short term effects (Peters et al., 1997). Exposures to nano sized TiO_2 via inhalation resulted in a higher tendency to cause inflammation in rat lungs when compared with exposures to fine particulate TiO_2 (Ferin et al., 1992). Furthermore, a study using polystyrene beads as model particles (Brown et al., 2001) has also demonstrated that a material with low toxicity can be toxic or bioreactive, when used in the nanoscale due to a much increased surface area. Comparable results have also been obtained in experiments with nanoscale carbon black (Koike and Kobayashi, 2006) and nickel (Zhang, 2003).

A different aspect is added by the exposure to carbon nanotubes (CNT). As NMs they could show enhanced toxicity when compared with larger particles. Nevertheless, they are also fibre shaped and could therefore behave like pathogenic fibres such as asbestos and cause toxicity associated with their needle-like shape (Donaldson et al., 2006). The length and type of the CNT can play a role in their toxicity. Bundled CNT are more likely to be treated by organisms or cells like larger materials and CNTs with lengths of more than 10-20 µm can pose a problem for alveolar macrophages by preventing clearance from tissue and causing continual activation of the macrophages leading to chronic inflammation as well as an accumulation of fibres due to a lack of sufficient clearance (Donaldson et al., 2006). A further study showed that long, multi walled CNTs showed pathogenic fibre like behaviour in mouse peritoneal (abdominal) mesothelial exposures, in contrast to, short and tangled multi-walled CNTs that did not show such behaviour (Poland et al., 2008). Exposure

of human keratinocytes to carbon nanotubes was associated with oxidative stress and apoptosis (Shvedova et al., 2003) and recent toxicity studies on nanotubes using mouse models suggested that workers exposed at the current permissible exposure level may be at risk of developing pulmonary fibrosis (Lin and Datar, 2006, Shvedova et al., 2005).

Here, in contrast, some studies have reported that there are also findings reported that discovered no cytotoxicity on cell cultures resulting from exposures to fullerenes (Levi et al., 2006) or to single wall carbon nanotubes (Wörle-Knirsch et al., 2006). Levi et al. (2006) concluded that a different mode of exposure might be responsible for those results. In the study of Levi et al. (2006) the pristine fullerenes were solubilised in methanol and plated on petri dishes before cells were introduced. Wörle-Knirsch et al. (2006) report interference of the CNTs with reagents used in the MTT assay, a common assay for cytotoxicity, and false positive effects due to that interference. Also impurities of nickel and iron in samples of carbon nanotubes were, according to this article, responsible for the observed toxicity. Both articles show that there is a great need for standardizing methods to make results comparable.

In section 1.1 of this chapter it was mentioned that nanomaterials have a relatively larger surface-area-to-mass ratio than their bulk material. Due to that, they can become more chemically reactive (Lauterwasser, 2005). Duffin et al. (2002) showed that by using an instillation model on acute inflammatory response of rats to low-toxicity poorly soluble particles (PSP) like titanium dioxide, carbon black or polystyrene that the acute inflammatory response can be attributed to their large surface area. Metal particles, like cobalt and nickel in nanoparticle form, also with a high surface area, were more inflammogenic on a mass dose basis. This study therefore suggests that the surface reactivity of such particles is a function of both their surface area and surface reactivity.

A higher surface area with more particles exposed on the surface and able to react, changes also the surface chemistry and surface charge. The charge at the surface influences how the substance will interact with other substances, for

example in which solvents it will dissolve (Royal Commission on Environmental Pollution, 2008). Surface charge also affects whether particles will remain dispersed or will aggregate and agglomerate in a specific medium (Royal Commission on Environmental Pollution, 2008). An aggregate is a cluster of particles held together by strong chemical bonds, while agglomerates are ruled by weaker forces, like hydrogen bonds or van der Waals forces and are defined as loose accumulations (Schulze Isfort and Rochnia, 2009). Aggregation and agglomeration processes play a role in the transport of the material in the environment (Boxall et al., 2007). Aggregation leads to the formation of larger particles which can get more easily trapped in soil or eliminated through sedimentation (Nowack and Bucheli, 2007). The sedimentation can lead in the aquatic environment to an elevated exposure of bottom feeding organism or that the larger particles appear in the prey pattern of animals feeding in the water column. On the other hand, aggregated particles can encounter surfactants in the water that might promote particle disaggregation and dispersion of nanoparticles in the environment (Tran et al., 2005). These surfactants could be manmade, like tensides in wastewater, or natural like humic and fulvic substances. Interactions of NMs with these compounds can influence bioavailability, mobility in the environment and toxicity due to availability to organisms or physical chemical modifications of NMs.

1.3 The ecology of *Daphnia magna* and their role in toxicity testing

Aquatic organisms are widely used in toxicity testing for a variety of reasons. Firstly, the aquatic environment is the ultimate sink for any chemicals which end up in the environment (van der Oost et al., 2003). Secondly, organisms can highlight effects which may not be detectable using standard chemical techniques. Thirdly, current risk assessment techniques require the deployment of standard assays and species for the detection of hazard in relation to specific scenarios (U.S. Environmental Protection Agency (EPA), 2002a). Often standard toxicity tests are also more cost effective than analytical methods that need to isolate the single compounds and give a quick answer that directly relates to the environment. Standard aquatic toxicity tests can be divided into

acute and chronic tests. Acute tests focus on one application or exposure, are short term tests and mostly have mortality as the endpoint. They can be divided into static, non renewable tests, static renewable and flow through tests (U.S. Environmental Protection Agency (EPA), 2002a). Static non-renewable tests are the simplest ones; the test water is not renewed over the test period. They are very cost effective, but are the least realistic and sensitive tests.

Chronic tests simulate several, or a continuous, application and cover a substantial part of the organisms' life cycle. They can have different endpoints that are affected by sublethal concentrations of the contaminant.

Furthermore, volatilisation, degradation and/or adsorption of the toxicant to the exposure vessel have to be taken into consideration (U.S. Environmental Protection Agency (EPA), 2002a). With respect to nanomaterials, sedimentation would play a major role in such studies. In the static renewal test, test organisms are exposed to a fresh solution of the same concentration of test sample at a defined time interval, either by transferring the test organisms from one test chamber to another, or by replacing all or a portion of solution in the test chambers. The risk of volatilisation and degradation is reduced in this method but it is still existent. The organisms are often fed in these tests to keep them in a healthier state (U.S. Environmental Protection Agency (EPA), 2002a). The most accurate method to ensure that the concentration of a pollutant, or effluent, is kept at the required level is the flow-through method. Here the test chambers are provided continuously with the solution or suspension to be tested to keep the concentrations stable. The estimate of the toxicity is more representative than in the other methods and volatilisation, degradation, adsorption to the exposure chamber walls and sedimentation are minimal. The downside of the flow-through method is that it is very labour intensive and requires large amounts of test solution. The method deployed in this study was a static renewal test, with a renewal period of the particle dispersed in water of 24 hours. This provided an acceptable trade off between labour intensity, feasibility in laboratory work and simulating possible natural occurrences.

There are several standard toxicity testing methods developed for a range of test species (Organisation for Economic Co-operation and Development

(OECD), 2006, U.S. Environmental Protection Agency (EPA), 2002a). The most prominent are the cladocerans *Daphnia magna* and *Ceriodaphnia dubia,* the freshwater fish *Pimephales promelas* (Fathead minnow) and *Oncorhynchus mykiss* (Rainbow trout), the midge *Chironomus tentans,* oligochaete like *Lumbriculus variegatus,* several marine and freshwater amphipods and the freshwater green algae *Pseudokirchneriella subcapitata* (formerly *Selenastrum capricornutum*). They are used, depending on their life cycle and habitat, for different types of toxicity tests. There are different protocols for testing pollutants in the water column and attached to the sediment, for freshwater as well as for saltwater (Organisation for Economic Co-operation and Development (OECD), 2006, U.S. Environmental Protection Agency (EPA), 2000, U.S. Environmental Protection Agency (EPA), 2002a, U.S. Environmental Protection Agency (EPA), 2002b). Cladocerans and the fish *Pimephales promelas* are fresh water organisms and live in the water column. They, as well as algae, are suitable for use in water toxicity tests, while amphipods, *Lumbriculus* and the midge *Chironomus tentans* in the larval stage live within or close proximity to the sediment and so are mostly used in sediment toxicity tests.

The cladoceran *Daphnia magna*, on which this study has concentrated, is a well studied planktonic invertebrate. As a member of the phylum Arthropoda, *D. magna* shares with all other members of this phylum an exoskeleton, jointed limbs and a hemocoel as primary internal cavity. The hemocoel accommodates their internal organs and has an open circulatory blood or hemolymph system (Ruppert et al., 2004). The exoskeleton forces members of the phylum Arthropoda to moult, shedding the old exoskeleton in favour of a new one, in order to grow (Rupert et al., 2004). *D. magna* belongs to the subphylum Crustacea. Crustaceans main differences to other Arthropods are the presence of a nauplius larvae, although often suppressed in favour of a more advanced larvae at the hatching stage (Rupert et al., 2004), and biramous (splitted) limps (Hejnol and Scholtz, 2004). The members of the class Branchiopoda and the subclass Cladocera share a two-valved carapace covering most of the body except the appendages. All Cladocerans have an unpaired compound eye which is the result of a fusion of two eyes in the late embryonic development (Ebert, 2005). An also unpaired nauplius eye is located between the compund eye and the mouth (Fig. 1.1).

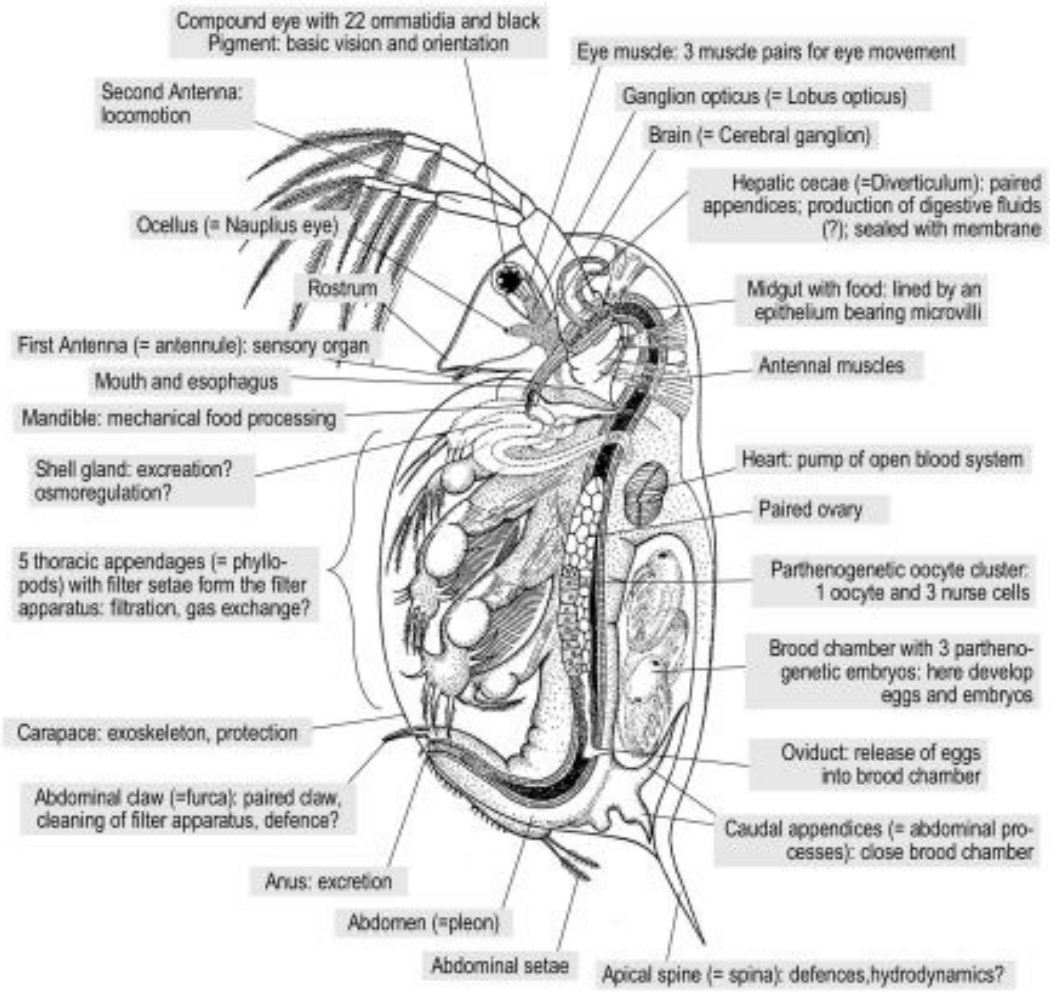


Figure 1.1: The anatomy of *Daphnia* (from Ebert, 2005)

Like other Crustaceans, *D. magna* has two pairs of antennae. The first pair of antennae is located beneath the rostrum and acts as a sensory organ (Ebert, 2005). In female *D. magna* the first pair of antennae is rudimentary and does not protrude over the rostrum, in male *D. magna,* however it is elongated and movable (Mitchell, 2001), Fig. 1.2). The second pair of antennae is used for locomotion.

Figure 1.2: Male *D.* magna with visible first antenna

During most of the year, populations of *D. magna* consist almost entirely of females that reproduce through parthenogenesis (Ebert, 2005). Self fertilized eggs are placed in the brood chamber or broodpouch, which is located dorsally beneath the carapace (Fig. 1.1). Production of males appears to be induced principally by stressful conditions (Mitchell, 2001), including low temperatures or high densities and subsequent accumulation of excretory products, and/or a decrease in available food (U.S. Environmental Protection Agency (EPA), 2002a). In those cases, a parthenogenetic male is produced alongside of females, as well as haploid (resting) eggs in cases called ephippia. Those eggs require fertilization by the male. This occurrence forms the sexual cycle in contrast to the parthenogenetic asexual cycle (Fig. 1.3).

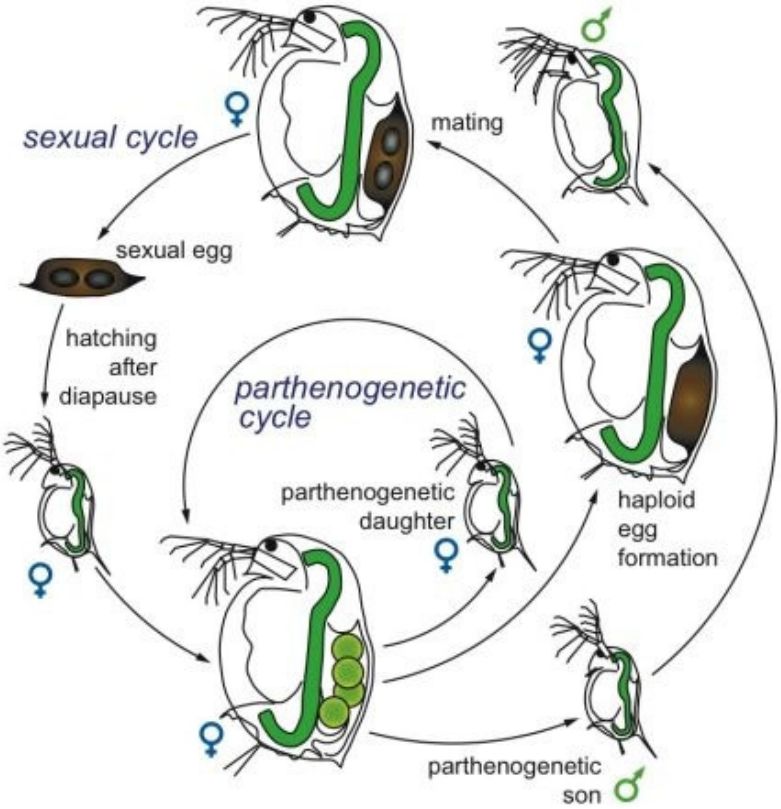


Figure 1.3: the sexual and the asexual (parthenogenetic) life cycle of a *Daphnia* (from Ebert, 2005)

The life history of *D. magna* can be divided into four different stages: the egg, juvenile, adolescent and adult. The life span of a single *D. magna* depends on environmental conditions like temperature or food supply and is roughly 40 days at 25°C, and about 56 days at 20°C (U.S. Environmental Protection Agency (EPA), 2002a). *D. magna* has three to five juvenile instars (developmental stage between moults), followed by a single adolescent instar and 6-22 adult instars. Each instar is terminated by a moult. Under favourable conditions an instar lasts two days but can last up to a week under unfavourable conditions. The average number of eggs per instar is approximately six to ten.

D. magna is principally a lake dweller and has a worldwide distribution in the northern hemisphere (Pennak, 1989, U.S. Environmental Protection Agency (EPA), 2002a). This invertebrate is restricted to waters that exceed a hardness of 150 mg/L (as $CaCO_3$) (Pennak, 1989) and it is mostly found in ponds with muddy bottoms, rich in organic matter and with low oxygen demand (3 to 4 mg/L).

Cladocerans are polyphagous feeders and find their food in the seston (living and non-living particulate matter, suspended in the water column). Daphnids, including *Daphnia magna*, are classified as fine mesh filter feeders (Geller and Muller, 1981), being able to filter particles with a minimum size as small as 200 nm. These fine mesh filter feeders are most abundant in eutrophic lakes during summer phytoplankton blooms.

D. magna plays a very important ecological role in freshwater habitats. This includes a role as a very efficient filter feeder that keeps algal blooms at bay or as a major food source for a whole range of aquatic invertebrates or vertebrates. It is sensitive to environmental conditions and to a whole range of contaminants and has a short life cycle that is observable in the laboratory. It is not surprising that several studies to date have focused on *D. magna* as test species when assessing the effects of nanomaterials in the aquatic environment.

Further aspects of nanomaterials and nanoparticles will be reviewed in later chapters. This will include the ecotoxicology of nanoparticles (chapter 3), uptake and translocation of nanoparticles (chapter 4) and bioindicators and oxidative stress (chapter 5).

1.4 Aims

The intent of this thesis was to assess the effects of selected nanomaterials on *D. magna*, so that data and information that can be used in environmental risk assessments of nanomaterials are generated. To achieve this, three different approaches were chosen, each with a different aim:

- initial acute toxicity assessments with mortality and moulting as endpoints, using *D. magna*, with a panel of particles of different sizes and type. From the results, adequate concentrations were selected for chronic tests with further physiological endpoints (mortality, reproduction, growth rate, moulting frequency) (chapter 3)

- histological experiments were conducted, to observe and quantify the accumulation of micro- and nanoparticles within specific organs of *D. magna* (chapter 4).
- biochemical investigations (oxidative stress) to determine the mechanisms of toxicity as well as sub lethal endpoints (chapter 5)

1.5 Hypothesis

The key testable **Null Hypothesis** in this project was that NPs tested have no different size dependent effects than larger particles of the same composition at the same mass dose on *D. magna.*

2. Culturing of *Daphnia magna*

D. magna were maintained and cultured according to US Environmental Protection Agency (EPA) guidelines (2002a).

2.1 Culture medium

The animals were held in hard reconstituted water as described in Table 2.1.

Table 2.1: *D. magna* culture water

Reagent added (mg/L of DI water)				Approximate final water quality		
$NaHCO_3$	$CaSO_4*2H_2O$	$MgSO_4$	KCl	pH	Hardness	Alkalinity
192.0	120.0	120.0	8.0	7.6-8.0	160-180	110-120

Hardness and Alkalinity expressed as mg $CaCO_3$/L.

The salts (Fisher Scientific, Loughborough, UK, Analytical reagent grade) were dissolved by stirring in deionized water (DI) in a 2L beaker. The salts were added in the following order $CaSO_4*2H_2O$ first, followed by the $MgSO_4$, then KCl and at least two hours later, the $NaHCO_3$. Once the salts were dissolved they were poured into the holding container and aerated with an air pump connected to an air stone with standard aquarium tubing, for at least two hours, preferably over night. The hardness and the pH value were measured after all the salts were dissolved in the reconstituted water. The water was used in experiments or for culturing *D. magna* for up to 14 days before new medium was prepared. The water was aerated with an air pump and an air stone while stored.

2.2 *D. magna* maintenance

D. magna were cultured in 2 L glass beakers containing 1600 ml of reconstituted water and covered with cling film to prevent evaporation of media. When handling the animals, a 5 ml disposable plastic pipette was used, with the tip cut off. The beakers were kept in a climate chamber that was set to 20°C ±1°C and a light/dark cycle of 16h/8h.

To harvest neonates within 24 h of hatching, all gravid (egg carrying) females were placed 24 h before harvest in new culture medium (to remove all older neonates). Within 24 h the newly hatched neonates were harvested. To prevent crowding, the neonates were transferred to fresh medium after approximately two days and the density was restricted to 50 neonates per 1600 ml of medium. After two weeks the animals were further reduced in number to 30 animals per 2 L beaker and 1600 ml of medium. The media in the culture was changed at least once per week to prevent degradation in water quality or overcrowding by neonates.

2.3 Feeding

Cultured single celled green algae of the species *Scenedesmus subspicatus* or *Chlorella sp.* were obtained from the University of Edinburgh and counted using a haematocytometer to obtain the number of cells per ml. An amount of $1x10^5$ cells/daphnid was fed daily to neonates; after 7 d this was increased to $5x10^5$ cells/daphnid. The container with algae concentrate was shaken before use, to guarantee a homogenous suspension. The algae were kept in a refrigerator at approximately 5°C.

3. Acute and chronic toxicity testing with *Daphnia magna*

3.1 Introduction

Relatively few studies have been published in the field of ecotoxicology of nanomaterials. They have all shown that generating a suspension of nanomaterials, such as fullerenes and NP carbon black, in aqueous medium poses a major difficulty. Three different methods have been cited so far in the literature. The first involves the suspension of the NP in tetrahydrofuran (THF) as a carrier solvent, which is subsequently removed by evaporation before suspension of the nanomaterials in water. A study using this method suggested that fullerenes induced oxidative stress in the brains of fish exposed to 0.5 ppm of fullerenes for 48 h (Oberdörster, 2004). A similar method was used to prepare particles when investigating mortality in acute 48h toxicity tests of *Daphnia magna* (Crustacea, Cladocera) to TiO_2 and fullerenes (Lovern and Klaper, 2006) (LC_{50} (48 h) = 5.5 ppm for TiO_2 and 460 ppb for fullerenes). There is evidence though, that residues of THF can remain in clusters of fullerene particles (Andrievsky et al., 2002), and that charge transfer occurs between THF and fullerenes (Brant et al., 2005), causing additional toxicity (Henry et al., 2007, Spohn et al., 2009). The other two methods of NP preparation are sonication for 30min and stirring in water for an extended period of time. *D. magna* did not exhibit any significant mortality when exposed to sonicated TiO_2 over 48 h (Lovern and Klaper, 2006) over a wide range of concentrations. Sonicated fullerenes induced mortality in 48 h exposures (LC_{50} (48 h) = 7.9 ppm) although with great variation and no clear dose response (Lovern and Klaper, 2006). When the fullerenes were suspended by water stirring, no significant mortality was observed within a 48 h exposure (LC_{50} (48 h) > 35 ppm), in contrast to what was observed for the same concentration when the THF method was used (LC_{50} (48 h) = 800 ppb) (Zhu et al., 2006), showing that the method used for suspending fullerenes has a major effect on mortality for both particles, TiO_2 and fullerenes. Nevertheless, an effect of fullerenes

prepared by water stirring can be observed in chronic, 21 day exposures where mortality and reduced offspring were reported in concentrations as low as 2.5 ppm (Oberdörster et al., 2006).

The standard endpoint in acute tests is mortality. The test is mostly conducted to estimate an LC_{50} value (concentration at which 50% mortality occurs or median mortality), a No Observed Adverse Effect Concentration (NOAEC), a Lowest Observed Adverse Effect Concentration (LOAEC) over a specified exposure time span (U.S. Environmental Protection Agency (EPA), 2002a) or to evaluate a concentration range for long term exposures. Mortality is normally assessed by observation of immobilization of the test animals. Mortality studies provide an indication of what would happen if a specific environment was exposed to a short pulse of contaminants, as often happens with pesticides in agricultural application (Pieters and Liess, 2006). Tests can be utilized with adults, juveniles, neonates (newborn), eggs or cell cultures. Mortality has of course the effect on an individual that the organism loses its chances to reproduce and contribute its genes to the gene pool of the population. At population level this can mean a decrease in abundance and fitness. An acute toxicity test with *D. magna* is normally conducted over a 48 h- 96 h period and it is normally too short to allow the assessment of endpoints such as growth or reproduction/fecundity.

A chronic toxicity test spans often over a wider range of the organisms' life cycle, and includes more than one single exposure due to water changes that normally are conducted at regular intervals. Each water change represents a distinctive exposure by replenishing the contaminant to its original concentration, making a long term exposure a string of multiple exposures. Endpoints used to assess the effect of chronic exposures depend mostly on characteristics specific to the organisms' life cycle. These include number of offspring for organisms with a short life cycle, since it is easily countable. Number of eggs or number of eggs hatched, locomotory activity, gill ventilation rate, heart rate, blood chemistry, histopathology, enzyme activity, weight increase or growth are also widely used (U.S. Environmental Protection Agency (EPA), 2002a).

Regarding the ecotoxicological effects of nanoparticles, mostly mortality has been used as an endpoint so far with a range of species (Lovern and Klaper, 2006, Oberdörster et al., 2006, Zhu et al., 2006). Oberdörster et al. (2006) assessed the chronic effects of fullerenes on *D. magna* fertility (number of viable offspring) over a 21 day period. In addition they also assessed effects on the number of moults and mortality over the time period of the experiment. Mortality was recorded at 2.5 ppm and 5 ppm after a lag phase of 4 days and occurred for two days. Afterwards mortality dropped to zero again. The animals reproduced and moulted as well, though at a lesser rate and had a reduced first brood. This could mean that although there seemed to be acclimatisation, the overall fitness of the animals was affected. Since *D. magna* moulting coincides with the release of neonates out of the broodpouch, a prolonged moulting frequency can mean a lower overall fecundity and an impact on population level (Oberdörster et al., 2006). One study so far measured the behavioural and physiological changes of *D. magna* when exposed to nanoparticles (Lovern et al., 2007). In this study the hopping rate (erratic swimming behaviour), heart rate, feeding appendage beat frequency (beating of the thoracic limps that produce the feeding current) and postabdominal curling rate (curling of the postabdomen with the postabdominal claws inwards) in relation to NP concentration were measured. Erratic swimming behaviour makes *D. magna* more visible to its predator and increases the predation risk (Lovern et al., 2007). Increased heart beat generally indicates a faster metabolism and is a sign of stress. A change in the feeding appendage beat frequency points to a change in the feeding rate and energy uptake.

The study here presented focuses on the endpoints of mortality, moulting frequency, fertility and growth, assessed through acute and chronic tests. These endpoints are easily addressed in the laboratory and provide a good overview on the effects nanoparticles may have on individuals and could be used to draw conclusions at population level. Toxicity effects observed in *D. magna* could also be similar in other invertebrate taxa and provide information on further research on the impacts of nanoparticles.

The particles tested in these studies, titanium dioxide, cerium dioxide, silver and carbon black, were chosen because all of them found their way into major

consumer applications. Although just one of the tested particles, titanium dioxide, was so far reported in the environment in nanoscale as a result of mining-wastes (Wigginton et al., 2007), realistic exposure concentrations are still unknown and have been only estimated by models so far (Boxall et al., 2007, Mueller and Nowack, 2008). Furthermore, there are uncertainties of the possible pathways of exposure, their fate to the environment, bioaccumulation and biomagnification (Crane et al., 2008), information that are vital for assessing the risk of nanomaterials to the environment. In the light of these facts it was considered a reasonable approach to assess the hazard of nanoparticles by choosing particles that have, due to a multitude of application, a reasonable likelihood of reaching elevated concentrations in the environment and therefore if a hazard is detected, would pose a threat to the environment. Also, in the case of cerium dioxide, silver and carbon black, particles of nano size and micro size were readily available in the laboratory and made exposures of different sized particles side by side possible.

The highest concentrations used (100 mg/L) in the preliminary 48 h acute studies (see section 3.2) were chosen based on mass doses used in studies published in the literature (Oberdörster et al., 2006, Lovern and Klaper, 2006, Zhu et al.,2006). Lovern and Klaper (2006) used 500 mg/L (500 ppm) of sonicated and unfiltered titanium dioxide in their 48 h acute exposures of *D. magna*, studies with C_{60} fullerenes (Oberdörster et al., 2006, Lovern and Klaper, 2006, Zhu et al.,2006) used mass doses of 10 mg/L and lower. The remaining concentrations were done by 1:10 dilutions to span over a wide range of concentrations. It was refrained from using the highest concentration, 100 mg/L, in the following 96 h acute exposures, due to difficulties described in section 3.3.1. In the 96 h acute exposures the highest concentrations used were 10 mg/L. Concentrations in the chronic 21 day exposures were dependent on the results of the 96 h acute studies since the aim was to assess sublethal endpoints. Therefore exposure concentrations were different for each of the four particles tested.

Since it was impossible at the time of the experiments to characterize the particles in solution, concentrations and size of particles in solution have to be seen as nominal.

The sections 3.1.1 to 3.1.4 give a brief review of the particles used:

3.1.1 Titanium dioxide

Titanium is a transition metal. Its naturally occurring oxide is titanium dioxide. Titanium dioxide accounts for 70% of the total production volume of pigments worldwide (International Agency for Research on Cancer, 2006). As a particle bigger than 100 nm, it is widely used to provide whiteness and opacity to products such as paints, plastics, papers, inks, foods, and toothpastes. It is also used in cosmetic and skin care products, and it is present in almost every sunblock, where it helps protect the skin from ultraviolet light. When used as a nanoparticle in sunscreen and cosmetics, TiO_2 has comparable UV protection abilities as the bulk material, but loses the cosmetically undesirable whitening as the particle size is decreased (Lauterwasser, 2005). As a nanoparticle, it is also used in air and water remediation (Long et al., 2006). Although studies showed that neither micro sized titanium dioxide, used for example in sunscreen, could penetrate the dermal layer of the skin (Lademann et al., 1999, Pflucker et al., 2001), nor nano sized titanium dioxide (Gamer et al., 2006) as already mentioned in chapter 1, an uptake was still considered possible through skin that might be damaged through disease (Lauterwasser, 2005, Royal Commission on Environmental Pollution, 2008). Nevertheless, titanium dioxide entering an organism via other pathways, such as inhalation or injection, is reported to have the potential to cause oxidative stress to a wide range of cell types not as monodispersed particle but often as aggregates of 800 - 1900 nm (Long et al., 2006). In addition, TiO_2 nanoparticles have been shown to cause pulmonary inflammation, tissue damage, and fibrosis at sufficiently high mass doses (Oberdorster et al., 1992, Oberdorster et al., 1994, Tran et al., 2000, Tran et al., 1999).

Due to the many applications of titanium dioxide, a release and possible accumulation in the environment is reported to be likely (Bergeron and Archambault, 2005). In fact, one report (Department for Environment Food and Rural Affairs (DEFRA), 2007) predicts titanium dioxide concentrations in the aquatic environment to be in the µg/l range. Although it is unclear if those

particles reach the aquatic environment in a nano form. When 66 nm particles are applied to water and suspended by vigorously shaking for a short amount of time, the particles aggregate to particles ranging in size from 175 – 810 nm with a mean of 330 nm (Adams et al., 2006, Department for Environment Food and Rural Affairs (DEFRA), 2007).

Lovern and Klaper (2006) assessed the effect of titanium dioxide prepared in two different ways, filtered in the presence of tetrahydrofuran (THF) and sonicated, and detected significant mortality of *D. magna* neonates in the filtered sample but not in the sonicated sample. As mentioned in the introduction (section 3.1), observed mortality could be due to residues of THF in the exposure water. A second study by the same authors (Lovern et al., 2007), which studied behavioural changes in the presence of sublethal concentrations of titanium dioxide, prepared in the same way as above, showed no significant changes at elevated concentration when compared to the control. A study with primary producers has demonstrated that titanium dioxide nanoparticle can also inhibit algal photosynthesis by producing reactive oxygen species that can oxidize the algal cell and stop activity of chlorophyll a within 30 min. if the algae come into contact with a film of TiO_2 coated on glass beads (Kim and Lee, 2005). This study supports the use of titanium dioxide for the remediation of eutrophic water but also demonstrates that toxic effects of titanium dioxide are not limited to invertebrates but also to primary producers.

A draft of a review of the Environmental Protection Agency of the United States about an assessment of potential ecological and health implications of nano sized titanium dioxide (U.S. Environmental Protection Agency (EPA), 2009) states that nano titanium dioxide could enter the environment in various ways, bathing in natural water bodies after application of sunscreen that contains titanium dioxide could be one of them. According to the same review was the annual global production of nano sized titanium dioxide estimated at 2000 metric tons around 2005, with about 65%, or 1300 metric tons, used in “personal care” products such as sunscreens and cosmetics.

3.1.2 Cerium dioxide

Cerium belongs to the lanthanide group of the rare earth metals. It has two common oxidative states, +3 and +4. While cerium also appears in its +3 oxidative state as for example as cerium (III) oxide (Ce_20_3), the most common occurrence is as cerium (IV) dioxide or CeO_2. CeO_2 is commonly used to polish glass, metallic jewellery or lenses (Masui et al., 2003). Additionally it is used as electrolyte in solid oxide fuel cells (Masui et al., 2003, Tok et al., 2007) and as a catalyst in catalytic converters of automobiles (Thill et al., 2006, Tok et al., 2007). Common synthesis approaches include hydrothermal and solvothermal synthesis, sol–gel synthesis and spray pyrolysis (Tok et al., 2007).

As a nanoparticle, cerium dioxide is used as an additive to diesel fuel to act as a fuel borne catalyst (Boxall et al., 2007). Boxall et al. (2007) states that nano sized cerium dioxide is added to diesel at a concentration of 5 - 10 ppm and is claimed to increase fuel efficiency by ~10%, but on the other hand is estimated to release 0.004 g/km of cerium dioxide emission. The same report states, that according to the current knowledge for catalysts, lubricants and additives, cerium dioxide is the most likely nanoparticle used for those applications to enter the aquatic environment.

A cytotoxic effect of cerium dioxide to *E. coli* was reported (Thill et al., 2006) as well as an uptake of ceria nanoparticles in vesicles within the cytoplasm of human fibroblast cells (Limbach et al., 2005). Also 20 nm cerium dioxide was held responsible to induce toxicity in human lung cancer cells caused by oxidative stress in a dose and time dependent pattern (Lin et al., 2006).

The role of cerium dioxide as fuel additive makes an exposure through inhalation the primary concern, but contamination of waterways by runoff produced by rain or spills of diesel fuel by handling make an exposure likely (Boxall et al., 2007).

3.1.3 Silver

Silver, like titanium, is also a transition metal which occurs mostly in the +1 oxidative state and has the highest electrical and thermal conductivity of all metals (Chen and Schluesener, 2008). As a precious metal it was and still is used in a wide range of applications like jewellery, tableware and coinage. Due to its physical properties, it is today also used in electrical contacts and conductors, in mirrors and in the catalysis of chemical reactions. Also, silver has been known, for a long time, to possess antimicrobial character, as silver ions are reported to kill bacteria by inhibiting the expression of enzymes and other proteins essential to ATP production (Soto et al., 2005). These attributes have resulted in the increased use of silver, in its nanoparticulate form, in wound dressings, water filters, food packaging and even clothing (Boxall et al., 2007). In 2003, Samsung introduced the first “Silver Sterilization Washing Machine” which claimed that 99.9% of bacteria would be killed, preventing bacteria and mould and suppress the odour and contamination that accompanies bacteria and mould formation (Samsung, 2003).

In toxicological studies, it has been reported that silver nanoparticles (15 nm) reduced mitochondrial function drastically and increased membrane leakage in mammalian germline stem cells (Chen and Schluesener, 2008) and that aggregated silver nanoparticles are cytotoxic to alveolar macrophage cells as well as epithelial lung cells. While there are studies showing that silver nanoparticles could be used in bone cement or other implantable devices as antimicrobial agents (Alt et al., 2004), other studies show that silver in nanoparticulate form could be toxic for the bone-lining cells and other tissues (Braydich-Stolle et al., 2005).

Concerns over release of nanoparticles in waste water treatment plants has already been mentioned in several reports (Department for Environment Food and Rural Affairs (DEFRA), 2007, Reijnders, 2006, Scientific Committee on Emerging and Newly-Identified Health Risks (SCENIHR), 2007) with waste water enriched with silver nanoparticles adding to these concerns (Luoma, 2008). Another possible source of silver nanoparticles in the environment is the leaching of particles out of food packages. According to a study, the most likely

nanomaterials entering the environment through disposal of food packaging will be clay, and silver nanoparticles (Department for Environment Food and Rural Affairs (DEFRA), 2007). Although the same study estimates the particle burden of silver nanoparticles in water would be, with 0.1 μg at 10% market penetration, low compared to other particles like titanium dioxide or latex, but if toxic effects to aquatic invertebrates could emerge at this concentration is not known.

3.1.4 Carbon black

Carbon black, as a nanoparticle, is in use since antiquity, and today is produced at a rate of 1.5 million tons every year, making it the most abundant nanoparticle (Lauterwasser, 2005). Other sources speak of more than 8 Mt/year of carbon blacks produced, mainly for tires (70%) and the rubber industries (20%) (European Commission Community Health and Consumer Protection, 2004) and for printing (Reijnders, 2006). Here the nanoparticles can improve abrasion resistance and toughness. (Reijnders, 2006) While accidentally produced particles, like in wildfires, are mostly larger, most nano sized carbon black particles are produced intentionally (Donaldson et al., 2005). Nevertheless, for both types, the most common source of carbon black is through incomplete combustion.

Research on inhaled ultrafine carbon black is ongoing in the area of air pollution research for over a decade. A study showed that exposure to ultrafine carbon black particles (surface area = 150 m^2/g; diameter < 0.1 μm) can increase respiratory virus infection risk in mice (Lambert et al., 2003) when mice were exposed to a virus in the presence of ultrafine carbon black as compared to exposures in the absence of ultrafine carbon black. Another study has shown that ultrafine TiO_2 (mean diameter = 29 nm) and carbon black particles (mean diameter = 14.3 nm) impaired phagocytosis by alveolar macrophages more strongly than fine particles of the same materials (TiO_2 mean diameter = 250 nm; carbon black mean diameter = 260.3 nm) (Renwick et al., 2001). A further study, using the same particles and looking at alveolar macrophages of rats, obtained from bronchoalveolar lavage after exposure, detected more epithelial damage and cytotoxicity in exposures to the ultrafine particles than in

exposures to their fine counterparts (Renwick et al., 2004). Increased workplace exposure to carbon black increases respiratory morbidity: increased coughing and sputum production, higher risk of chronic bronchitis and an increase in lung cancer risk (Reijnders, 2006). It has also been shown that carbon black induces inflammation (Donaldson et al., 2005), epithelial injury and that the particles are retained in the lung, allowing dose accumulation (Renwick et al., 2004). One study suggested that differences of inflammogenicity between ultrafine carbon black and larger carbon black particles may be explained through increased surface area or particle number of the ultrafine particle (Brown et al., 2000). Also the amount of oxidative stress induced in epithelial cells might be, at least partially, mediated by surface area of particles (Koike and Kobayashi, 2006).

In the aquatic environment it is predicted that carbon black has only a low toxic effect with thresholds ranging from tens to thousands of parts per million (parts per million = mg/L) (U.S. Environmental Protection Agency (EPA), 2007). But these data are based on 24 hour tests and concentrations for chronic effects are predicted. The actual long term effects of carbon black nanoparticles on aquatic invertebrates are still to be determined. A study focussing on the marine macroalgae *Fucus serratus* showed, that effects of carbon black nanoparticles on sperm concentration and fertilization, body axis alignment, germination and rhizoid elongation were likely to be primarily physical (Nielsen et al., 2008).

3.1.5 Aims

The **aim** of the experiments described in this chapter is to assess the effects of particles of different composition and size on *D. magna* by measuring lethal and sublethal endpoints in acute, short term exposures and chronic, long term exposures at different mass doses. This enables an estimation of whether composition and size play an important role in toxicity.

3.2 Methods:

In this section, the different particles used in the exposures are described, as well as the methodology and the assessment of endpoints for acute, 48h or 96h tests and chronic, 10 day or 21 day tests. In the end, the statistical methods used to verify the results and the models used for quantifying and interpreting the effects are described.

3.2.1 Acute toxicological tests

In the acute exposures, two different regimes were followed. Preliminary studies were conducted in 100 ml beakers with 5 neonates per replicate, exposed for 48 h. These preliminary tests were conducted with the nanoparticle form only. In these acute preliminary studies neonate *D. magna* were exposed to NP titanium dioxide (Degussa P25, average size 25 nm) or NP carbon black (Degussa Printex 90, average size 14 nm).

Later tests were conducted by exposing the neonates to nano sized particles alongside micro sized particles at the same mass dose. Furthermore the animals were exposed individually in 20 ml Scintillation vials with 10 animals per treatment. This allowed a better tracking of mortality and especially moulting since the single animal could be observed over the exposure duration. The test duration was 96 h and all tests were repeated three times. The tested particles were:

- nano sized carbon black (average size 14 nm, Printex 90, Degussa, Frankfurt, Germany);
- micro sized carbon black (average size 260 nm, Huber 990, Degussa, Frankfurt, Germany);
- nano sized silver (average size 35 nm) (NanoAmor, Houston TX, USA);
- micro sized silver (average size 0.6 - 1.6 μm) (NanoAmor, Houston TX, USA);
- nano sized CeO_2 (average size <25 nm) (Sigma- Aldrich, Gillingham, Dorset, UK);

- micro sized CeO_2 (average size <5 μm) (Sigma- Aldrich, Gillingham, Dorset, UK).

The size information provided for silver and CeO_2 was derived from supplier information, while the carbon black particle sizes were derived from Stone et al. (1998).

3.2.1.1 Preparation of the exposure medium in 48h tests

The concentrations chosen for the experiment were 100 mg/L, 10 mg/L, 1 mg/L, 0.1 mg/L of carbon black or titanium dioxide nanoparticles and a medium control (*Daphnia* medium only). The nanoparticles were weighed in a glove box, to avoid contamination of the environment. The material required to make up the highest concentration used in the experiment, 100 mg, was weighed in a scintillation vial. The nanoparticles were transferred into a 1 L volumetric flask and the scintillation vial washed with *Daphnia* medium several times to insure a quantitative transfer. The volumetric flask was then filled 2/3 with reconstituted water (*Daphnia* medium) and sonicated for 30min, then filled up to the 1 L mark to give a final concentration of 100 mg/L. The other concentrations were prepared by 1:10 serial dilution.

3.2.1.2 Exposure protocol for 48 h tests

For acute tests neonates younger than 24 h were used. They were harvested prior to the experiment and pooled to avoid possible differences arising between neonates from different culture beakers. One hundred ml beakers were used as exposure chambers. They were filled with 40 ml of nanoparticles suspended in reconstituted water at the selected concentrations. Three replicates per treatment and one control treatment were used with 5 neonates exposed per replicate. The animals were not fed during the test duration. Mortality and moulting were recorded on a daily basis. Mortality was assumed when no movement could be detected and moulting when a shed carapace was observed. Moulting and mortality were assessed every day at the time equivalent to the time of test setup. The beakers were kept in a climate

chamber that was set to 20 °C ±1 °C and a light/dark cycle of 16 h/8 h. The test duration was 48 h.

3.2.1.3 Preparation of the exposure medium in 96 h tests

The exposure media were prepared as described in section 3.2.1.1, but the concentrations chosen for all particles (carbon black, cerium dioxide and silver) were 10 mg/L, 1 mg/L, 0.1 mg/L and 0.01 mg/L and a control. The exposure to nano and micro sized carbon black additionally included a treatment of 5 mg/L.

3.2.1.4 Exposure protocol 96 hour tests

The exposure chambers were 20 ml scintillation vials (Fisher Scientific Loughborough, UK). Those were loaded via a 20 ml syringe with exposure media and algae. Neonate *D. magna* were then transferred into the exposure chamber. There were 10 replicates per treatment, arrayed on a plastic rack that was able to accommodate 5 treatments. The exposure media was changed daily and *D. magna* was fed with approximately $5X10^5$ algae cells alongside with the water change. Data were collected as described in section 3.2.1.2.

3.2.2 Chronic toxicological tests

The chronic tests were set up in the same way as the acute tests. There was a preliminary 10 day test with carbon black nanoparticles (Degussa Printex 90, average size 14 nm) at concentrations of 1 mg/L, 0.1 mg/L, 0.01 mg/L, 0.001 mg/L and a control. This test was conducted following the protocol described for the acute preliminary tests but with a duration of 10 days.

Like for the acute 96 hour test, a second set of tests was conducted with the animals individually exposed, as described in 3.2.1.4. The particles here were carbon black, cerium dioxide and silver in nano size and micro size as described in section 3.2.1. The test concentrations chosen were dependent upon the outcome in the 96 hour acute tests, and were different for different

particles. The exposure duration was 21 days. The exposure media was changed daily and *D. magna* was fed with approximately $5X10^5$ algae cells alongside with the water change in both types of test.

The endpoint of mortality was recorded by assessing immobilization of the organism, whereas the endpoint of moulting was assessed by registration of a cast of the carapace as described in the previous section.

The length measurement was carried out by preserving the animals after the end of the experiment in 4% formalin and then mounting them on a glass slide under a dissecting microscope (Carl Zeiss, Welwyn Garden City, UK). Photographs were taken with a digital camera mounted on the dissecting microscope. These pictures were analyzed with Image tool for windows (UTHSCSA San Antonio, US). The length of the animals was measured from the base of the spina to the top of the head above the complex eye (see Figure 3.1). The number of offspring (fertility) was assessed by counting the newly hatched neonates in each treatment.

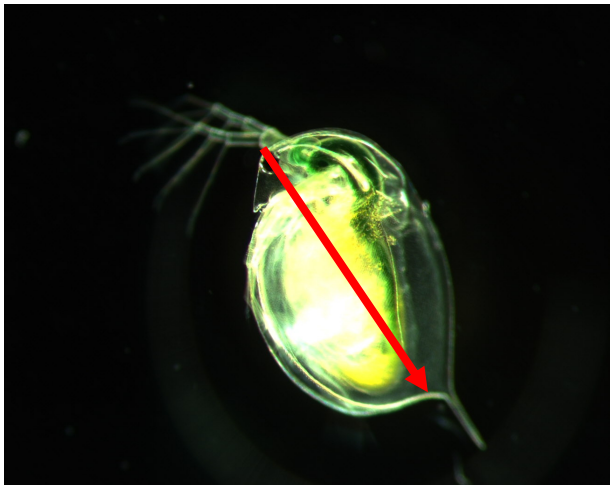


Figure 3.1: Length measurement of *D. magna*

For the test to be valid, at least 80% of the animals of the control treatment needed to survive the test duration (U.S. Environmental Protection Agency (EPA), 2002a). The test was repeated if these criteria were not met.

3.2.3 Statistical methods

Statistical tests were carried out with SPSS release 16 (SPSS Incorp., Chicago, Il, USA). Data were checked for normality and homogeneity of variances.
Mortality was analyzed by comparing the different treatments to the control treatment by means of a Chi square test. Cumulative moulting and cumulative offspring were analyzed by calculating the cumulative effect for each individual on a daily bases. Then the different treatments were compared against each other for each day by one way ANOVA. A Tukey post hoc test was carried out for multiple comparisons of means if the variances of the residuals were homogenous, otherwise a Games-Howell post hoc test was used for the same purpose.

For evaluating and modelling the effects of the different nanoparticles the assumptions described below were made.

<u>For survival</u>

To describe survival due to exposure, a model should include the concentration of particles used, as well as exposure time. These two factors should interact, since the rate of survival decreases faster, the higher the concentration and the longer the exposure duration is. Leaving one factor out of the model (time or concentration) would leave the survival rate unchanged. The assumption was made that a change in the survival rate (d(%Survival)) is best represented by the function of the exponential decay with d(%Survival) proportional to the concentration and exposure time and the decrease of survival dependent from the rate of survival at every time point. These assumptions lead to the differential equations described below:

(1) $d(\%Survival) = (\pm const * Concentration * \%Survival) * d(Time)$

Dividing equation (1) by %Survival and integrating it delivers the following equation:

(2) $Ln(\%Survival) = const_0 \pm const_1 * Concentration * Time$

Or

(3) $$\%Survival = e^{(const0 \pm const1 * Concentration * Time)}$$

The $const_0$ in equation (2) represents Ln(%Survival) at t = 0 and should be close to 4.6 (= Ln(100%Survival)), the term would stay constant if no exposure would take place.

The second term in equation (2), ($\pm const_1$ *Concentration*Time) represents the influence of the exposure to %Survival over time. It shows the strength of the interaction between concentration and exposure time for each of the particles studied. The sign of this term shows the direction of the interaction. A positive sign would indicate a beneficial effect on survival, a negative sign a harmful effect.

Furthermore, assuming the single factors concentration and time additive instead of multiplicative in the equation did not improve the fit of the model significantly. Other models, such as linear models were tested, but showed worse fit results than the logarithmic model.

<u>For moulting</u>

It can be assumed that, contrary to mortality or survival, a base moulting frequency, independent from exposure, has to be taken into consideration. This base moulting frequency is only dependent on time and in this model it was assumed it would increase linearly over time if undisturbed. An exposure to nanoparticles would influence this base moulting frequency positively or negatively, depending on the concentration of the particles and the duration of exposure. Moulting is given after integration as Cumulative(%moult), i.e. the numbers of moults of each animal are added up and only animals alive at the specific time point are taken into consideration. These assumptions lead to the differential equations described below:

(4) $$d(\%moult) = (const_1 \pm const_2 * Concentration) * d(Time)$$

Integrating the equation delivers the following equation:

(5) Cumulative(% moult) = $const_0$ + $const_1$*Time ± $const_2$ *Concentration*Time

Since $const_0$ >0 in equation (5) would mean there is moulting before the start of the experiment and this constant proved to be insignificant in all regressions, equation (5) can be re-written as:

(6) Cumulative(%moult) = $const_1$*Time ± $const_2$ *Concentration*Time

This model assumes that under normal conditions, as previously mentioned, moulting of *D. magna* should increase linearly in time, given by the term "$const_1$*Time". The interaction with nanoparticles that may interfere with moulting is given by the term "± $const_2$ *Concentration*Time" with ± $const_2$ indicating the strength of the interaction. A positive sign would indicate an increase in moulting, a negative sign a decrease.

<u>For offspring production</u>

It was assumed, that the production rate for offspring would behave similarly to moulting in a linear way if undisturbed, dependent on time. An exposure to nanoparticles would influence the production of offspring positively or negatively, dependent on the concentration of the particles and on the time of exposure. As with moulting, offspring is given after integration as Cumulative (%offspring), i.e. the numbers of offspring of each animal are added up and only animals alive at the specific time point are taken into consideration. These assumptions lead to the differential equations described below:

(7) d(%offspring) = ($const_1$ ± $const_2$*Concentration)*d(Time)

Integrating equation (7) delivers the following equation:

(8) Cumulative(%offspring) = $const_0$ + $const_1$*Time ± $const_2$ *Concentration*Time

The cumulative offspring was assumed to increase linearly from the day the first offspring was observed (day 10). The term "$const_1$*Time" in equation (7) gives the increase in offspring dependent on time without an interaction with nanoparticles, while the term "±$const_2$ *Concentration*Time" in equation (7) adds the effect of exposure to nanoparticles, with ±$const_2$ showing the strength of the interaction as well as the direction. The constant $const_0$ in equation (7) is needed here to accommodate the fact that the animals were neonates at the beginning of the experiments and started to reproduce in the course of the exposures from day 10 onwards. To model the production of offspring from the start of the experiment, a negative constant $const_0$ has to be implemented so the equation reaches zero on day 10.

Significance was assumed with a $p \leq 0.05$.

3.3 Results

The results in the following chapter are presented with the preliminary 48 h acute exposures first, than the 10 day chronic study, followed by the 96 h acute studies, the 21 day chronic studies and in the end the modelling of the effects of the various nanoparticles. Within these sections, results for carbon black will be presented first, than cerium dioxide (or titanium dioxide) and silver last.

3.3.1 Acute 48 h toxicity tests

3.3.1.1 Mortality

In the acute exposure of *D. magna* to TiO_2 nanoparticles (25 nm), only 10% mortality was observed in the highest concentration of 100mg/L after 48 h (n=20 per treatment in 4 replicates) (data not shown). Mortality was not significantly elevated when compared to the control at any concentration over a 48 h period. It was observed, that *D. magna* readily took up suspended particles at even low concentrations and these could clearly be seen in the gut (Fig. 3.2).

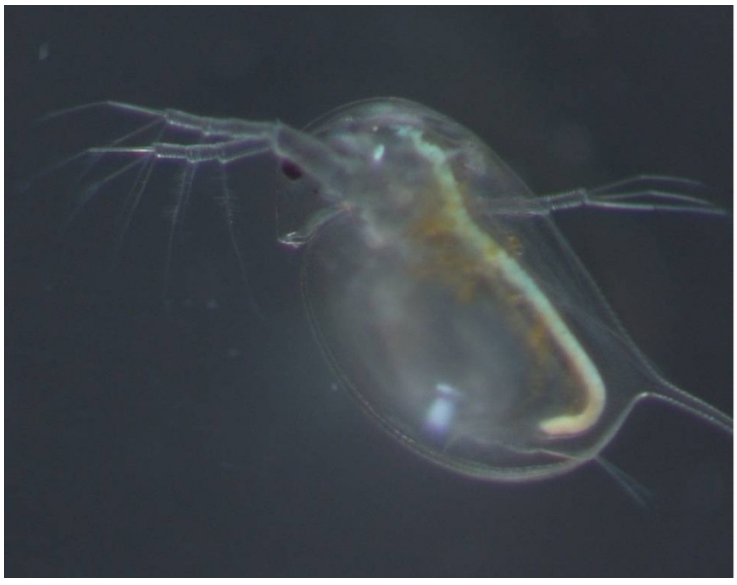


Figure 3.2: *D. magna* after exposure to 0.1 mg/L nano sized titanium dioxide for 48 h

In the acute exposure to NP carbon black (14 nm), 13.33% mortality was observed in the 10 mg/L concentration after 48 h. Due to agglomeration of carbon black mainly in the 100 mg/L treatment, 40% of the total animals could not be accounted for, that is they were not easily visible (n=15 per treatment in 3 replicates). The neonate *D. magna* individuals were often not clearly distinguished from the agglomerates of carbon black nanoparticles. Therefore, no LC_{50} could be calculated (data not shown). As with titanium dioxide, an uptake and accumulation of particles in the gut was observed (Fig. 3.3). It was also observed, that animals exposed to carbon black and titanium dioxide NPs were covered in particles, mainly in the higher concentrations of 10 mg/L and 100 mg/L (Fig. 3.4). In these treatments, a clear impairment of the animals swimming ability was observed.

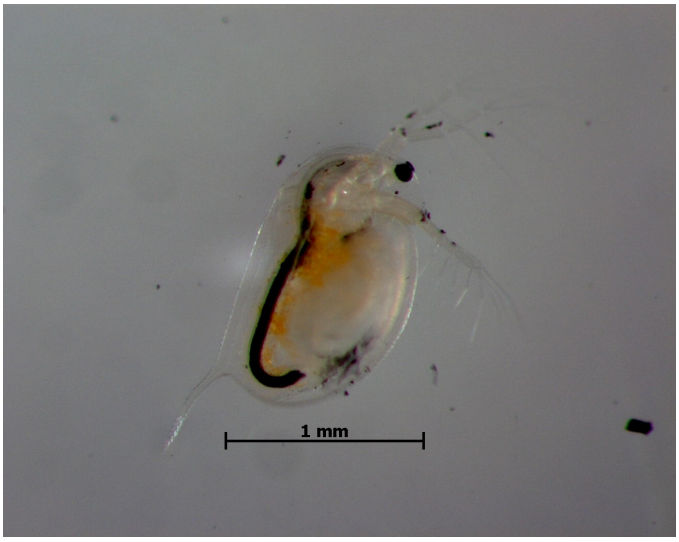


Figure 3.3: *D. magna* neonate exposed to 1 mg/l 14 nm carbon black for 48 h. The carbon black particles had been ingested and can be seen in the gastrointestinal tract

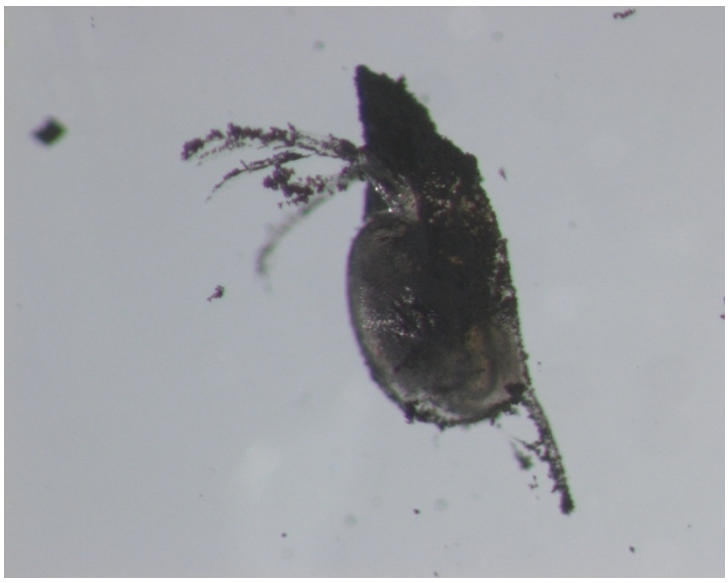


Figure 3.4: *D. magna* after exposure to 10 mg/L nano sized carbon black for 48 h

3.3.1.2 Moulting

A concentration dependent increase in moulting was observed on treatment of *D. magna* with TiO_2 nanoparticles (25 nm) over a 48 h period (Fig. 3.5). After 24 h the 100 mg/L of TiO_2 treatment resulted in the highest occurrence of moulting, while after 48 h, 10 mg/L showed a higher moulting frequency. After testing the data for a normal distribution, a one way ANOVA was done along with Tukey's pairwise comparisons. For the moulting frequency there was a significant increase in moulting, dependent on the TiO_2 concentration. After 24 h the moulting frequency of the animals in the treatment exposed to 100 mg/L of TiO_2 was significantly higher ($p= 0.001$) when compared to the control, the 0.1 mg/L and the 1 mg/L treatment. In the time period of 24 h to 48 h the 100 mg/L treatment was no longer significantly higher than the control, the 0.1 mg/L and 1 mg/L, but the 10 mg/L treatment was significantly higher than all other treatments ($p= 0.001$) including the 100 mg/L treatment. The cumulative moult (Fig. 3.6) showed a similar trend with 10 mg/L being significant higher after 48 h than the control, the 0.1 mg/L and 1 mg/L treatment. The exposure to 100 mg/L of TiO_2 resulted in a significant higher moulting frequency after 48 h than the control and the 0.1 mg/L treatment.

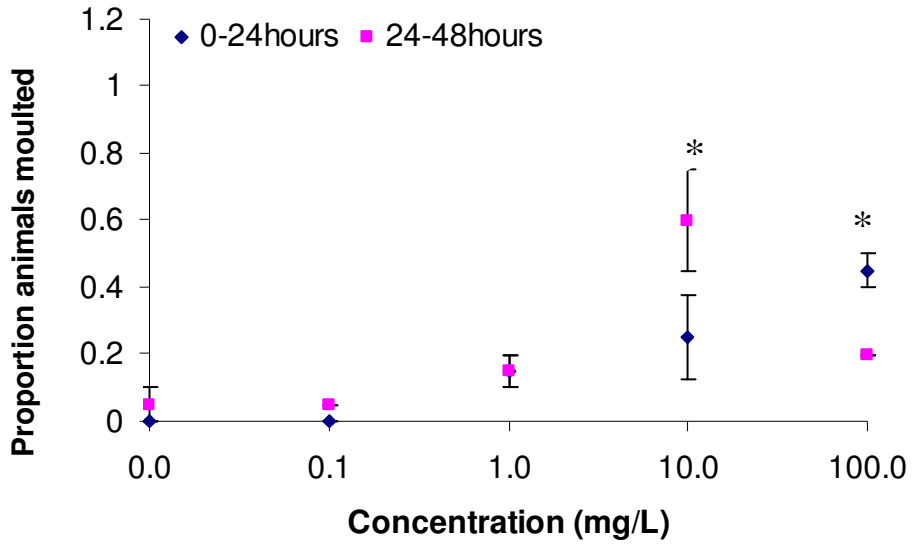


Figure 3.5: Moulting frequency of *D. magna* treated with TiO_2 particles of 25 nm calculated on a day to day basis (four replicates per treatment with n= 5 per replicate, values are means +/- standard error, * indicates a result with $p \leq 0.05$)

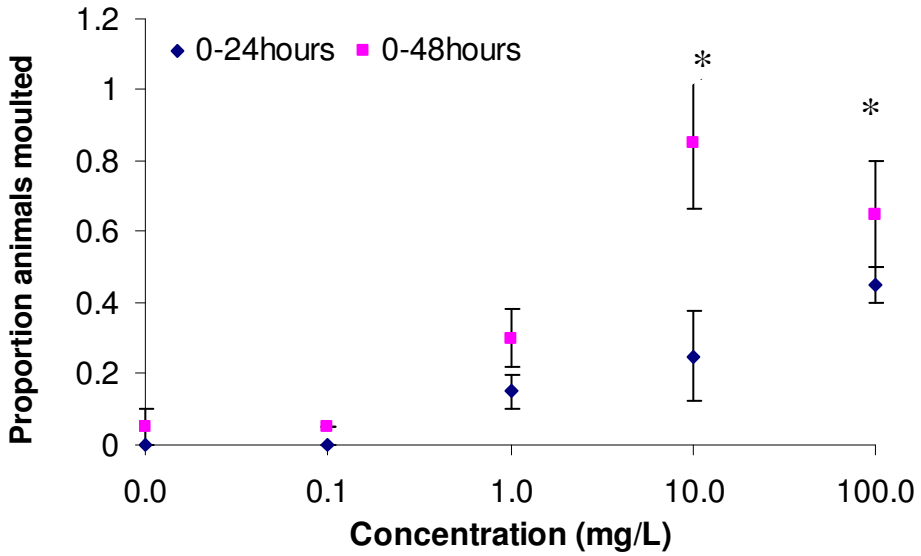


Figure 3.6: Cumulative moults of *D. magna* treated with TiO_2 particles of 25 nm. Values are the total number of animals moulting over the indicated time periods (four replicates per treatment with n= 5 per replicate, values are means +/- standard error, * indicates a result with $p \leq 0.05$)

3.3.2 Chronic 10 day toxicity test

3.3.2.1 Mortality

In a 10 day chronic test with nano sized carbon black no mortality was observed for the first 48 h (Fig. 3.7). Mortality of animals was observed at day 3 in the 1 mg/L treatment and the 0.1 mg/L treatment. A one way ANOVA for day four shows that there is a significant difference between the treatments (p= 0.002), with mortality in 1 mg/L being significantly higher than in the other treatments. A comparison of the remaining treatments at day 10 shows no further significant difference between the treatments.

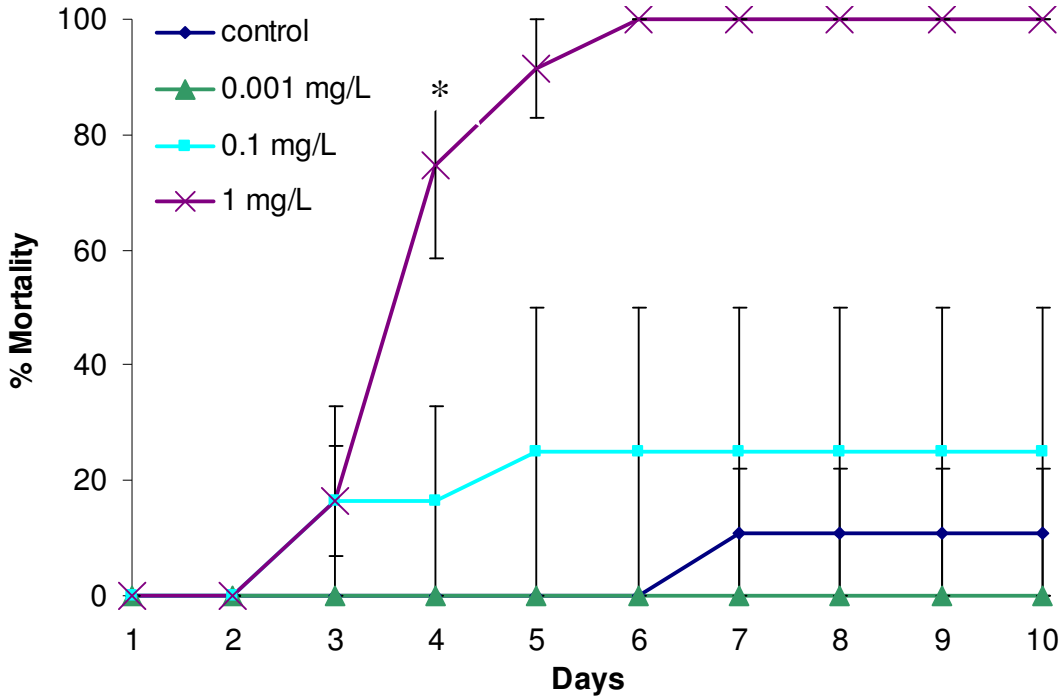


Figure 3.7: % Mortality of *D. magna* treated over time with several concentrations of NP carbon black (four replicates per treatment with n= 3 in each replicate, values are means +/- standard error, * indicates a result with p ≤ 0.05)

3.3.2.2 Moulting

When comparing the moulting frequency due to exposure to titanium dioxide across the different concentrations on the different days (Fig. 3.8), a one way ANOVA along with Tukey's pairwise comparison indicates that just day one

showed a significant difference (p= 0.007) between the animals in the treatments, with the animals in the 1 mg/L treatment having a significantly higher amount of moults than the animals in the control and the 0.001 mg/L treatment. A comparison of the cumulative moult of *D. magna* (Fig. 3.9) between the different concentrations over the test period showed that there was a significant difference in moulting between the animals in the 1 mg/L treatment, the control animals and the animals in the 0.001 mg/L treatment on day one (p= 0.007) and a significant difference between the moults of the animals in the 1 mg/L treatment and the control at day two (p= 0.018), but no significant difference between the 1 mg/L treatment and the 0.001 mg/L treatment. On day 3 there was no longer a significant difference between the treatments. Mortality was too high in the 1 mg/L treatment on day 5 to detect any significant differences between this treatment and the other treatments.

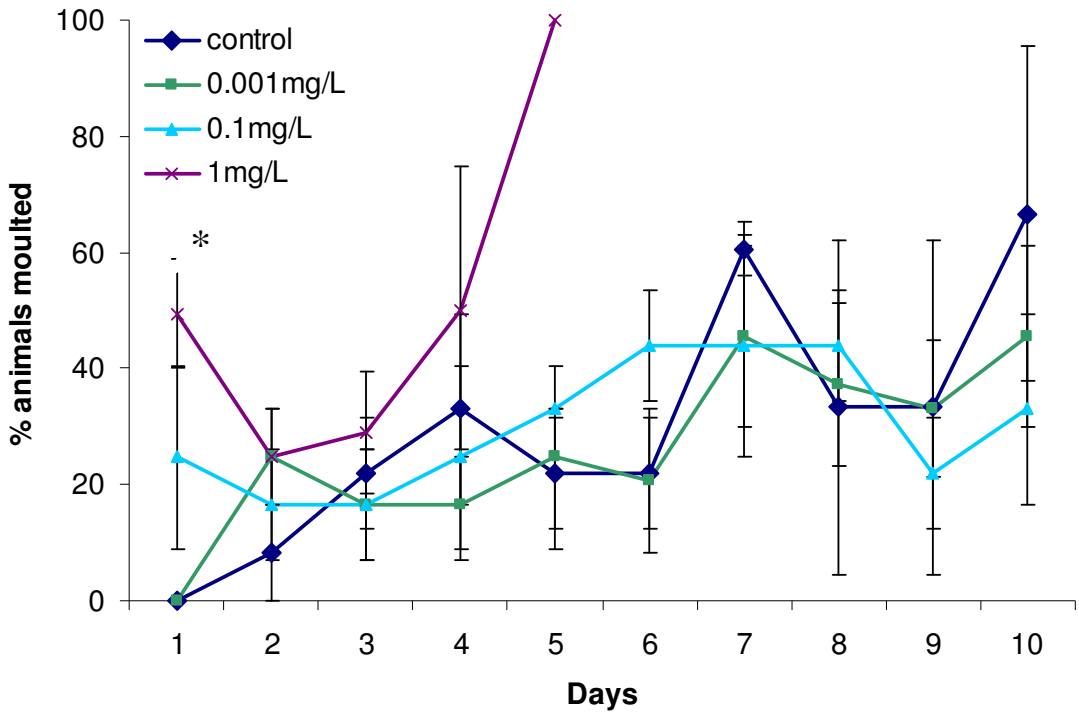


Figure 3.8: moulting frequency due to exposure to titanium dioxide calculated on a day to day basis (four replicates per treatment with n= 3 per replicate, values are means +/- standard error, * indicates a result with p ≤ 0.05)

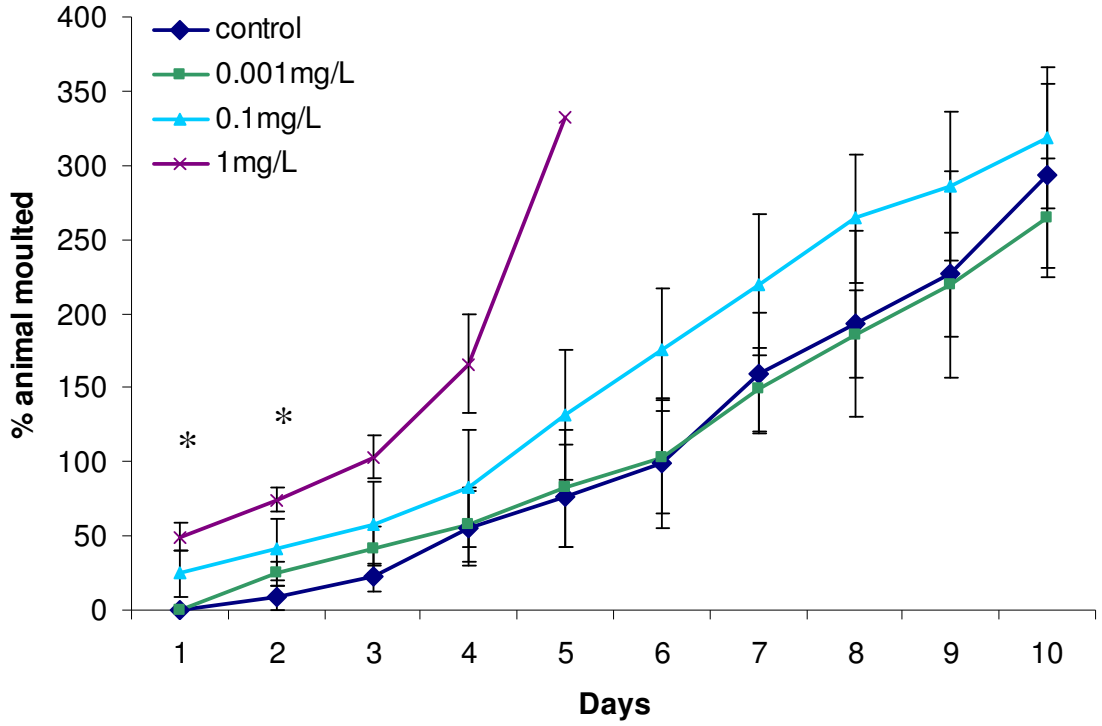


Figure 3.9: Cumulative moults due to exposure to titanium dioxide added up per treatment (four replicates per treatment with n= 3 per replicate, values are means +/- standard error, * indicates a result with $p \leq 0.05$). One hundred % means every animal per treatment moulted once on average

3.3.2.3 Length measurement

A length measurement of the remaining animals at the end of the exposure to titanium dioxide showed that there is a significant difference between the control treatment and the 0.001 mg/L treatment (Fig. 3.10), with the animals from the 0.001 mg/L treatment being larger ($p= 0.018$). There was no significant difference between the 0.1 mg/L and the control or 0.001 mg/L treatment. The data were tested by a one way ANOVA and a Tukey's pairwise comparison test.

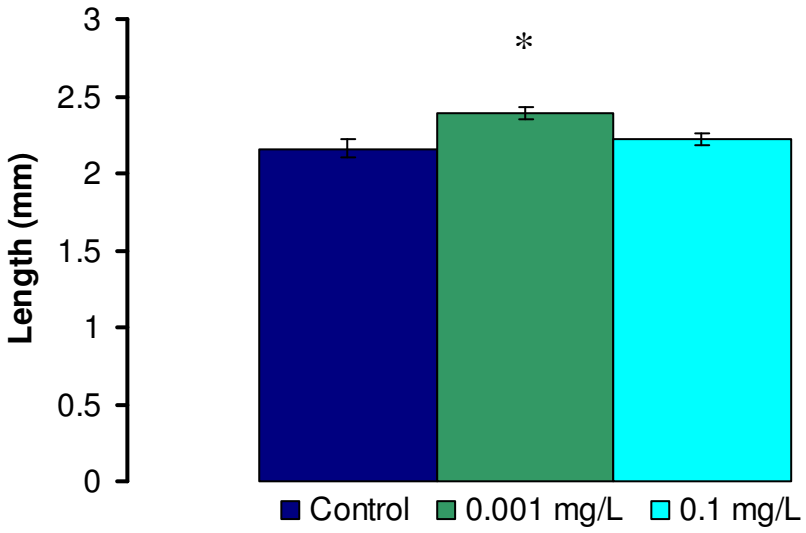


Figure 3.10: Length measurements (control and 0.001 mg/L n= 6, 0.1 mg/L n= 8, values are means +/- standard error, * indicates a result with $p \leq 0.05$)

3.3.2.4 Offspring

The release of offspring was not observed in the different replicates during the duration of the exposure. Nevertheless, the occurrence of animals carrying eggs was observed at day 10 in all remaining treatments independent of exposure concentration.

3.3.3 Acute 96 h exposures to micro and nano sized carbon black

3.3.3.1 Mortality

Mortality of *D. magna* in the nano sized (14 nm) and the micro sized (260 nm) treatments differed greatly when compared at the same mass dose. While animals in the micro sized treatment did not show any significant mortality over the test period (Fig. 3.11), there was significant mortality of *D. magna* in the nano treatments for the two highest concentrations (Fig. 3.12). Animals in the 10 mg/L and 5 mg/L concentrations showed significant higher mortality at day 2 when compared to the control (10 mg/L $p < 0.005$, 5 mg/L $p = 0.009$) with 100%

mortality at day 4. Mortality differed significantly in the three repetitions of the different nano carbon black exposures for the 5 mg/L and to a lesser extent for the 10 mg/L exposure on days two and three as represented by the standard error. On day four, all replicate experiments reached 100% mortality in the 5 mg/L exposure as well as the 10 mg/L exposure.

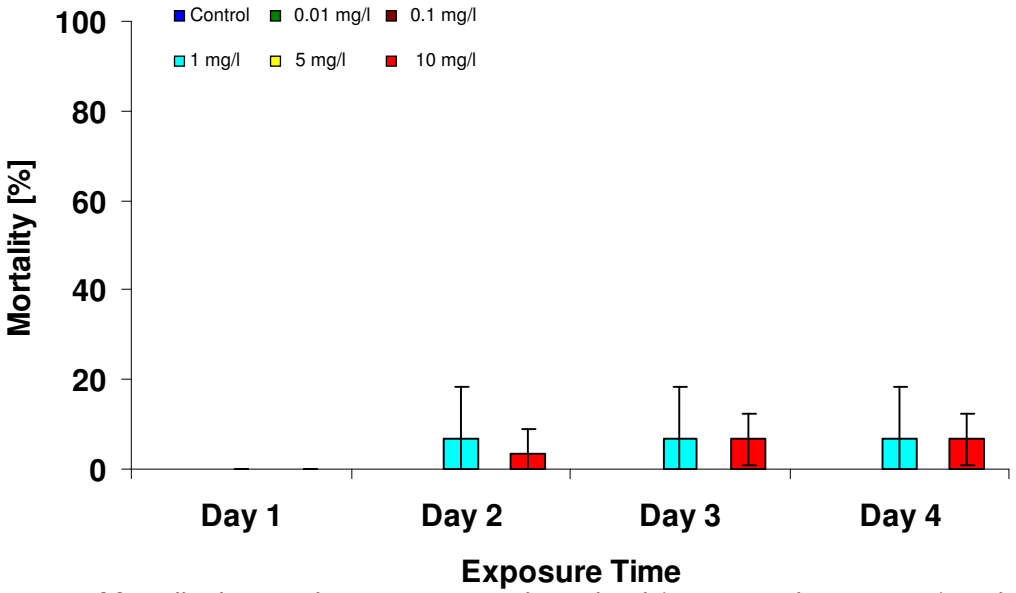


Figure 3.11: Mortality in a 96 h exposure to micro sized (average size 260 nm) carbon black (0.01 mg/L n= 10; 5 mg/L n= 20; otherwise n= 30, values are means +/- standard error). No significant mortality was observed.

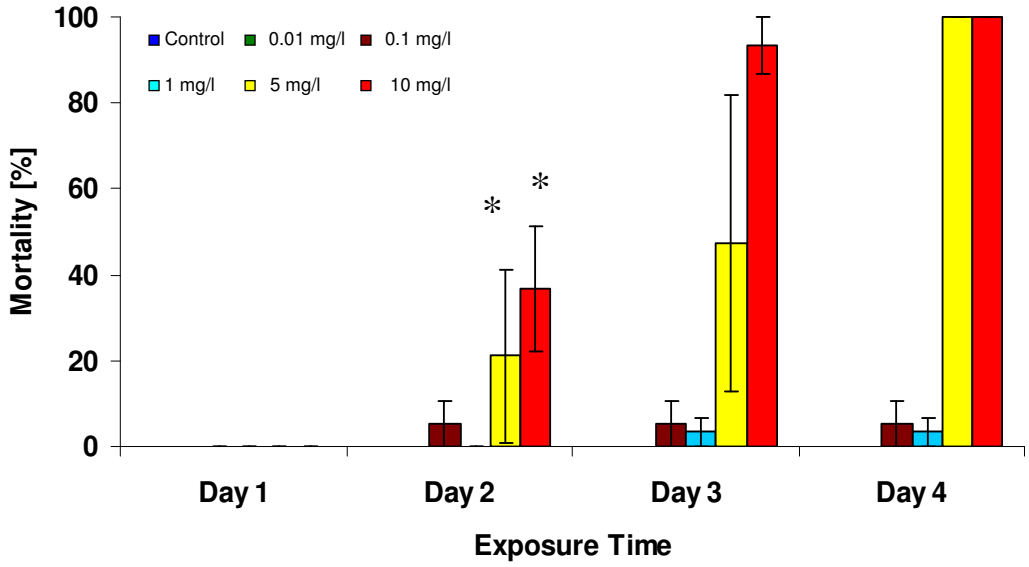


Figure 3.12: Mortality in a 96 h exposure to nano sized (average size 14 nm) carbon black (0.01 mg/L n= 10; 5 mg/L n= 20; otherwise n= 30, values are means +/- standard error, * indicates a result with $p \leq 0.05$). Since the data had no homogenous variances, a non parametric post hoc test was used.

3.3.3.2 Moulting

In order to assess any differences in moulting across treatments, the cumulative moulting was calculated. The percentage of moults of surviving animals was noted on a day to day basis and added up. While an exposure to 260 nm carbon black particles seemed to have no effect at the tested concentrations on the moulting frequency (Fig. 3.13), there was a significant reduction in the moulting frequency of the animals for the exposures to nano sized carbon black (Fig. 3.14). Animals in the 5 mg/L treatment and 10 mg/L treatment showed a significant reduced moulting frequency already at day 1 (5 mg/L p = 0.001, 10 mg/L p = 0.029) which stayed significantly lower on day 2 as well (5 mg/L p = 0.024, 10 mg/L p = 0.006). A cease in moulting of the animals at day 3 in the 5 mg/L treatment and 10 mg/L treatment of nano sized carbon black was observed. Since 100% mortality was recorded in these two treatments, no moulting could be observed on day 4. The data were tested by a one way ANOVA and a Tukey's pairwise comparison test on a day to day basis.

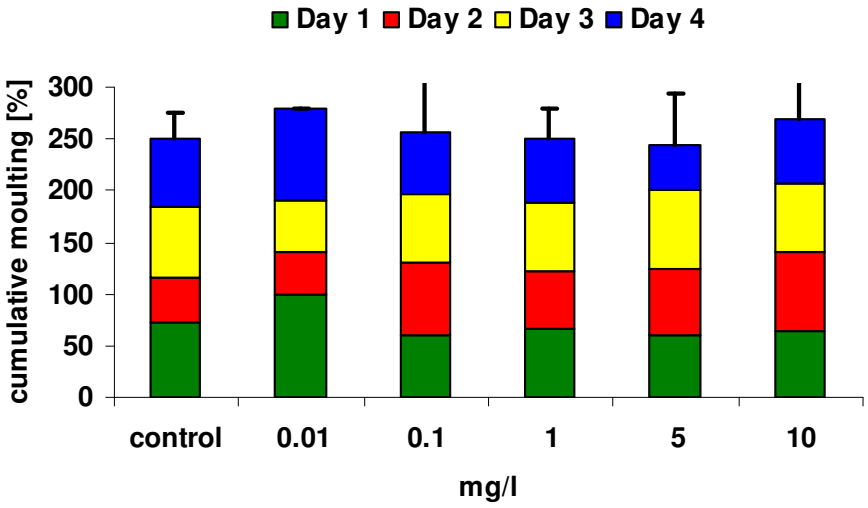


Figure 3.13: Cumulative moulting in a 96 h exposure to micro sized (average size 260 nm) carbon black (0.01mg/L n= 10; 5mg/L n= 20; otherwise n=30, values are means +/- standard error). No significant difference in the cumulative moulting between treatments was observed.

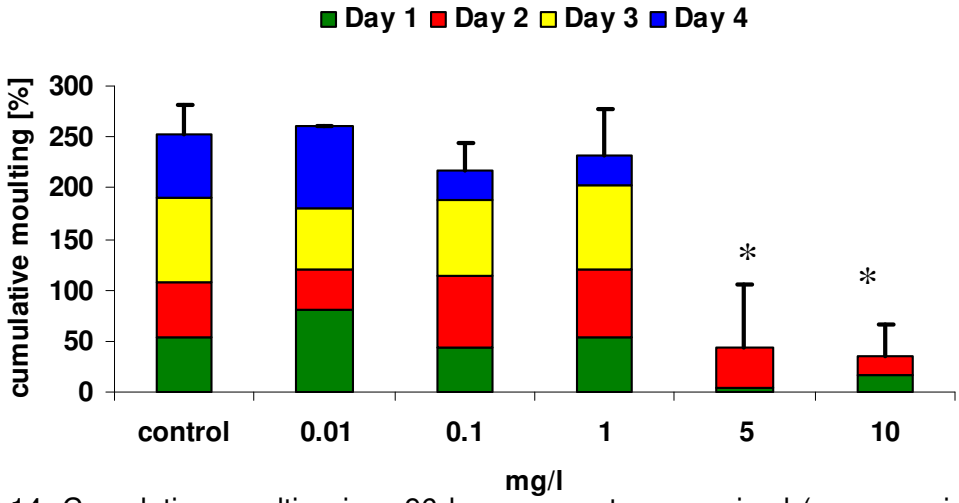


Figure 3.14: Cumulative moulting in a 96 h exposure to nano sized (average size 14 nm) carbon black (0.01 mg/L n= 10; 5 mg/L n= 20; otherwise n= 30, values are means +/- standard error, * indicates a result with $p \leq 0.05$). Since the data had no homogenous variances, a non parametric post hoc test was used.

3.3.4 Acute 96 h exposures to micro and nano sized cerium dioxide

3.3.4.1 Mortality

There was no significant mortality observed in the 96 h test period for either CeO_2 particle size at any concentration and time point tested (Fig. 3.15 and Fig. 3.16)

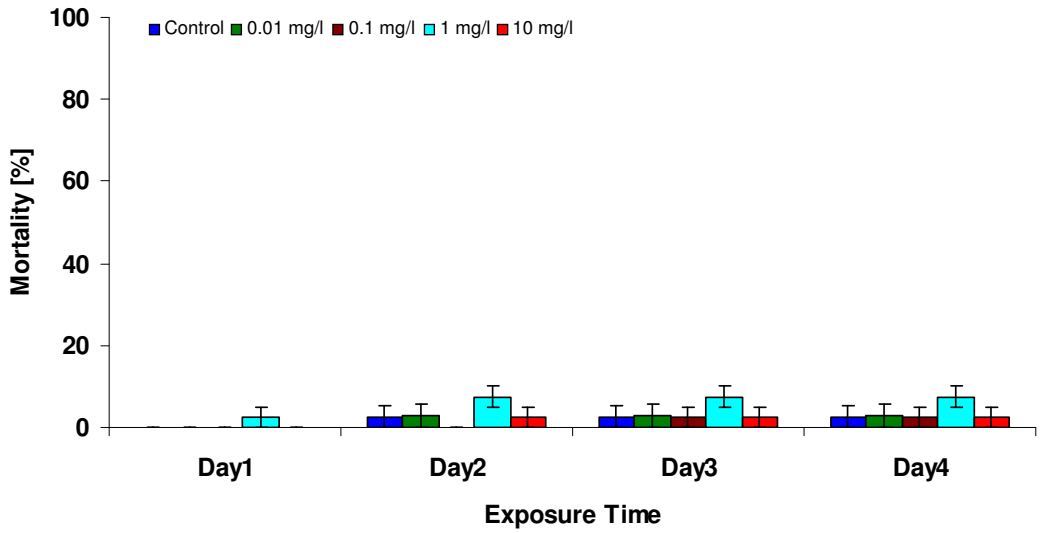


Figure 3.15: Mortality in a 96 h exposure to micro sized (average size < 5000 nm) cerium dioxide (n=30, values are means +/- standard error). No significant mortality was observed in all treatments when compared to the control.

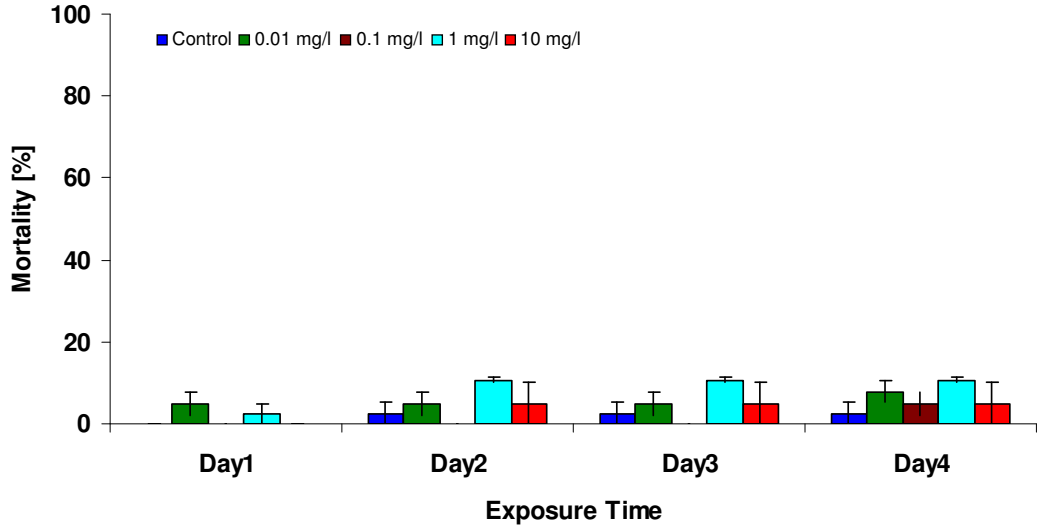


Figure 3.16: Mortality in a 96 h exposure to nano sized (average size < 25 nm) cerium dioxide (n=30, values are means +/- standard error). No significant mortality was observed in all treatments when compared to the control.

3.3.4.2 Moulting

An exposure to micro sized (average size <5000 nm) CeO_2 particles had no significant effect at the tested concentrations on the moulting frequency of *D. magna* (Fig. 3.17).

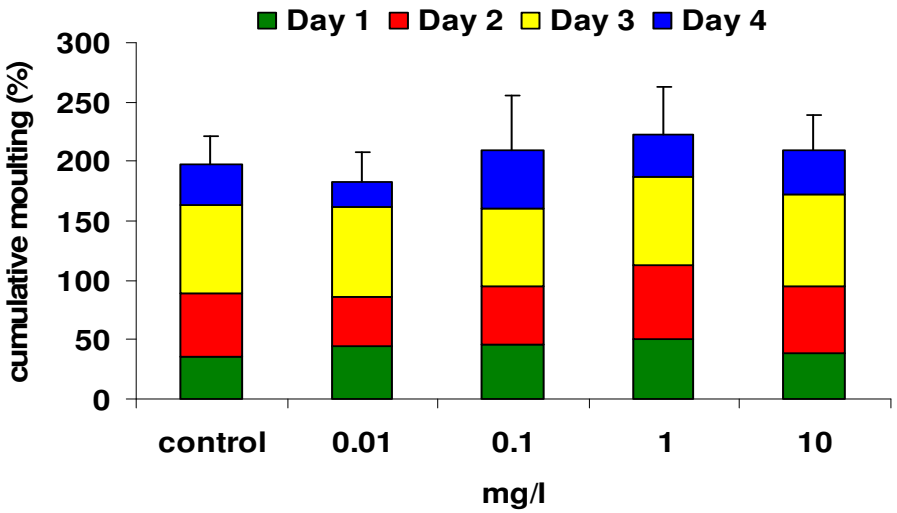


Figure 3.17: Cumulative moulting in a 96 h exposure to micro sized (average size <5000 nm) cerium dioxide (n=30, values are means +/- standard error). No significant difference in the cumulative moulting between treatments was observed.

In the exposure to nano sized cerium dioxide (average size <25 nm) there was a significant reduction in the cumulative moulting frequency of *D. magna* for the highest concentration of 10 mg/L (Fig. 3.18) from day 3 onwards ($p < 0.001$) when compared to the animals in the other treatments or the control.

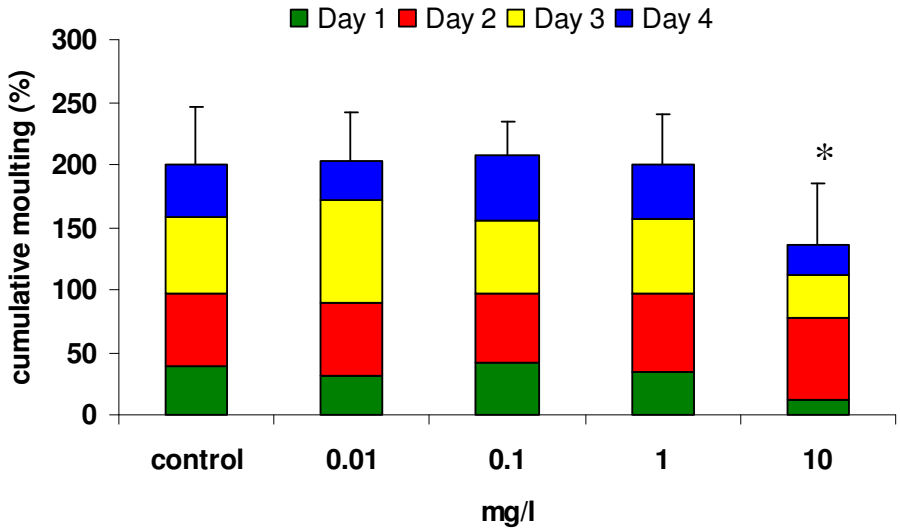


Figure 3.18: Cumulative moulting in a 96 h exposure to nano sized (average size <25 nm) cerium dioxide (n= 30, values are means +/- standard error, * indicates a result with $p \leq 0.05$).

3.3.4.3 Length measurement

Due to an obvious length difference in the exposed animals, the animals were measured as described in section 3.2.2. A significant reduction was observed in the size of *D. magna* after 96 h for micro-sized CeO_2 (0.01 mg/L $p = 0.008$) and nano-sized CeO_2 (0.01 mg/L $p = 0.003$ and 10 mg/l $p < 0.001$), when compared to the control treatment (Fig. 3.19). The 10 mg/L nano exposure also induced a significant decrease in animal size compared to all other treatments ($p < 0.001$).

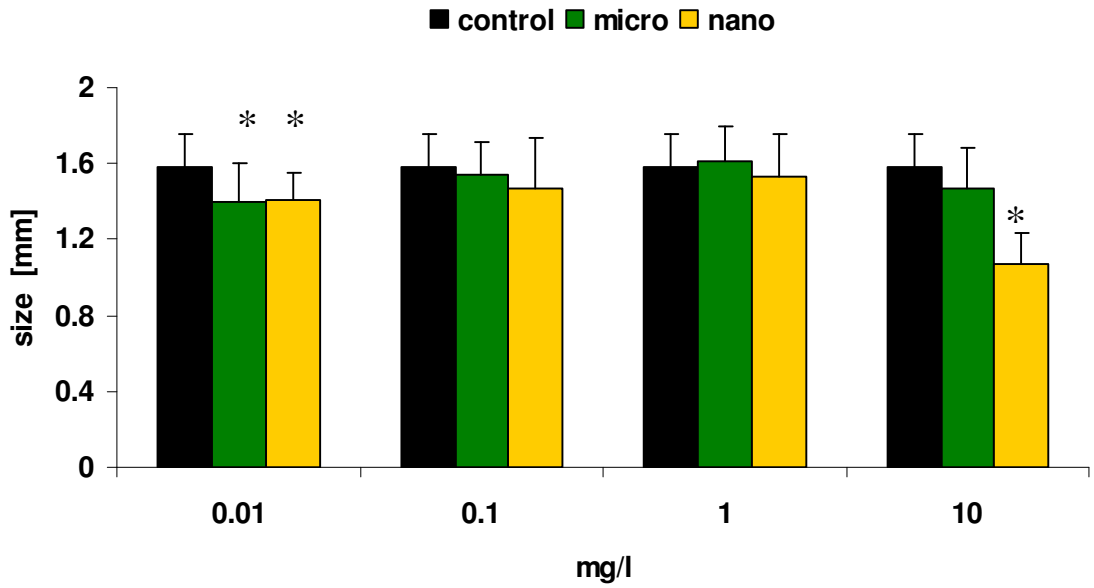


Figure 3.19: Size measurement to determine growth after a 96 h exposure to nano (average size <25 nm) and micro (average size <5000 nm) sized cerium dioxide (micro 0.01 mg/L; 0.1 mg/L; 10 mg/L and nano 0.1 mg/L; 10 mg/L n= 28; micro 1 mg/L n= 27; nano 1 mg/L n= 25; nano 0.01 mg/L n= 24; pooled control from nano and micro exposure n= 32, values are means +/- standard error, * indicates a result with $p \leq 0.05$).

3.3.4.4 High concentration exposure

Due to the results in the first acute tests with cerium dioxide in which no significant mortality was observed, a further test with higher concentrations was set up. The procedure was the same as in the earlier tests. The concentrations used were 50 mg/L, 40 mg/L, 20 mg/L, 10 mg/L and a control (Fig. 3.20). The test was repeated twice. No significant mortality was observed when the treatments were compared to the control.

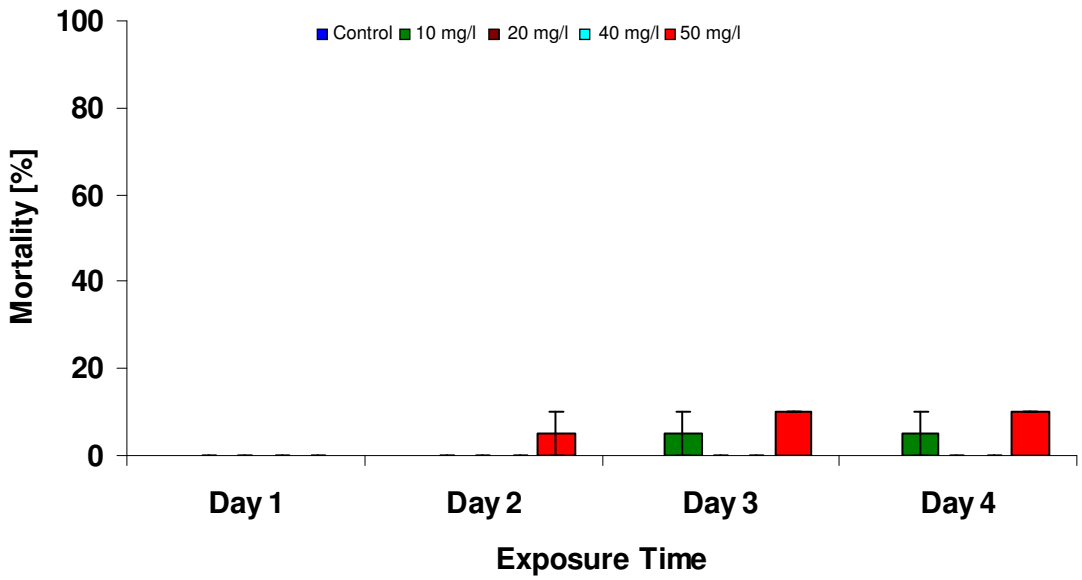


Figure 3.20: Mortality in a 96 h exposure to nano sized (average size < 25 nm) cerium dioxide (n= 20, values are means +/- standard error).

3.3.5 Acute 96 h exposures to micro and nano sized silver

3.3.5.1 Mortality

The study with Ag particles (35 nm nano-silver and 600-1600 nm micro-sized silver) showed dose- and size-dependent toxicity to *D. magna*. Significant mortality of *D. magna* was observed in the 1 mg/L and 10 mg/L treatments of the exposure to micro sized silver already on the first day, when compared to the control ($p < 0.001$) (Fig. 3.21). On day four, the animals exposed to 0.1 mg/L of micro sized silver showed significant higher mortality than the control treatment as well ($p = 0.038$).

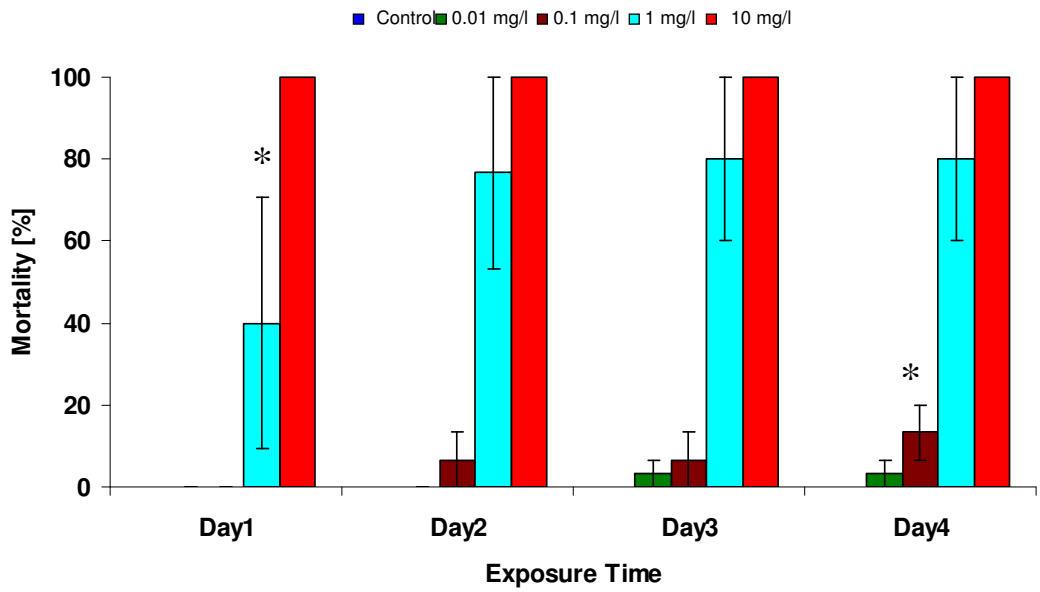


Figure 3.21: Mortality in a 96 h exposure to micro sized (average size 600-1600 nm) silver (n=30, values are means +/- standard error, * indicates a result with $p \leq 0.05$). Since the data had no homogenous variances, a non parametric post hoc test was used.

The nano silver exposure resulted in higher mortality than the micro silver exposure (Fig. 3.22). Significant mortality was observed in the 1 mg/L and 10 mg/L ($p < 0.001$) and the 0.1 mg/L ($p = 0.038$) treatments already on the first day when compared to the control.

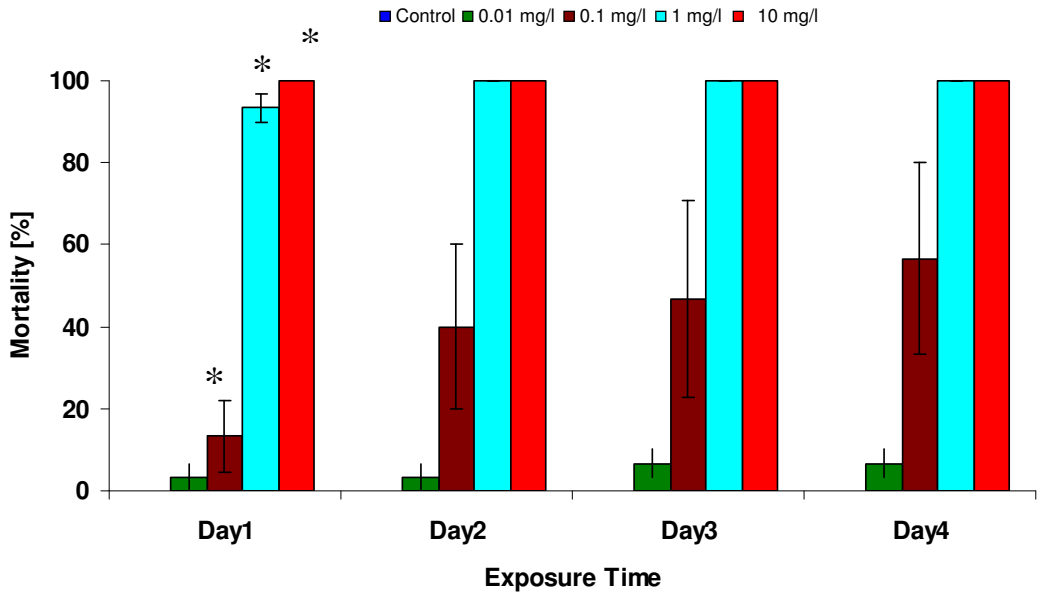


Figure 3.22: Mortality in a 96 h exposure to nano sized (average size 35 nm) silver (n= 30, values are means +/- standard error, * indicates a result with $p \leq 0.05$). Since the data had no homogenous variances, a non parametric post hoc test was used.

3.3.5.2 Moulting

The assessment of cumulative moulting in exposures to silver micro and nano sized particles was heavily influenced by mortality. The animals in the 10 mg/L treatment reached 100% mortality in the micro sized silver exposures, in less than 24 h and could therefore not be assessed. No other significant differences could be observed in the silver micro particle exposures, when compared to the control (Fig. 3.23).

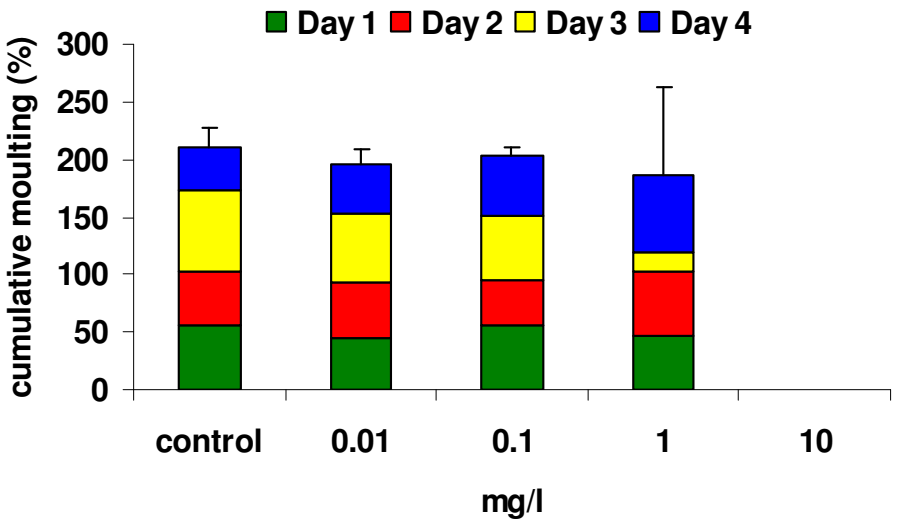


Figure 3.23: Cumulative moulting in a 96 h exposure to micro sized (average size 600-1600 nm) silver (n=30, values are means +/- standard error).

Similar influences of mortality on cumulative moulting were observed in the exposures to nano sized silver particles (Fig. 3.24). Here, the 10 mg/L treatment also reached 100% mortality in less than 24 h and could therefore not be assessed. The 1 mg/L treatment had 93% mortality after 24 h and 100% mortality after 48 h. The animals surviving the first 24 h in this treatment did not moult. No other significant differences could be observed, when compared to the control.

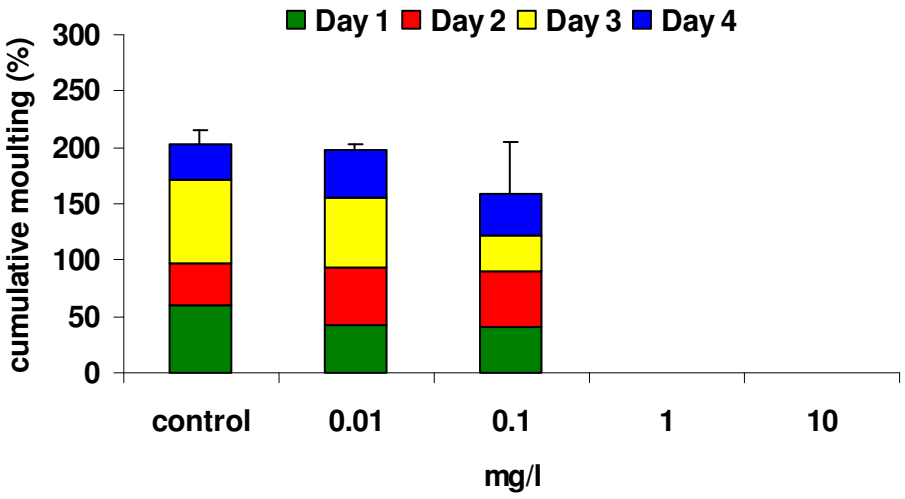


Figure 3.24: Cumulative moulting in a 96 h exposure to nano sized (average size 35 nm) silver (n= 30, values are means +/- standard error).

3.3.5.3 Length measurement

The surviving *D. magna* of the exposure were measured to assess differences in growth over the exposure duration (Fig. 3.25). A significant reduction of length was observed in the *D. magna* after 96 h of treatment to the micro-sized silver particles of a concentration of 1 mg/l ($p = 0.004$) and nano-sized silver treatment of 0.1 mg/l ($p < 0.001$), when compared to the control treatment. The 1 mg/L and 10 mg/L nano treatment as well as the 10 mg/L micro treatment could not be assessed, due to 100% mortality at these concentrations after 96 h.

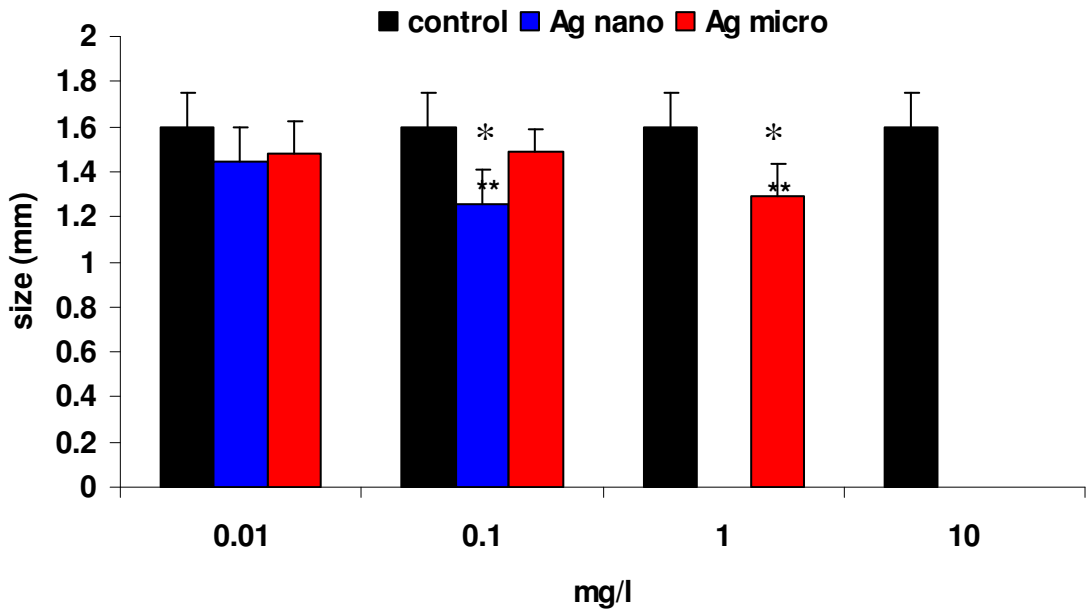


Figure 3.25: Size measurement to determine growth after a 96 h exposure to nano (average size 35 nm) and micro (average size 600-1600 nm) sized silver (micro 0.01 mg/L n= 27; 0.1 mg/L n= 26; 1 mg/L n= 6 and nano 0.1 mg/L n= 12; 0.01 mg/L n= 28; pooled control from nano and micro exposure n= 34; values are means +/- standard error, * indicates a result with $p \leq 0.05$).

3.3.6 Chronic 21 day exposure to micro and nano sized carbon black

The chronic 21 day test, in contrast to the acute 96 h tests, was just conducted once. The concentrations chosen were lower than the ones used in the acute tests, given that sub lethal endpoints were being assessed. These were 2 mg/L, 1 mg/L, 0.5 mg/L and 0.1 mg/L for both sized particles (260 nm and 14 nm).

3.3.6.1 Mortality

No mortality due to exposure to micro and nano sized carbon black was observed in either the nano (average size 14 nm) or micro (average size 260 nm) treatment.

3.3.6.2 Moulting

The moulting frequency was analyzed as cumulative moulting over the duration of 21 days. In the exposure to micro sized carbon black (Fig. 3.26) it was observed, that there was no difference in cumulative moulting of *D. magna* between the various concentrations. The same is true for the exposure to nano sized carbon black (Fig. 3.27). No significant reduction in the cumulative moulting between treatments was observed on day 21, although animals in the 2 mg/L treatment showed significant lower cumulative moulting than the control on day 5, 8 and day 11 ($p < 0.05$). Nevertheless, it reaches approximately the same number of moults after 21 days and the differences at the end of the experiment were not found to be significant.

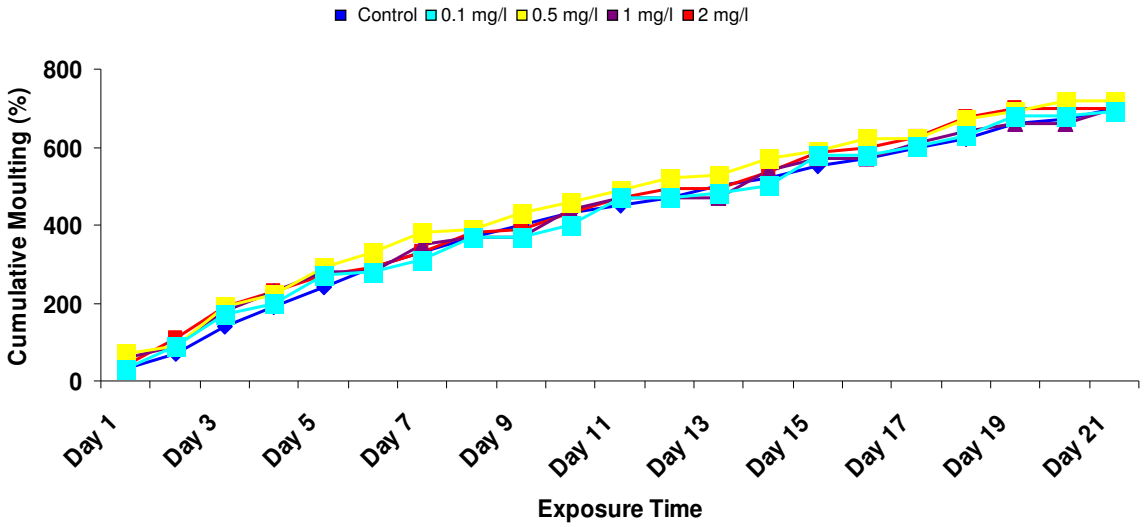


Figure 3.26: Cumulative moulting in a 21 day exposure to micro sized (average size 260 nm) carbon black (n= 10 per treatment). No significant reduction in the cumulative moulting between treatments was observed.

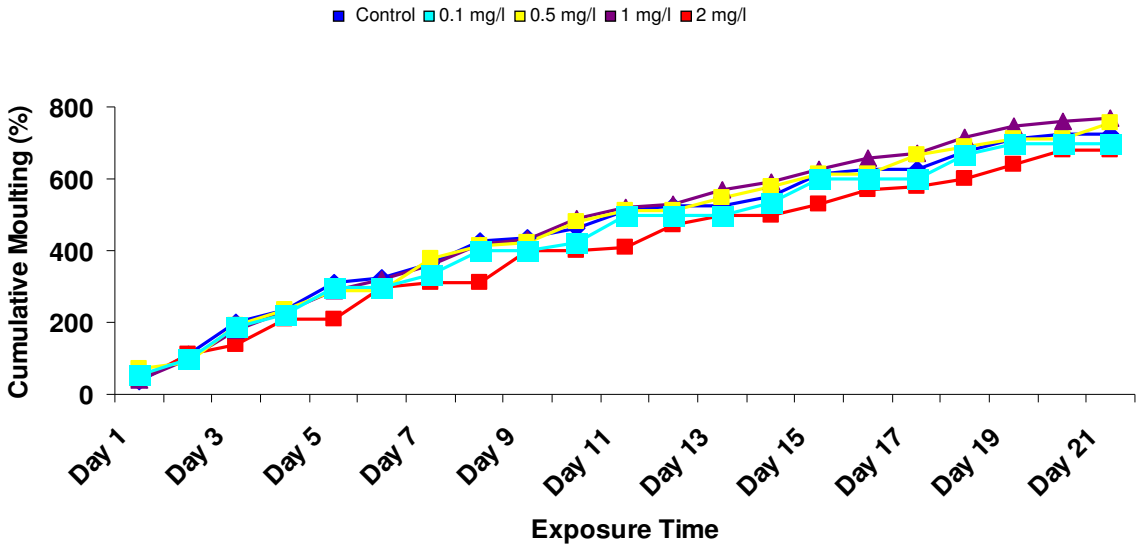


Figure 3.27: Cumulative moulting in a 21 day exposure to nano sized (average size 14 nm) carbon black (n= 10, per treatment). Since the data had no homogenous variances, a non parametric post hoc test was used.

3.3.6.3 Length measurement

Assessing growth after exposure to micro sized carbon black (average size 260 nm) it was observed that there was no significant difference in size found between animals in the different 21 day treatments (Fig. 3.28). The same mass dose led to different results in the exposure to nano sized carbon black (average size 14 nm) (Fig. 3.29). Here, a significant reduction was observed in the size of *D. magna* exposed to nano sized carbon black at a concentration of 2 mg/L, when compared to the length of the animals in the control treatment and all other treatments ($p < 0.001$). Animals in the 1 mg/L treatment were significantly reduced in length when compared to animals in the control treatment ($p = 0.003$), but this value was not significantly different from what was recorded for animals in the 0.1 mg/L or 0.5 mg/L treatments.

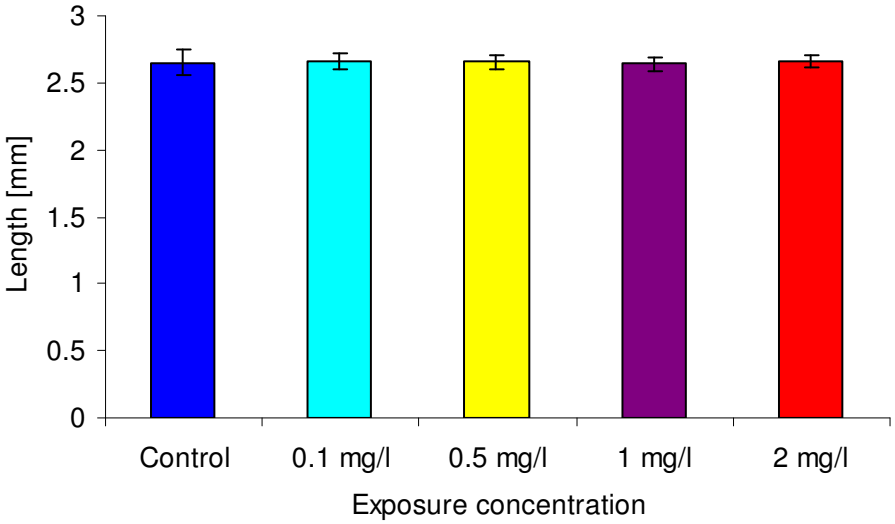


Figure 3.28: Size measurement to determine growth after a 21 day exposure to micro (average size 260 nm) sized carbon black (control, 1 mg/L and 0.5 mg/L n= 10; 0.1 mg/L, 2 mg/L n= 8; values are means +/- standard error).

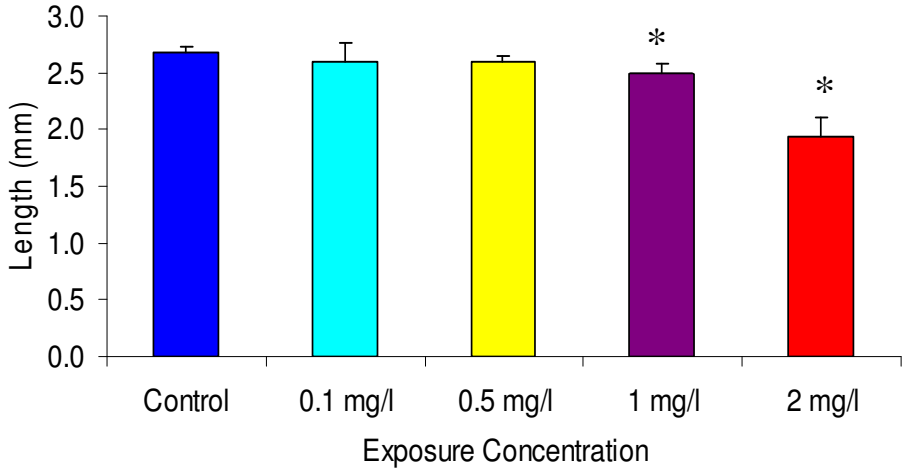


Figure 3.29: Size measurement to determine growth after a 21 day exposure to nano (average size 14 nm) sized carbon black (control n= 8, 1 mg/L and 0.5 mg/L n= 9; 0.1 mg/L, 2 mg/L n= 10; values are means +/- standard error, * indicates a result with $p \leq 0.05$).

3.3.6.4 Offspring

The assessment of effects on offspring was done by counting released neonates in the different replicates. This gave a good indicator of influence of exposures to particles on fertility. The value measured was the quantity of offspring as well as the time point of release. This was done to enable the assessment of differences not only at the end, but also during the exposure. The treatments were compared by a one way ANOVA with the single animal in the replicates of a treatment as data points. A Tukey post hoc test was carried out for multiple comparisons of means if the variances of the residuals were homogenous, otherwise a Games-Howell post hoc test was used for the same purpose.

The exposure to micro sized carbon black revealed no significant difference in cumulative offspring production between the different treatments (Fig. 3.30). Although the graph indicates that animals in some treatments seemed to start producing offspring later than the control, this was not found to be significantly different at any time. In the exposure to the nano sized particles (Fig. 3.31), the animals treated with the highest concentration of 2 mg/L did not reproduce through the duration of the experiment. From day 18 to day 21 the animals in the 1 mg/L treatment produced significantly lower numbers of offspring than animals in the control ($p < 0.05$). It has to be noted, that the number of offspring produced by animals in the control treatment of the nano exposure was higher than the ones produced by animals in the control of the micro exposure.

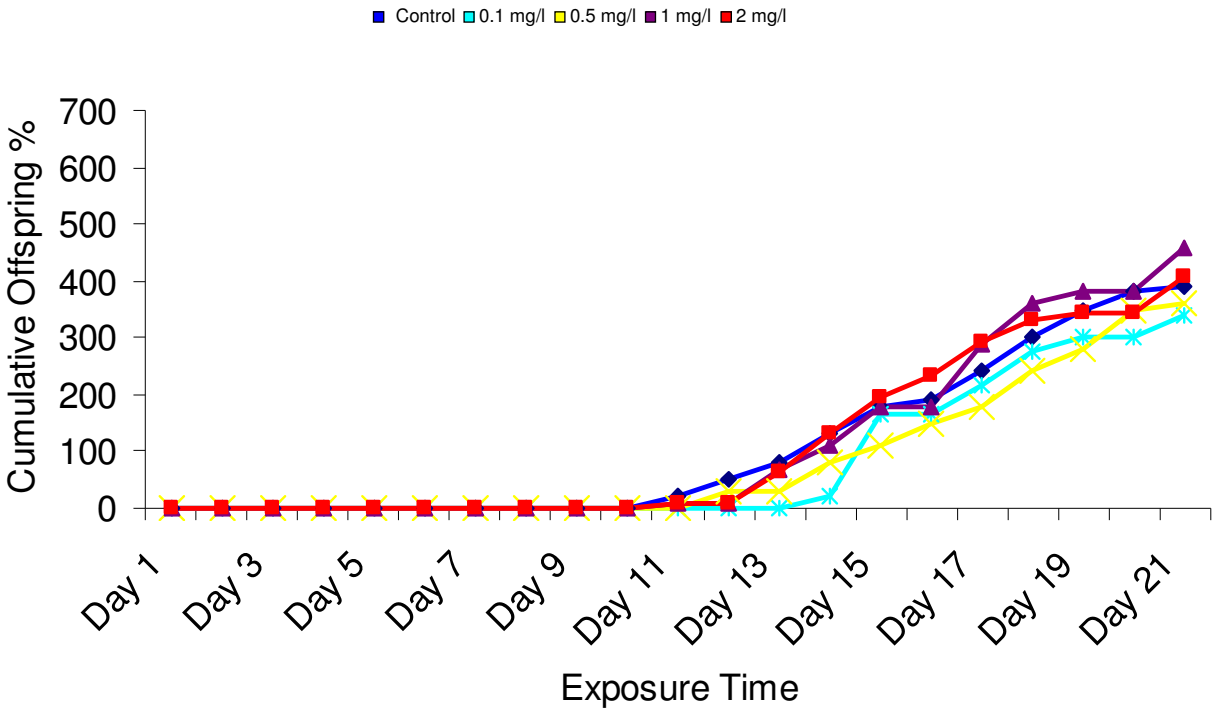


Figure 3.30: Cumulative offspring during a 21 day exposure to micro sized carbon black (average size 260 nm). No significant difference between the treatments was observed.

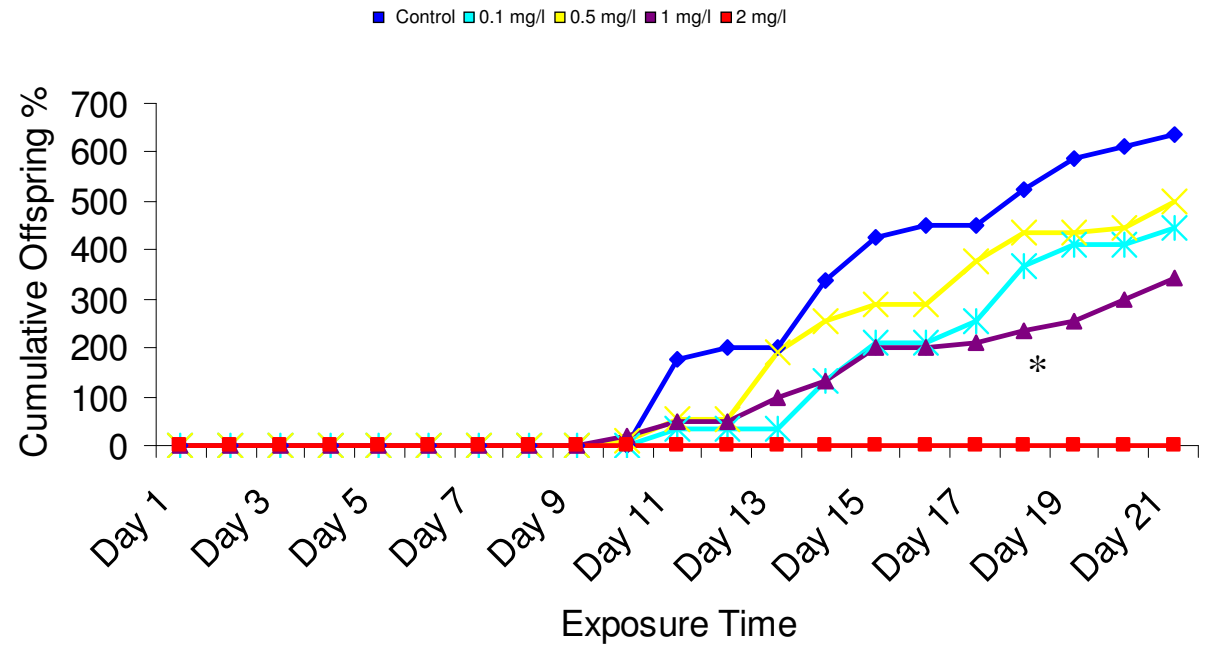


Figure 3.31: Cumulative offspring during a 21 day exposure to nano sized carbon black(average size 14 nm). Since the data had no homogenous variances, a non parametric post hoc test was used (* indicates a result with $p \leq 0.05$).

3.3.7 Chronic 21 day exposure to micro and nano sized cerium dioxide

Since there was no mortality observed in the acute 96 h tests to cerium dioxide (see section 3.3.4.1), the same concentrations were chosen for the chronic 21 day test. The concentrations were 10 mg/L, 3 mg/L, 1 mg/L and 0.1 mg/L.

3.3.7.1 Mortality

As for the 96 h studies, exposures to micro sized cerium resulted in no significant mortality over the 21 day exposure period (Fig. 3.32). The 3 mg/L treatment showed 30% mortality after day 16 but this was not statistically significant (p= 0.06) when compared to the control or the other treatments. The 21 day exposure to nano sized cerium dioxide showed a different pattern of mortality (Fig. 3.33). The treatment with the highest concentration, the 10 mg/L treatment showed significant mortality of *D. magna* after day 5 (p= 0.05) when compared to the control or the other treatments and the mortality increased on the following day, to reach a 100 % at day 7. All other treatments with lower concentrations showed no mortality.

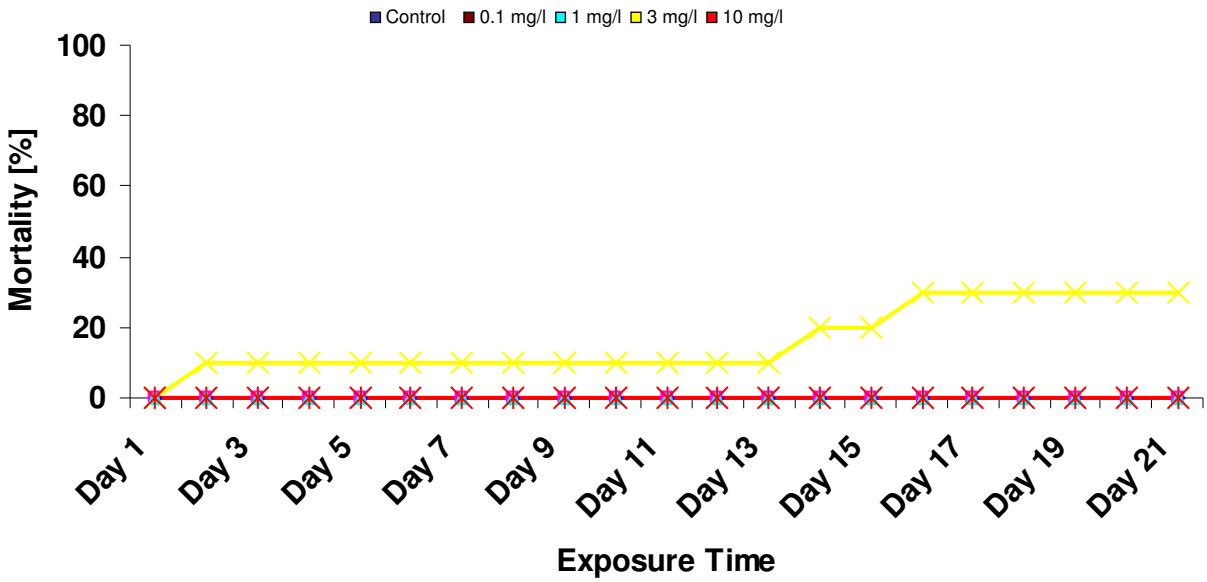


Figure 3.32: Mortality in a 21 day exposure to micro sized (average size <5000 nm) cerium dioxide (n= 10).

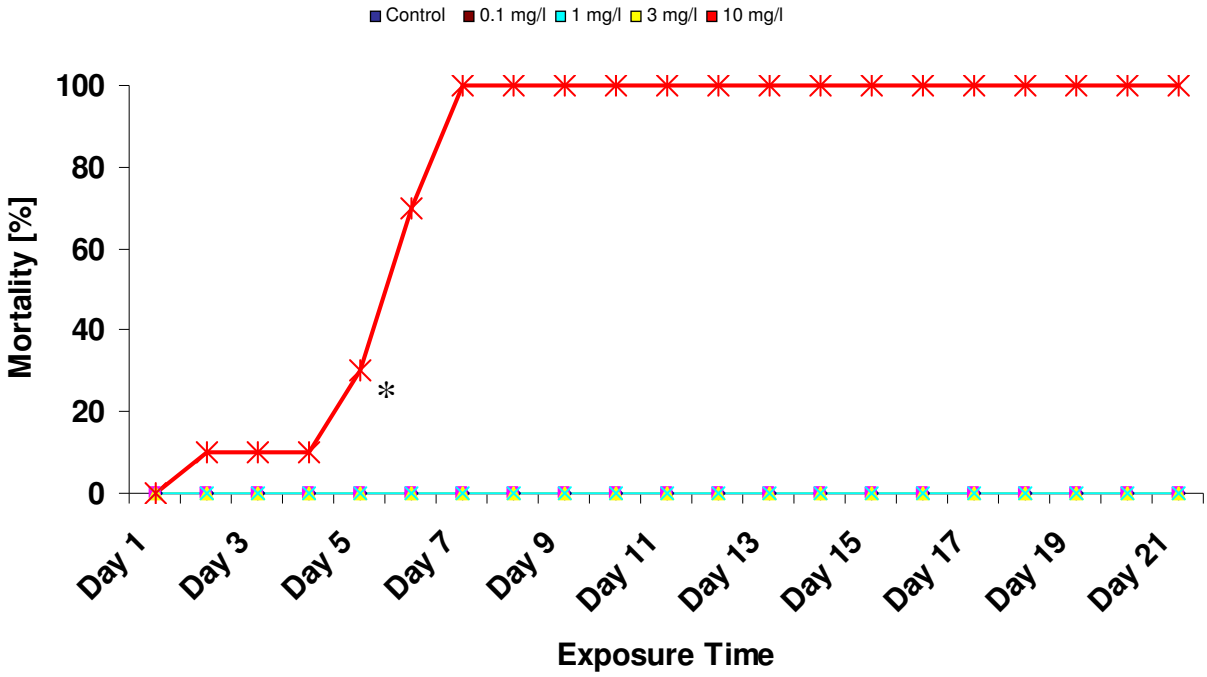


Figure 3.33: Mortality in a 21 day exposure to nano sized (average size <25 nm) cerium dioxide (n= 10) (* indicates a result with $p \leq 0.05$).

3.3.7.2 Moulting

The results of the assessment of cumulative moulting of animals exposed to cerium dioxide micro- and nanoparticles looked similar to the results of the mortality experiments. No significant difference between the treatments was observed in the exposure to micro sized cerium dioxide (Fig. 3.34). In the nano exposure to cerium dioxide, there was an effect on moulting of *D. magna* visible in the treatment with 10 mg/L, which also showed mortality (Fig. 3.35). Animals in the 10 mg/L treatment had from day 4 onward a significantly reduced cumulative moulting ($p= 0.002$) when compared to the control. The cumulative moulting could just be assessed until day 7, due to 100% mortality following this time point. Cumulative moulting, at day 4, was found to be more sensitive than mortality in detecting changes due to exposure.

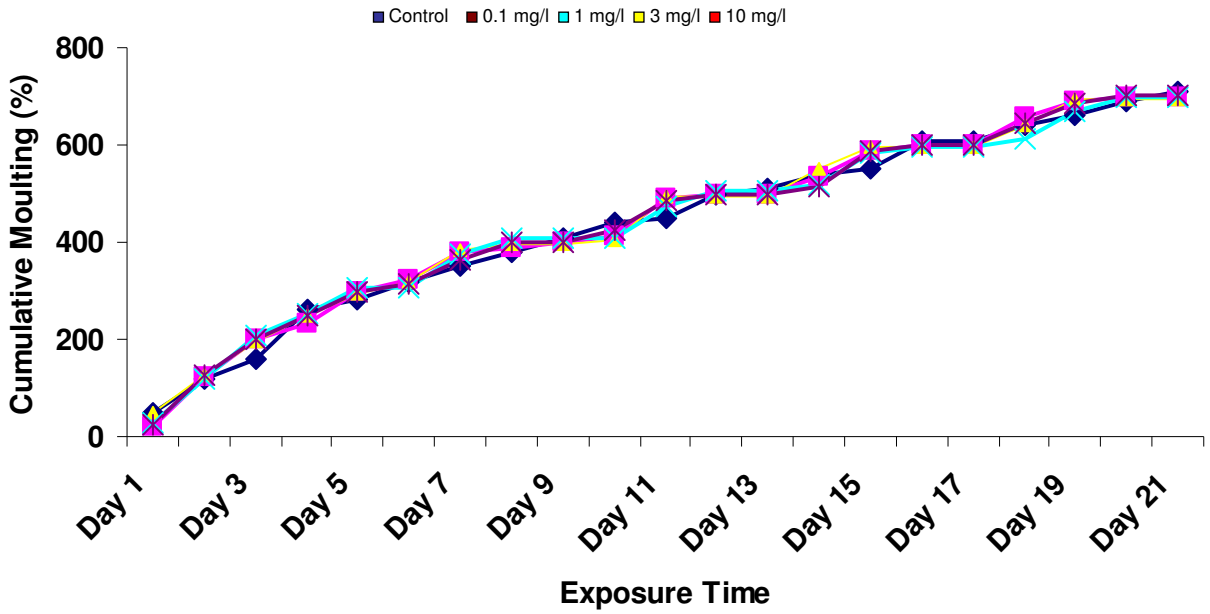


Figure 3.34: Cumulative moulting in a 21 day exposure to micro sized (average size <5000 nm) cerium dioxide (n= 10).

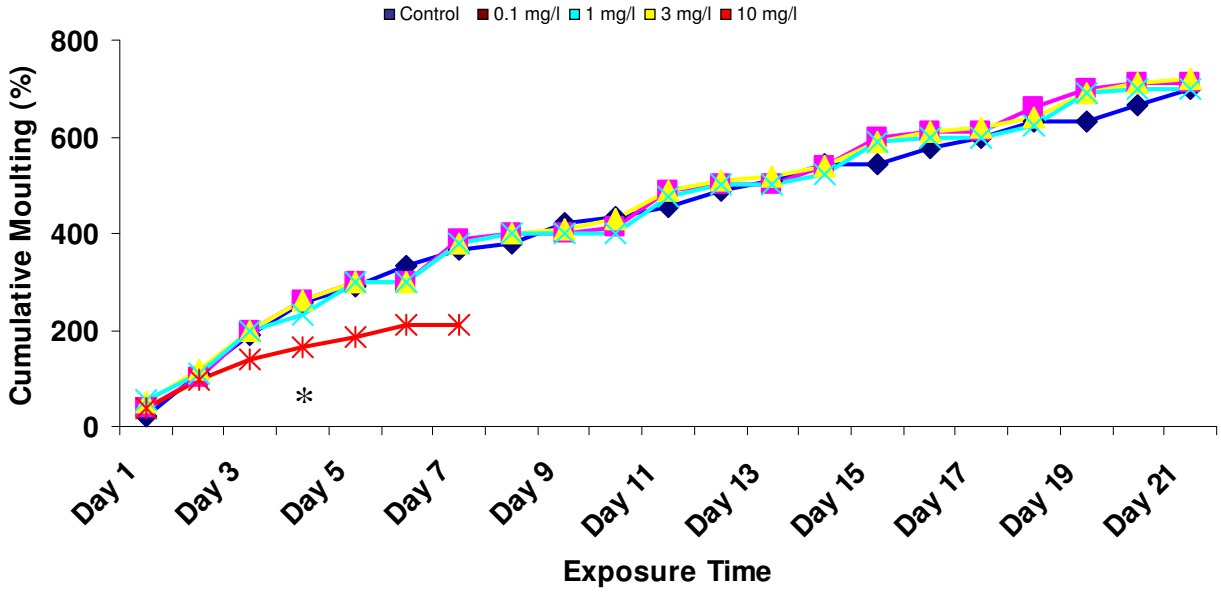


Figure 3.35: Cumulative moulting in a 21 day exposure to nano sized (average size <25 nm) cerium dioxide (n= 10) (* indicates a result with $p \leq 0.05$).

3.3.7.3 Length measurement

The assessment of growth after the 21 day exposures to cerium dioxide showed that neither the micro sized particles (Fig. 3.36) nor the nano sized particles (Fig. 3.37) had any significant effect on growth in the exposure period. The 10 mg/L treatment in the nano exposure to cerium dioxide could not be assessed, due to 100% mortality at day 7.

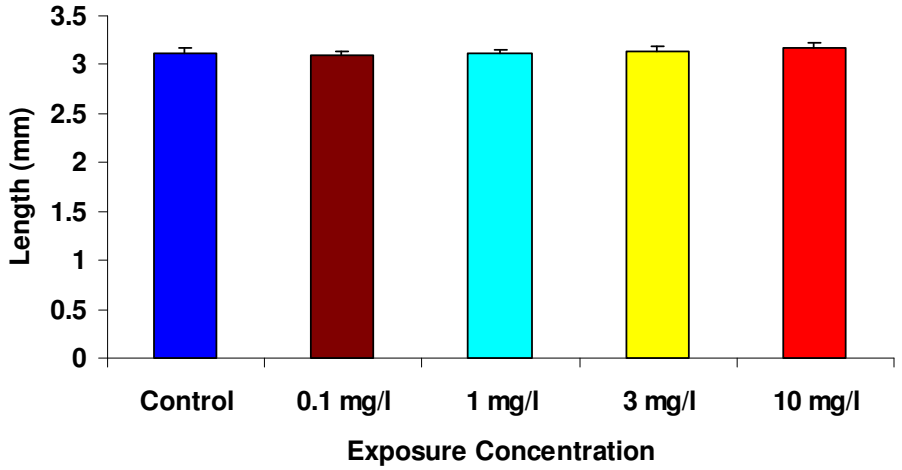


Figure 3.36: Size measurement to determine growth after a 21 day exposure to micro (average size <5000 nm) sized cerium dioxide (control, n= 10; 0.1 mg/L and 1 mg/L n= 9; 3 mg/L and 10 mg/L n= 7; values are means +/- standard error).

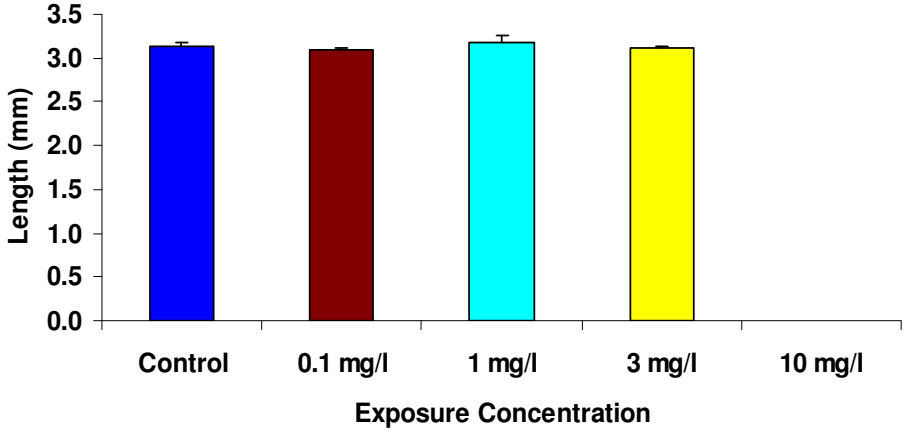


Figure 3.37: Size measurement to determine growth after a 21 day exposure to nano (average size <25 nm) sized cerium dioxide (1 mg/L n= 10; control and 3 mg/L n= 9; 0.1 mg/L n= 8; values are means +/- standard error).

3.3.7.4 Offspring

The assessment of offspring after the 21 day exposures to cerium dioxide showed, similarly to moulting, that neither the micro sized particles (Fig. 3.38) nor the nano sized particles (Fig. 3.39) had any significant effect on fertility of *D. magna* in the exposure period. The 10 mg/L treatment in the nano exposure to cerium dioxide could not be assessed, due to 100% mortality at day 7 before any offspring was produced.

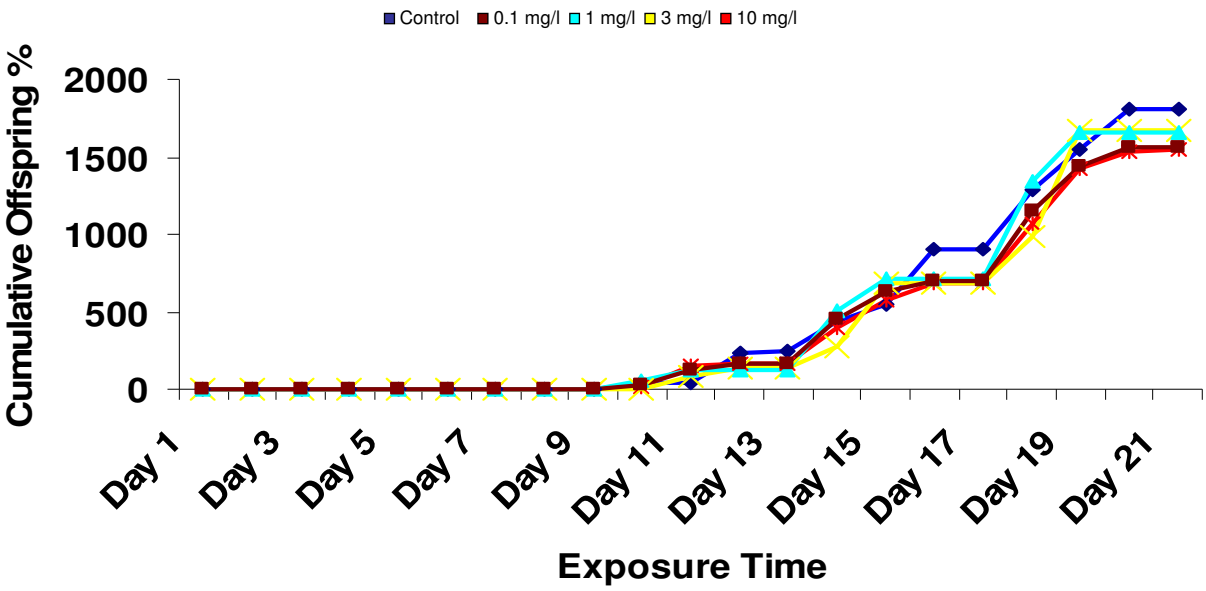


Figure 3.38: Cumulative offspring during a 21 day exposure to micro (average size <25 nm) sized cerium dioxide (n= 10).

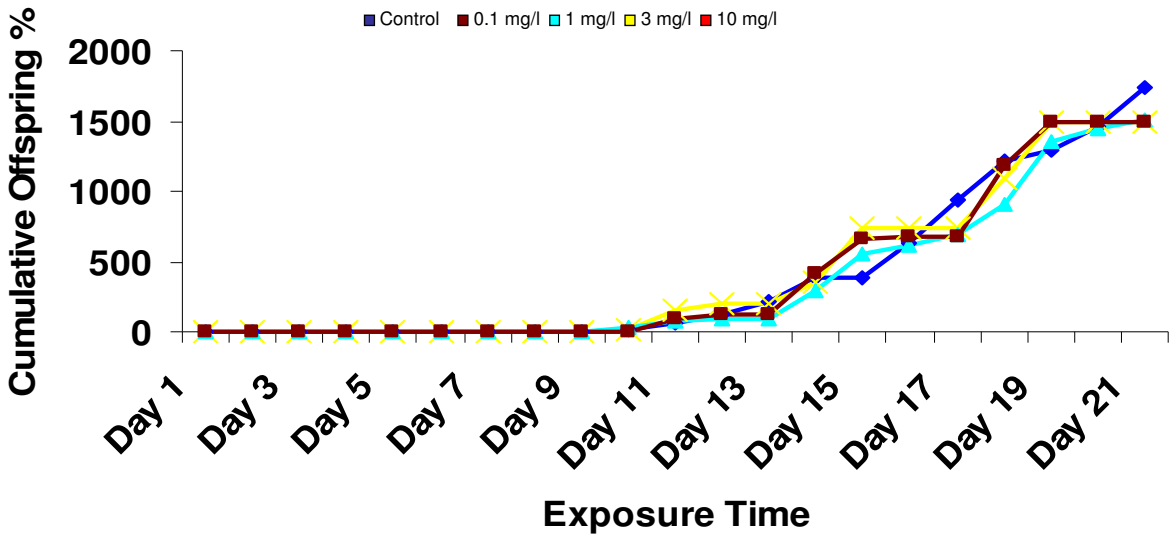


Figure 3.39: Cumulative offspring during a 21 day exposure to nano sized (average size <25 nm) cerium dioxide (n= 10).

3.3.8 Chronic 21 day exposure to micro and nano sized silver

The chronic 21 day test involving exposure of *D. magna* to silver particles was conducted once. The concentrations chosen were lower than the ones used in the acute tests, due to the goal to study sub lethal endpoints, as described above. These were 0.05 mg/L, 0.01 mg/L, 0.005 mg/L and 0.001 mg/L for both sized particles (600-1600 nm and 35 nm).

3.3.8.1 Mortality

Exposures to micro and nano sized silver resulted in no significant mortality over the exposure duration. Although the animals exposed to 0.005 mg/L micro sized silver expressed 20% mortality from day 8 onward, these were not significantly different, since all the other treatments, including the control treatment, also experienced 10% mortality (Fig. 3.40). The exposure to 0.001 mg/L silver nanoparticles resulted in the highest mortality (30%) of *D. magna* (Fig. 3.41), but this was not significantly different from all other treatments or the control. The control treatment showed no mortality.

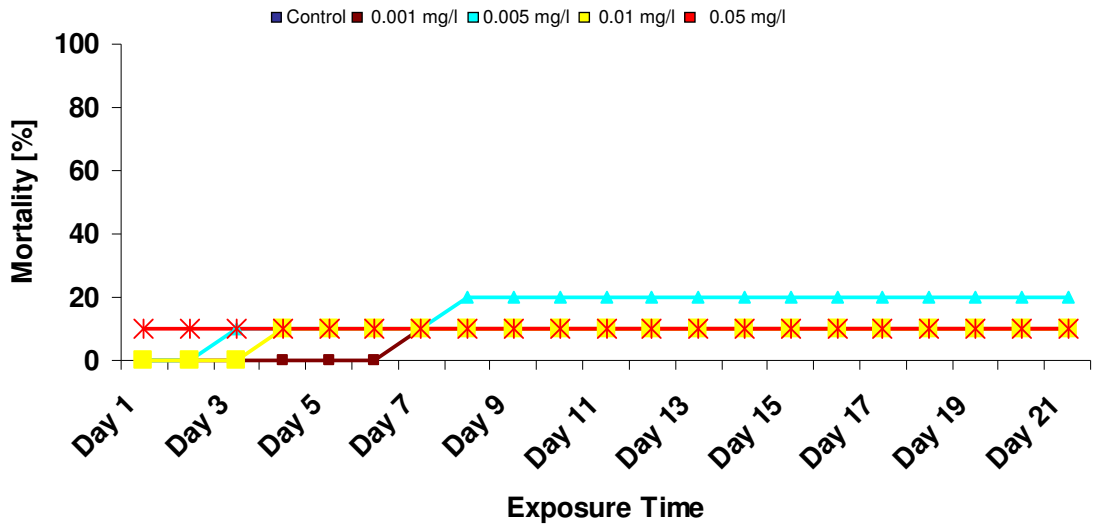


Figure 3.40: Mortality in a 21 day exposure to micro sized (average size 600-1600 nm) silver (n= 10).

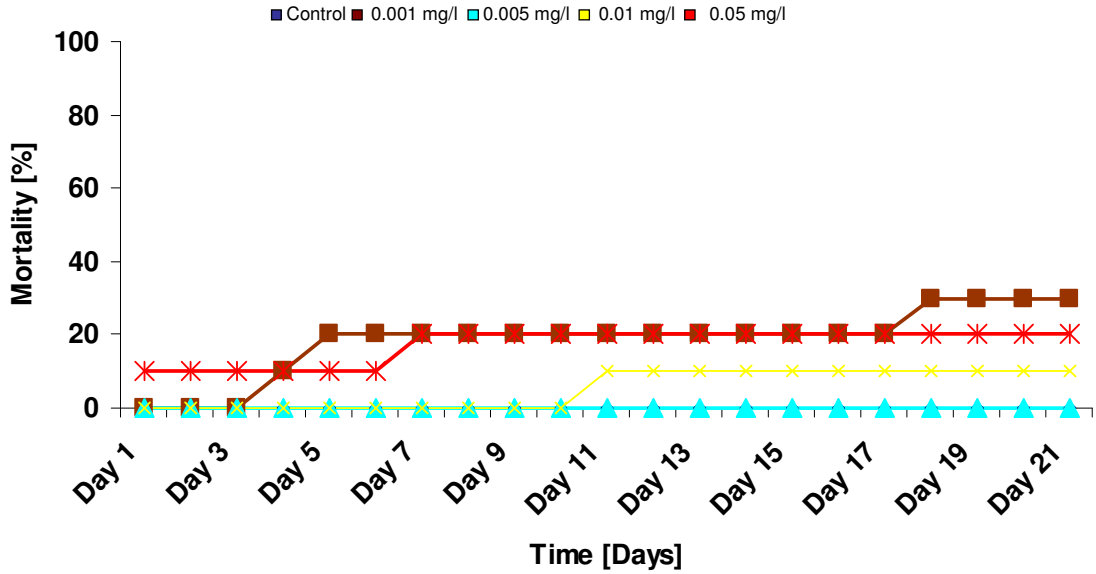


Figure 3.41: Mortality in a 21 day exposure to nano sized (average size 35 nm) silver (n= 10).

3.3.8.2 Moulting

The assessment of cumulative moults in exposures to micro and nano sized silver particles revealed that just the organisms exposed to the highest exposure of micro sized silver (Fig. 3.42), the 0.05 mg/L exposure, had less cumulative moults than the control throughout the exposure period, although it was only

significantly lower on day 21 (p = 0.023). No significant reduction in moulting of *D. magna* was observed in the nanoparticle exposure to silver (Fig. 3.43).

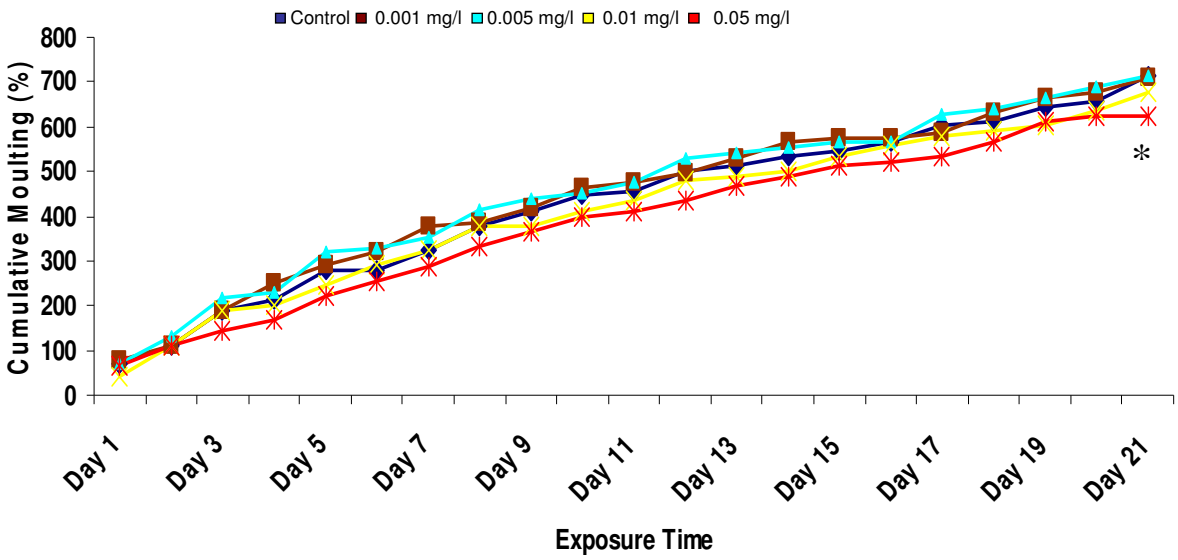


Figure 3.42: Cumulative moulting in a 21 day exposure to micro sized (average size 600-1600 nm) silver (n= 10) (* indicates a result with p ≤ 0.05).

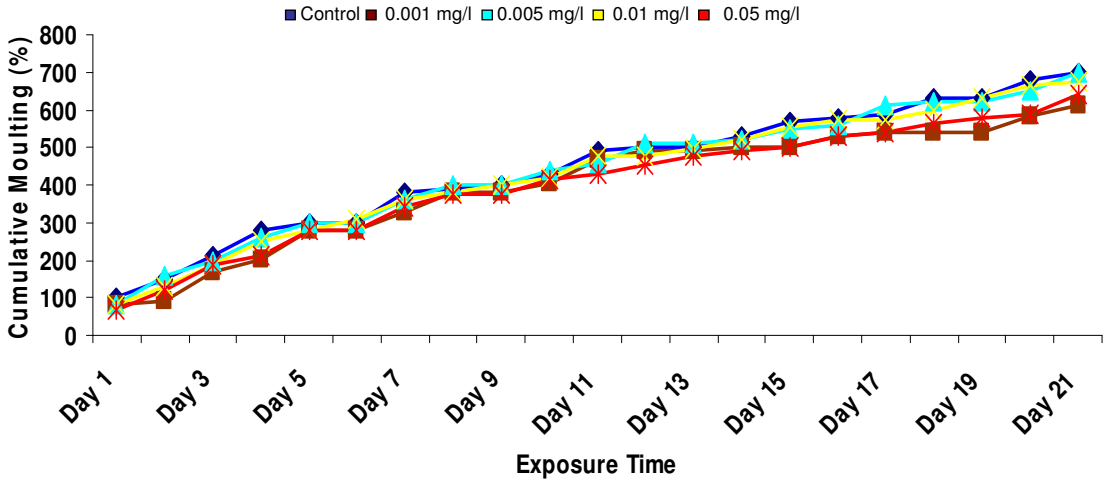


Figure 3.43: Cumulative moulting in a 21 day exposure to nano sized (average size 35 nm) silver (n= 10).

It was observed on several occasions for animals in the 0.001 mg/L and 0.05 mg/L treatment, that release of neonates and moulting were occurring several days apart. These two processes are normally closely linked. In one occasion, in the 0.001 mg/L treatment, it was observed that the animal had the old

carapace still intact, while a new one had build underneath (Fig. 3.44). This specific animal had released neonates 4 days earlier and failed to moult.

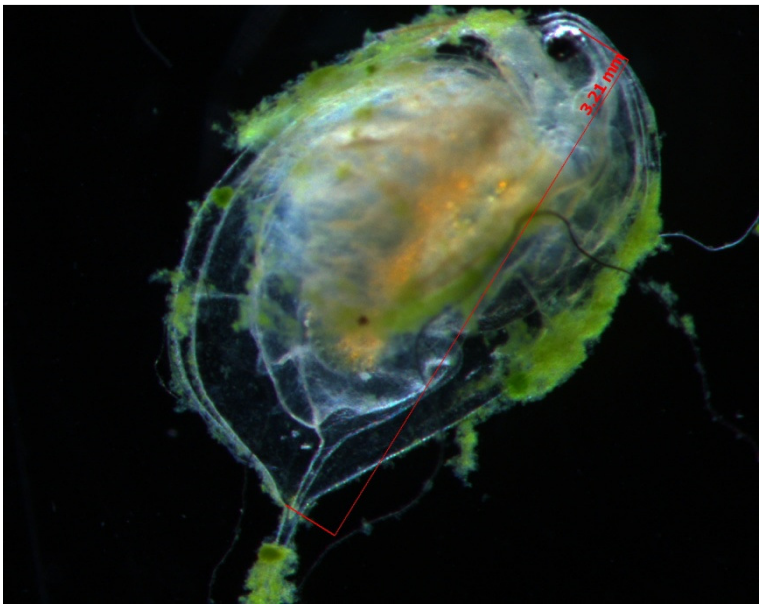


Figure 3.44: Moulting affected at an exposure to nano sized silver (average size 35 nm) at a concentration of 0.001mg/L after 21 days

3.3.8.3 Length measurement

The length measurement after the exposure to micro silver showed no significant difference between the treatments (Fig. 3.45). The exposures to nano silver resulted in the 0.001 mg/L treatment showing a significant increase in growth when compared to the control ($p = 0.05$), the 0.005 mg/L and 0.01 mg/L treatment ($p = 0.01$) (Fig. 3.46). Comparing the 0.001 mg/L to the 0.05 mg/L treatment indicated no significant difference ($p = 0.061$).

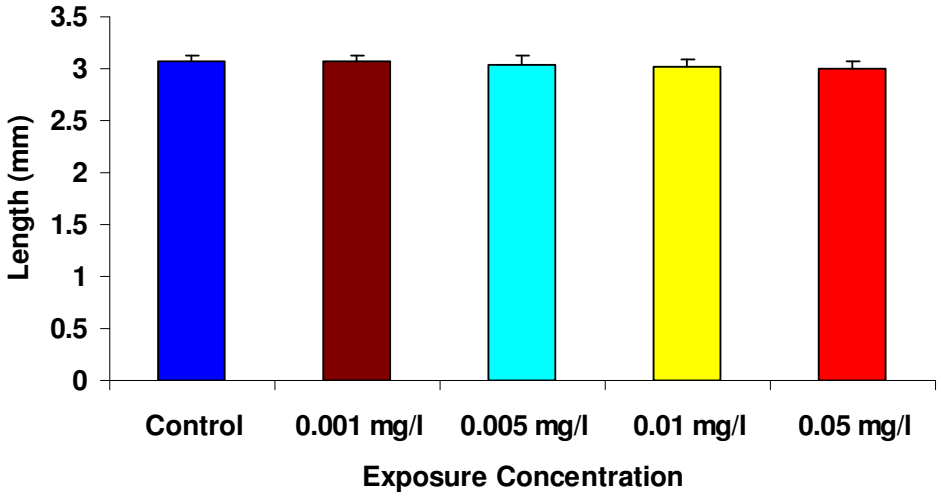


Figure 3.45: Size measurement to determine growth after a 21 day exposure to micro (average size 600-1600 nm) sized silver (control, 0.001 mg/L, 0.01 mg/L and 0.005 mg/L n= 9; 0.005 mg/L n= 8; values are means +/- standard error).

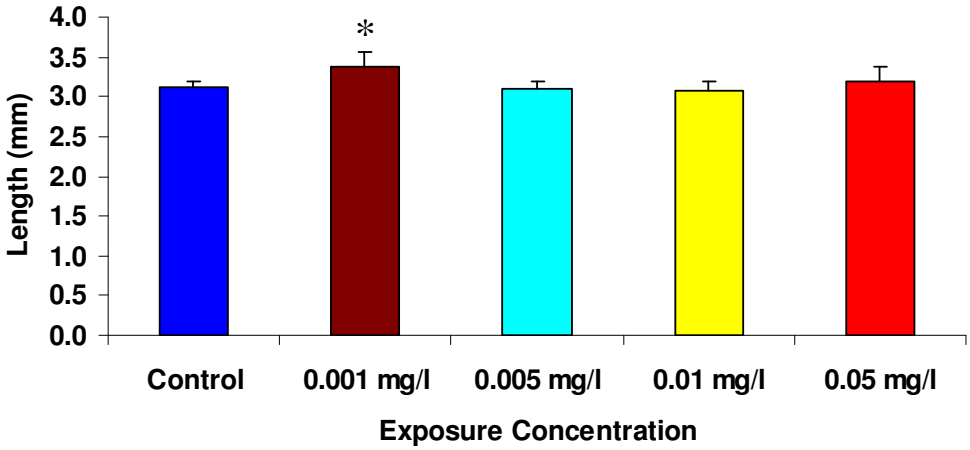


Figure 3.46: Size measurement to determine growth after a 21 day exposure to nano (average size 35 nm) sized silver (control and 0.005 mg/L n= 10; 0.01 mg/L and 0.05 mg/L n= 8; 0.001 mg/L n= 7; values are means +/- standard error, * indicates a result with $p \leq 0.05$).

3.3.8.4 Offspring

The assessment of offspring during the exposure to micro and nano sized silver showed concentration-dependent decreases in offspring number in both experiments. In the micro silver treatment it was observed that the animals on the 0.05mg/L treatment generated significantly less offspring than the animals in the other treatments, except in the 0.005 mg/L treatment, on day 21 ($p <$

0.05) (Fig. 3.47). In the nano exposures (Fig. 3.48), the organisms exposed to 0.01 mg/L and 0.05 mg/L produced significantly less offspring than the control from day 19 onwards ($p < 0.05$). Although the 0.001 mg/L group also had much less offspring than the control, this difference was not found to be significant ($p = 0.062$ on day 21).

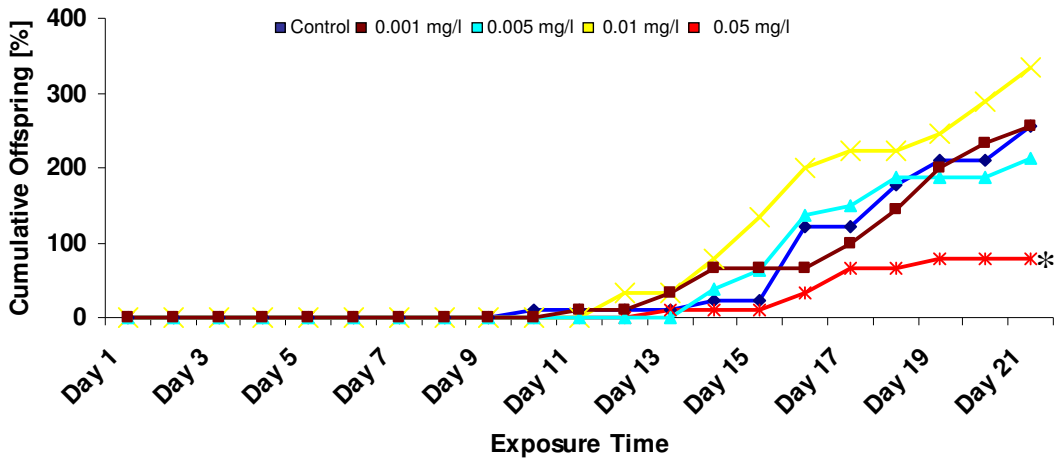


Figure 3.47: Cumulative offspring during a 21 day exposure to micro sized (average size 600-1600 nm) silver (n= 10) (* indicates a result with $p \leq 0.05$).

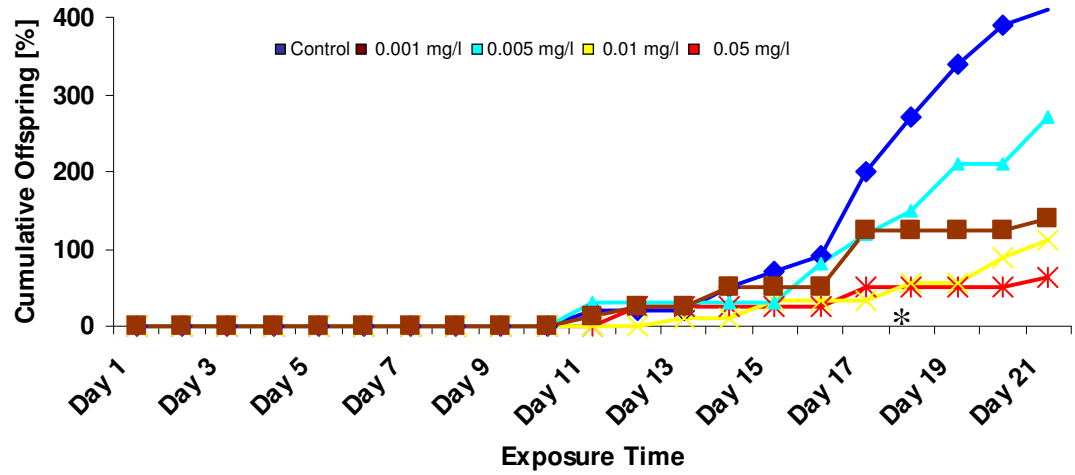


Figure 3.48: Cumulative offspring during a 21 day exposure to nano sized (average size 35 nm) silver (n= 10) (* indicates a result with $p \leq 0.05$).

3.3.9 Modelling the effects of nanoparticle exposure

In the subsequent models, the abbreviation Conc is used for Concentration in [mg/L]. Time is given in Days, since the measurement was made in all experiments on a daily basis. The p -value underneath each constant indicates the significance level of this specific constant in the model. The $p_{equation}$ -value indicates if regressions carried out were found to be statistically significant. R^2 is the unadjusted square of the multiple correlation coefficients, and is a measure of the fit of the model. The t –value is the value of the t- distribution. The absolute number of this value gives an indication of the precision of the specific constant in the model. The higher the value, the more precisely does the constant reflect the relationship between measured values and the model. A t- value below 2 with the number of data points used here generally means that the constant does not contribute to the model (See table for t- distribution in (Box et al., 2005)). The numerical values in the equation are regression coefficients and give with their algebraic sign the direction and with their absolute value the strength of their interaction.

3.3.9.1 Mortality

Modelling mortality of *D. magna* exposed for 96 h to micro sized carbon black delivers the following equation:

(9) $$\ln(\%\text{Survival}) = 4.598 - 0.001 * \text{Conc} * \text{Day}$$

$$p < 0.001 \quad p = 0.013$$

$$\text{t-Values} \qquad (749.2) \quad (-2.7)$$

$$R^2 = 0.250 \quad F(1,22) = 7.3 \quad p_{equation} = 0.013$$

A significant effect of the concentration and exposure time of micro sized carbon black was observed on survival. However, with a coefficient of 0.001 this effect was found to be very weak.

For nano sized carbon black the equation is:

(10) $$\ln(\%\text{Survival}) = 4.910 - 0.186 * \text{Conc} * \text{Day}$$

$$p < 0.001 \quad p < 0.001$$

$$\text{t-Values} \qquad (12.0) \quad (-5.7)$$

$$R^2 = 0.598 \quad F(1,22) = 32.7 \quad p_{equation} < 0.001$$

The equation indicates a significant negative effect of concentration and time on *D. magna* survival exposed to nano sized carbon black particles, given the coefficient value of 0.186 and the negative algebraic sign.

Micro sized cerium dioxide and nano sized cerium dioxide yielded the equations:

(11) $$\text{Ln(\%Survival)} = 4.572 - 0.000*\text{Conc}*\text{Day}$$

$$p < 0.001 \quad p = 0.131$$

$$\text{t-Values} \qquad (1057.1) \quad (-1.6)$$

$$R^2 = 0.122 \qquad F(1,18) = 2.5 \quad p_{equation} = 0.131$$

for micro sized cerium dioxide and

(12) $$\text{Ln(\%Survival)} = 4.564 - 0.001*\text{Conc}*\text{Day}$$

$$p < 0.001 \quad p = 0.210$$

$$\text{t-Values} \qquad (393.4) \quad (-1.3)$$

$$R^2 = 0.086 \quad F(1,18) = 1.7 \quad p_{equation} = 0.210$$

for nano sized cerium dioxide. Due to very low mortality in both exposures over the duration of 96 h the model fit is not very good and the effects of exposure are very low or none existent.

Exposure to micro sized silver shows a much stronger influence of particle concentration on mortality:

(13) $$\text{Ln(\%Survival)} = 4.595 - 0.485*\text{Conc}*\text{Day}$$

$$p < 0.001 \quad p < 0.001$$

$$\text{t-Values} \qquad (94.6) \quad (-13.7)$$

$$R^2 = 0.931 \quad F(1,14) = 188.6 \quad p_{equation} < 0.001$$

With nano sized silver having the strongest effect:

(14) $$\text{Ln}(\%\text{Survival}) = 4.502 - 2.813*\text{Conc}*\text{Day}$$

$$p < 0.001 \quad p < 0.001$$

$$\text{t-Values} \qquad (13.9) \quad (-11.9)$$

$$R^2 = 0.911 \quad F(1,14) = 142.6 \quad p_{equation} < 0.001$$

Both models showed a significant effect of concentration and exposure time on survival. The coefficients obtained showed that the decrease in survival due to nano sized silver particles was much stronger than the decrease in survival due to micro sized silver particles.

Since mortality in the chronic 21 day, exposures was generally low no model could be fitted to those data.

3.3.9.2 Moulting

The same modelling approach was followed using moulting data obtained during the exposures. Modelling moulting of *D. magna* exposed to micro sized carbon black resulted in the following equations:

(15) $$\text{Cumulative}(\%\text{moult}) = 64.075*\text{Day} + 0.239*\text{Conc}*\text{Day}$$

$$p < 0.001 \quad p = 0.319$$

$$\text{t-Values} \qquad (59.68) \qquad (1.02)$$

$$R^2 = 0.996 \quad F(2,22) = 2766.8 \quad p_{equation} < 0.001$$

Although the formula suggests a beneficial effect on moulting (increased moulting, since the coefficient sign is positive) due to exposure to micro sized carbon black, the t- value associated with the second coefficient of 1.02 and its p = 0.319 indicate that the interaction of concentration and time do not play a significant role.

For nano sized carbon black the interaction seems to be much stronger, showing that the exposure resulted in a decrease in moulting of the animals with a significant negative effect of concentration and day obtained:

(16) $$\text{Cumulative(\%moult)} = 62.682*\text{Day} - 5.515*\text{Conc}*\text{Day}$$

$p < 0.001 \quad p < 0.001$

t-Values (34.12) (-10.41)

$R^2 = 0.984 \quad F(2,20) = 603.4 \quad p_{equation} < 0.001$

Micro sized cerium dioxide has a similar effect on cumulative moulting as micro sized carbon black:

(17) $$\text{Cumulative(\%moult)} = 51.188*\text{Day} + 0.323*\text{Conc}*\text{Day}$$

$p < 0.001 \quad p = 0.306$

t-Values (37.10) (1.05)

$R^2 = 0.990 \quad F(2,18) = 937.2 \quad p_{equation} < 0.001$

The formula also suggests a beneficial effect on moulting but as in micro sized carbon black, the t-value of 1.05 and the p- value of p = 0.306 suggest that moulting was not significantly affected by the exposure to micro sized cerium dioxide.

In contrast to the mortality results obtained after exposure to nano sized cerium dioxide**,** a negative effect can be seen on moulting during a 96 h exposure time to nano sized cerium dioxide:

(18) $$\text{Cumulative(\%moult)} = 50.612*\text{Day} - 1.690*\text{Conc}*\text{Day}$$

$p < 0.001 \quad p < 0.001$

t-Values (49.60) (7.44)

$R^2 = 0.994 \quad F(2,18) = 1423.2 \quad p_{equation} < 0.001$

As with mortality, the strongest effect on cumulative moulting can be seen after exposures to silver. Micro sized silver exposures resulted in comparable effects to nano sized carbon black:

(19) $$\text{Cumulative(\%moult)} = 51.982*\text{Day} - 7.323*\text{Conc}*\text{Day}$$

$$p < 0.001 \quad p = 0.001$$

$$\text{t-Values} \quad (55.83) \quad (-3.95)$$

$$R^2 = 0.997 \quad F(2,14) = 2078.2 \quad p_{equation} < 0.001$$

Nano sized silver had, like in mortality, the strongest overall effect on cumulative moulting when compared to the other particles:

(20) $$\text{Cumulative(\%moult)} = 51.630*\text{Day} - 91.12*\text{Conc}*\text{Day}$$

$$p < 0.001 \quad p = 0.002$$

$$\text{t-Values} \quad (41.53) \quad (-4.25)$$

$$R^2 = 0.996 \quad F(2,10) = 1265.0 \quad p_{equation} < 0.001$$

Due to an absence of significant changes, only changes in moulting due to nano sized carbon black and nano sized cerium dioxide (until day 6 of exposure)were modelled.

For nano sized carbon black in a 21 day exposure the interaction seemed to be less strong than for the 96 h exposure:

(21) $$\text{Cumulative(\%moult)} = 40.093*\text{Day} - 1.463*\text{Conc}*\text{Day}$$

$$p < 0.001 \quad p = 0.029$$

$$\text{t-Values} \quad (59.12) \quad (-2.21)$$

$$R^2 = 0.984 \quad F(2,103) = 3269.2 \quad p_{equation} < 0.001$$

For nano sized cerium dioxide values similar to the 96 h exposure were obtained. Here cumulative moulting was modelled until day six, the day before the highest treatment showed 100% mortality, and this resulted in the following equation:

(22) $$\text{Cumulative(\%moult)} = 58.909*\text{Day} - 1.939*\text{Conc}*\text{Day}$$

$$p < 0.001 \quad p < 0.001$$

$$\text{t-Values} \quad (41.59) \quad (-6.42)$$

$$R^2 = 0.988 \quad F(2,28) = 1135.4 \quad p_{equation} < 0.001$$

3.3.9.3 Offspring

The model for micro sized carbon black showed, like the model for moulting, a beneficiary effect for cumulative offspring when exposed for 21 days:

(23) Cumulative(%offspring) = - 434.212 + 38.686*Day + 1.159*Conc*Day

	$p < 0.001$	$p < 0.001$	$p = 0.005$
t-Values	(-20.37)	(28.18)	(2.90)

$R^2 = 0.939$ $F(2,57) = 437.6$ $p_{equation} < 0.001$

For nano sized carbon black it showed a negative interaction indicating a decrease in offspring due to exposure:

(24) Cumulative(%offspring) = - 331.098 + 42.732*Day – 10.901*Conc*Day

	$p < 0.001$	$p < 0.001$	$p < 0.001$
t-Values	(-7.52)	(14.93)	(-13.09)

$R^2 = 0.851$ $F(2,57) = 163.4$ $p_{equation} < 0.001$

Exposure to micro sized cerium dioxide induced a small, negative but with $p = 0.127$ not significant interaction with the equation of the cumulative offspring from *D. magna*:

(25) Cumulative(%offspring) = - 1824.950 + 167.624*Day – 0.539*Conc*Day

	$p < 0.001$	$p < 0.001$	$p = 0.127$
t-Values	(-19.14)	(27.55)	(1.55)

$R^2 = 0.931$ $F(2,57) = 383.7$ $p_{equation} < 0.001$

Nano sized cerium dioxide showed a small positive effect on cumulative offspring due to concentration and time, but also, as in equation 25, not significant:

(26) Cumulative(%offspring) = - 1703.171+ 154.329*Day + 0.270*Conc*Day

	$p < 0.001$	$p < 0.001$	$p = 0.8$
t-Values	(-18.24)	(25.80)	(0.25)

$R^2 = 0.939$ $F(2,45) = 345.5$ $p_{equation} < 0.001$

As for mortality and moulting, the effects on offspring were strongest when exposed to silver. Exposure to micro sized silver seemed to have a strong negative effect on offspring production:

(27) Cumulative(%offspring) = - 254.656 + 23.970*Day – 101.823*Conc*Day

$p < 0.001$ $p < 0.001$ $p < 0.001$

t-Values (-10.61) (15.67) (-5.80)

$R^2 = 0.819$ $F(2,57) = 128.77$ $p_{equation} < 0.001$

Nano sized silver still causeds the strongest effect on the cumulative number of offspring *of D. magna* but it was not much stronger than micro sized silver:

(28) Cumulative(%offspring) = - 210.933 + 20.209*Day – 113.705*Conc*Day

$p < 0.001$ $p < 0.001$ $p < 0.001$

t-Values (-5.74) (8.63) (-4.23)

$R^2 = 0.594$ $F(2,57) = 41.6$ $p_{equation} < 0.001$

A comparison of the constant before the interaction term Conc*Day permits predictions about the significance and magnitude of the effects of the different particles but also on the impacts of different particle sizes on the various endpoints.

Table 3.1 shows that the particles can be ranked in their toxicity with cerium dioxide having nearly no negative effect on the endpoints tested, ranging to silver having very strong effects in all endpoints observed. It can also be concluded that particle size contributes to toxicity, micro sized particles only showed adverse effects in case of the silver while nano sized particles showed negative effects for all endpoints in the case of silver and carbon black and at least in one endpoint, moulting, for cerium dioxide.

Table 3.1: List of constants obtained by linear or logarithmic regression of test results from exposures of *D. magna* to different particles in the nano or micro range. Constants in red show values not significant in the equation.

Endpoint	Particle	Constant
Mortality	Carbon black micro	– 0.001
	Carbon black nano	– 0.186
	Cerium dioxide micro	– 0.000
	Cerium dioxide nano	– 0.001
	Silver micro	– 0.485
	Silver nano	– 2.813
Moulting	Carbon black micro	+ 0.239
	Carbon black nano	– 5.515
	Cerium dioxide micro	+ 0.323
	Cerium dioxide nano	– 1.690
	Silver micro	– 7.323
	Silver nano	- 91.12
	Carbon black nano 21 days	– 1.463
	Cerium dioxide nano 6 days	– 1.939
Offspring	Carbon black micro	+ 1.159
	Carbon black nano	– 10.901
	Cerium dioxide micro	– 0.539
	Cerium dioxide nano	+ 0.270
	Silver micro	– 101.823
	Silver nano	– 113.705

3.4 Discussion

3.4.1 Acute 48h toxicity tests

Although mortality is the key endpoint in acute toxicity tests with *D. magna*, no significant mortality was observed in the concentrations tested during this study for neither NP carbon black nor NP TiO_2. This is consistent with another study which has investigated NP TiO_2 (Lovern and Klaper, 2006) and suggests that NP TiO_2 and most likely NP carbon black both have a low acute toxicity. Mortality observed in the here presented study occurred only in the highest concentration (100 mg/L) and seemed to be caused by the particles covering the entire animal thereby reducing their mobility. However, it has been proposed that predicted exposure concentrations in the water for titanium dioxide are rather in the low µg/L range (Boxall et al., 2007), so the tested concentrations for this particle may be irrelevant or most likely would just appear near heavily contaminated sites (e.g. slude dumping sites). Similar predictions for carbon black NP could not be obtained.

It was also observed that both nanoparticles were readily ingested and accumulated in the gut within 30 minutes. If there was a translocation of the tested particles through the gut wall barrier into the body cavity this could not be determined by light microscopy due to the resolution of the microscope, but also the titanium dioxide and carbon black covered the carapace and therefore hindering, even after washing, the detection of particles underneath the carapace. In the case of titanium dioxide, studying the uptake and translocation via stable isotope tracing could be a possibility (Gulson and Wong, 2006). For silver and cerium dioxide neutron activation and detection by gamma spectroscopy or autoradiography, could be a promising approach (Oughton et al., 2008). Verification through transmission electron microscopy might be difficult with the particles used in this study due to a low contrast and presumably low concentrations in the observed slides.

Moulting frequency was increased at higher concentrations for TiO_2 in a dose dependent manner as demonstrated by the count of the cumulative moulting frequency. The cumulative moulting frequency at 100 mg/L of TiO_2 was very high in the first 24 h, but was reduced in the second 24 h. This was accompanied by the first occurrence of mortality and could mean that moulting is an important line of defence for *D. magna* in the attempt to get rid of particles that restrict their fitness. In contrast, Oberdörster et al. (2006) reported that moulting decreased with exposure to fullerenes at concentrations of 2.5 and 5 mg/L. A change in the moulting frequency can lower the number of offspring since release of the neonates out of the broodpouch and moulting concur (Oberdörster et al., 2006). With an increased moulting frequency, as observed in this study, the production of neonates could be reduced and the energy rather invested in the production of a new carapace or the neonates released prematurely, lowering the survival of the offspring. The uptake of nanoparticles in the gut could mean decreased uptake of food particles and increased potential for nanoparticle crossing membranes and entering the body cavity. The form in which nanoparticles are taken up, as agglomerates or single particles, and their behaviour in the intestinal tract needs to be studied further. In order to improve the assessment of the moulting frequency, single animals were exposed individually in smaller exposure chambers. This study also demonstrated the difficulties associated with assessing moulting during exposures to NP carbon black (data not shown) since agglomerates in suspension make the detection of shed carapaces hard.

3.4.2 Chronic 10 day toxicity tests

The conclusions for acute toxicity tests are also true for the chronic tests. Mortality was observed in relatively low concentrations (1 mg/L) of NP carbon black, starting after 48 h after the beginning of exposure. This is the time point at which most acute toxicity tests for nanoparticles in the literature end. This could mean that any delayed toxicity of nanoparticles would make it necessary to assess these data again with a longer duration, for example 96 h. Animals exposed to 1 mg/L of NP carbon black showed a clear dose dependent response regarding mortality, while animals exposed to 0.1 mg/L showed

mortality from day 3 to day 5. Then mortality returned to zero. This could be a sign of acclimatization, adaptation or selective survival to the particle exposure. The assessment of the moulting frequency suggests that it is elevated at the 1 mg/L treatment at day one and two. In the cumulative moulting frequency the animals in the 0.1 mg/L treatment also showed an elevated cumulative moulting frequency but studying the day to day data shows that this is due to the elevated moults in day one and six. Otherwise the pattern followed the control and 0.001 mg/L treatments. The length measurement data show no difference between the remaining animals from the different treatments. This could either be because treatment with nanoparticles has no effect on growth, or the animals affected experienced mortality rather than reduced growth. It is also possible that the exposure period was not sufficient. This needs to be further investigated by expanding it to a 21 day instead of a 10 day test. No released offspring were detected, although in most cases embryos in the broodpouch could be observed. As with the length assessment, longer test duration would be advisable to give the animals time to produce successive broods.

3.4.3 Acute 96 h tests

The three tested materials showed differences in mortality between the different particles as well as between the different sizes tested. While animals exposed to cerium dioxide showed no significant mortality in 96 h at the tested concentration and sizes, animals exposed to silver showed high mortality at very early time points for nano sized particles as well as their micro sized counterpart. Carbon black seems to be intermediary concerning toxicity between the two other particles. Carbon black also showed the biggest difference in mortality when the different sized particles were compared at the same mass dose. While micro sized carbon black induced no significant mortality, significant mortality can be seen at exposures to nano sized carbon black at the same mass dose.

The high mortality in the silver nano as well as the silver micro exposures could be an indication that a second mode of toxicity, such as the release of free silver ions might be also responsible for the observed toxicity. Navarro et al. (2008) also indicated the importance of free silver ions as a major source of

toxicity in their studies of nanoparticulate silver. In their study the inhibition of silver nanoparticles and $AgNO_3$ on photosynthetic rates of *Chlamidomonas reinhardtii* were measured and compared. It was concluded that free Ag^+ ions present in the media were responsible for observed decreases in photosynthetic activity on treatment with both, Ag nanoparticles and $AgNO_3$. The silver nanoparticles though showed an effect that could not be explained solely by free ions in the media. Navarro et al. (2008) hypothesized, that interactions of particles with metabolic products like H_2O_2 at the surface of the algae could lead to oxidation and release of ions. This could explain why exposures to silver micro particles resulted in toxicity at lower surface area doses compared to the nanoparticle exposed *D. magna* in the here presented study. Nano and micro sized particles would both release silver ions in the media, causing toxicity. Two different scenarios would be possible to explain the toxicity seen in the exposures. First, toxicity of silver was just dependent on free ions released by the two different particles into the media. The higher toxicity of nanoparticles, when compared to the micro particles, could be due to the higher surface area of the nanoparticles and hence a greater formation of ions. The second scenario, in addition to the release of ions, would include an interaction of particles with the organism for example by ingestion. Here as well, higher surface area or particle number of nano silver, at the same mass dose, could be responsible for the increased toxicity observed. Which scenario applied to the exposures here, could not be determined. An exposure alongside with cysteine, which binds free silver ions, as described in Navarro et al. (2008), could provide further clues. A second publication (Miao et al., 2009) attributed toxicity observed in exposures of the marine diatom *Thalassiosira weissflogii* to silver nanoparticles solely on free silver ions, with nanoparticulate silver having negligible toxic effects.

From the results presented here, a gradient of material toxicity can be drawn, with cerium dioxide being the least toxic, to silver being the most toxic. Also a size dependent increase in toxicity was observed when significant negative effects were observed, with exposures to nano sized particles being more toxic than micro sized particles.

When studying the cumulative moulting due to nanoparticle and microparticle exposure it can be said that this endpoint is more sensitive than mortality. Moulting frequency in exposures to nano sized carbon black showed that in the two highest concentrations (5 mg/L and 10 mg/L) there was a reduction of moulting at day one, even before mortality occurred, and a reduction on day two of the 10 mg/L treatment. The micro treatment followed the same pattern as described for mortality with no effect observed on moulting at the concentrations tested, implying that either no sub lethal stress occurs at the tested concentrations, or moulting frequency is not sensitive enough to detect it.

Exposure to cerium dioxide showed no change in moulting frequency of *D. magna* exposed to micro particles, leading to the same conclusions as for the carbon black micro treatments. In contrast, the animals in the highest concentration in the nano treatment showed a significant reduction in moulting over the tested period although no significant mortality occurred. This leads to the conclusion, that moulting frequency is a more sensitive endpoint to detect stress than mortality on exposure to CeO_2.

Looking at growth by measuring the length of animals it can be seen in the case of cerium dioxide and silver that effects were observed at the same concentrations at which *D. magna* was already affected in the moulting frequency. Additionally, significant reduced growth when compared to the control could be seen when *D. magna* were treated with nanoparticle silver at a concentration of 0.1 mg/L. A reduced growth was also observed in *D. magna* that were exposed to 0.01 mg/L of micro and nano sized cerium dioxide although no lower moulting frequency or higher mortality occurred at those concentrations. A similar incidence of a low concentration having an equal effect as a high concentration, with intermediate concentrations not being affected, was observed in the chronic, 21 day exposure to nano silver (section 3.3.8) and will be discussed in section 3.4.4 of this chapter.

A length measurement after 96 h of exposure to carbon black was not conducted, since the carbon black exposures were the first to be carried out and this endpoint was added after obvious length differences were seen in the exposure to micro and nano sized cerium dioxide.

3.4.4 Chronic 21 day tests

Although no mortality was observed in the carbon black chronic test, it was clearly observed that the organisms exposed to the 2 mg/L nanoparticle treatment and, to a lesser degree the 1 mg/L treatment, were subject to stress. While cumulative moulting only showed significant reduction in the 2 mg/L treatment on certain days and reached approximately the same level of moulting after the 21 day exposure period, the reduction in growth and the absence of offspring showed that the animals were nevertheless under considerable stress.

The chronic tests with cerium dioxide revealed no toxic effects for the exposures to micro sized cerium dioxide in a mass dose dependent manner. Although mortality was increased in the 3 mg/L treatment, this effect might be attributed to experimental reasons rather than effects due to particle exposure since the same concentration showed no effect in moulting, growth or offspring. The increased mortality might have been the result of some animals floating in the beginning of the experiment, therefore being handicapped due to prevention of food uptake as a consequence. However, even if the mortality was elevated in this treatment, it showed not to be significantly higher than mortality of *D. magna* in the other treatments.

The results from the nano exposure to cerium dioxide confirm effects already seen in the acute tests. At a concentration of 10 mg/L the animals showed a decreased moulting frequency over the 96 h exposure, as well as reduced growth. It seems that a prolonged exposure to this concentration leads to mortality. However looking at the results from the high concentration exposure, it can be hypothesised that above a threshold concentration, duration of exposure is more important than concentration of exposure since no significant mortality after 96 h was observed at 50 mg/L of nano sized cerium dioxide. This could be explained by the particles interfering with food uptake for example rather than having toxic effects by themselves. Animals exposed to carbon black, titanium dioxide and cerium dioxide were completely covered when exposed to higher concentrations of particles such as 5 mg/L or 10 mg/L (Fig. 3.4). This would lead to an impaired swimming ability as well as a reduced

filtering rate due to the filtering apparatus being clogged up by particles. Additionally, suspended particles were taken up readily at even low concentrations and accumulated in the gut (Fig. 3.2 and Fig. 3.3).

It is known, that suspended sediment particles like silt and clay can have effects in cladocerans on various parameters, like ingestion rate (Kirk, 1991), feeding rate, body growth and brood size (Kirk, 1992). But those effects were just observed at concentrations as high as 50 mg/L of suspended sediment in water (Kirk, 1991, Rellstab and Spaak, 2007). With concentrations below 25 mg/L a beneficial effect to fitness of *Daphnia* species was in fact observed at low food concentrations (Rellstab and Spaak, 2007). It was hypothesized, that lower concentrations could be beneficial to a certain point due to higher turbidity that decreases predatory pressure in natural habitats, or the ability of clay and silt particles to reduce the bioavailability of hydrophobic toxicants (Rellstab and Spaak, 2007). However, these factors are not applicable to laboratory experiments and no significant beneficial effect due to low concentration exposures to particles was observed. The reasons given for a reduction of fitness were an inhibition of algal feeding rate due to a decreased ingestion rate (Kirk, 1991), since nutritional algal cells often get rejected accidentally alongside clay particles in the filtering process (Kirk, 1992). The high proportion of inorganic material in the gut forces the animals to filter more water to obtain their required energy (Rellstab and Spaak, 2007). The ingested clay particles, that accumulate in the gut, cause the animal to reduce the beating rate of the feeding appendages which even further reduces the rate of food collection (Kirk, 1992). As stated earlier, these studies pointed out that negative effects were not observed below 50 mg/L and also the particles used for those assessments had a larger mean diameter than the nano sized particles used here. In fact, it was even stated, that particles < 1 µm had no inhibitory effect on *Daphnia* at all (Rellstab and Spaak, 2007). While the cerium dioxide micro sized particles with < 5 µm fit within the size range that could have adverse effects on fitness of the studied species *D. magna*, the highest concentration seems to be, at 10 mg/L not high enough to cause stress in the endpoints observed. The nano sized cerium dioxide particles, however, are of a size below 25 nm, which is well below the size that should affect the animals. Notwithstanding effects were observed at the highest concentration in the acute studies (moulting and

growth) as well as the chronic study (mortality). It is possible (in fact, likely), that the actual sizes of the nano sized particles suspended in water was, due to agglomeration, much higher than the size of the pristine particles and might fall in the size of > 1 μm which may lead to the effect of decreased nutrient uptake. On the other hand, it might be possible that with cerium dioxide nanoparticles other mechanisms unique to nanoparticles take effect.

Chronic exposures to silver showed that this material, even at low concentrations for both sized particles, affected fecundity of *D. magna.* With a strongly reduced reproduction rate, a population exposed to even low concentrations of μg/L of silver might not be able to sustain itself. Nevertheless, it is important to note that exposures to silver in the laboratory may not reflect fully results obtained in the field. For example, a study (Gao et al., 2009) indicated that toxicity of nano sized silver may be reduced in the presence of increased levels of dissolved organic carbon, which is likely to be higher in the field than in laboratory exposures. In addition, Luoma (2008) has highlighted the importance of silver ligands in the natural environment.

Another remarkable observation for the chronic exposure to silver nanoparticles was, that the lowest concentration tested, 0.001 mg/L showed similar trends in mortality, growth and fertility as the highest concentration tested. The 0.001 mg/L nano silver treatment had the highest mortality of *D magna* in the exposure, although this was not significantly higher than the mortality observed in the control. Exposed to 0.001 mg/L nano silver, *D. magna* also experienced a lower cumulative moulting rate than animals in the other exposure concentrations, except animals in the 0.05 mg/L treatment, but again this effect proved to be not significant. Lastly, with $p = 0.062$, a strong trend towards less offspring than the control could be observed for animals exposed to 0.001 mg/L nano silver. The growth of the animals was significantly higher for animals exposed to 0.001 mg/L nano silver than the control, with animals exposed to 0.05 mg/L having an increased growth as well when compared to the control, although not significant. Increased growth in *Daphnia* does not necessarily mean increased fitness (Rellstab and Spaak, 2007). It can also mean that energy was diverted from other activities, for example from reproduction. This actually would mean a decrease in fitness, since less offspring would be

contributed to the next generation. In contrast, the juvenile growth rate can be directly seen as a measure of fitness (Lampert and Trubetskova, 1996, Trubetskova and Lampert, 2002), since growth to reach maturity, in order to reproduce, is essential. Growth in long term exposures like the chronic 21 day exposures has to be set in context with other endpoints like fertility since increased growth and decreased fertility could also be a not beneficial effect as already mentioned.

The dose response pattern emerging in growth related to exposure to nano sized silver particles, was also observed for growth in treatments exposed to nano sized cerium dioxide (see Fig. 3.19) and can arguably be ascribed to hormesis (Calabrese, 2005, Calabrese and Baldwin, 2003) which was also observed by two more recent studies (Barrena et al., 2009, Drobne et al., 2009). While here also a U- shaped dose- response occurs when growth after 21 days of exposure is plotted against concentration, it is different from hormesis because hormesis is generally understood as low- dose stimulation and high-dose inhibition (Calabrese and Baldwin, 2003). One explanation for the effects seen might be that nano sized particles might be better dispersed at low concentrations, due to less particles in the media and less chance of agglomeration, and thus higher probably of organism-particle contact.

It was also noted in the 0.001 mg/L and 0.05 mg/L treatments to silver particles on several occasions that release of neonates and moulting were occurring several days apart. These two processes are normally closely linked. In one occasion in the 0.001 mg/L treatment it was observed that one individual had the old carapace still intact, while a new one had built underneath (Fig. 3.44). This specific animal had released neonates 4 days earlier and failed to moult. It is known that metal ions as silver can inhibit Na^+ uptake by competing with Na^+ for the Na^+ channels (Bianchini and Wood, 2008, Lam and Wang, 2006). An interference of silver ions in the moulting process and a lower Na^+ body concentration could have those effects, since active Na^+ uptake in crustaceans is highest immediately before and after the moult, in order to balance the water uptake that occurs to allow growth (Zare and Greenaway, 1998). It could even be speculated, that the increased growth is a result of the animal taking up more water in order to counterbalance the Na^+ deficiency and thus grows larger.

This uptake of water is an active, drinking like process, and will be discussed further in chapter 4.

Estimated environmental concentrations of silver are predicted to be in water at 0.1 µg if 10% market penetration of nano sized silver in consumer products takes place (Boxall et al., 2007). That means if 10% of consumer products like fabrics (e.g. clothing), shampoo, soap and toothpaste, for example, that are already widely available and sold commercially, include silver nanoparticles. This predicted concentration is one tenth of the concentration where adverse effects were observed. A 100% marked penetration would exceed this concentration (Boxall et al., 2007) and could lead to problems, assuming that nanotechnology becomes as established in future to reach this market penetration.

3.4.5 Modelling the effects of nanoparticle exposure

The models used for analyzing effects in the short term, 96 h exposures seem to fit reasonably well the data obtained. The greater the differences between effects induced by different concentrations of the same particle, the better the model is able to describe the events. In the case of measurement of mortality in the cerium dioxide exposures, where no mortality occured over a wide range of concentrations, models will have naturally a lower significance. Still, the factors listed in table 3.1 give a good summary of the effects and their respective strength. The statement that the different particles can be classified due to their toxicity as cerium dioxide, as the least toxic, and silver as the most, are confirmed. Moulting and mortality in the chronic tests showed to have less influence on the model. In the case of mortality, this poor link was mostly due to the lower concentrations used in the exposures and therefore a low mortality obtained. This resulted in differences being less significant between treatments of different concentrations of particles.

In the case of moulting, the difficulty with modelling these effects lies in the life history of *D. magna*. The animals pass through their whole life cycle in the chronic experiments described here. The tests are set up with neonates < 24 h

old which, during the experiment, reach maturity and reproduce. *D. magna* has 5-6 neonatal instars before it reaches maturity. During those instars, moulting frequency is very rapid, often 4-5 times during 6 days. Once the animal becomes an adult and starts to reproduce, the moulting frequency reduces to once every 3-4 days. Although both situations follow a linear increase of cumulative moults, the frequency will be different and a representation by a single equation might not be an optimal approach.

A second problem of the models for chronic studies was that the effects seen in the 21 day exposure to nano sized silver were similar to both the low and high concentrations, with intermediate concentrations not showing any effect. A linear model describing an increase or decrease over time for different concentrations will underestimate the overall effect if the single effects have a U- shaped dose-response. This can be seen in the factors concerning offspring generated in the silver nano and silver micro treatments.

It can be concluded from the results shown in this chapter that a gradient of toxicity can be drawn from cerium dioxide having a low toxicity at the tested concentrations, carbon black an intermediate and silver a high toxicity. Furthermore, nano sized particles induced greater effects, when effects were observed, than micro sized particles. Toxicity and moulting frequency seems to be affected in a surface area dose related pattern with carbon black, while silver showed a different mode of toxicity that might be due to Ag^+ ions released into the media. Long term, chronic exposures showed effects at lower concentrations than the short term, acute exposures for all three particles tested. Silver nanoparticles in chronic exposures and cerium dioxide micro- and nanoparticles showed that growth can be negatively affected at low concentrations, while intermediate concentrations showed no effect. Also abnormalities in moulting were observed at high as well as low concentrations of silver nanoparticles. Both endpoints, growth and moulting, should be an aim for future studies, taking low concentrations into account as tested in this work.

4. Micro- and nanoparticle uptake by *Daphnia magna*

4.1 Introduction

Due to the small size of a nanoparticle, the active uptake into organisms needs to be investigated since the size of particles is contradictory to the feeding strategy of the organism. Nevertheless, uptake of NPs has been reported in several publications including fullerenes in *Daphnia* (Oberdörster et al., 2006), polystyrene microspheres in medaka (Kashiwada, 2006), gold particles in *Daphnia* (Lovern et al., 2008) and lipid coated carbon nanotubes in *Daphnia* (Roberts et al., 2007). The assessment of uptake is therefore essential as it relates to consequences of exposure and dose.

The most likely route of uptake of nanoparticles by *D. magna* is through ingestion, including active selection by the feeding apparatus, as well as passive diffusion or uptake alongside larger particles. The maximum diameter of particles that can be actively ingested are determined by the size of the animal (Burns, 1968), which for a fully grown *D. magna* can be approximately particles with the size of 70 μm and above. The minimum size is believed to be dependent on the distances between the setulae on the thoracic limbs of *D. magna*, which is independent from age or size due to the gap being constant (Geller and Muller, 1981). Setulae act like a comb and filter particles out of the feeding current along the opening of the carapace and channel them to the mouth. *D. magna* is classified as a fine filter feeder (Geller and Muller, 1981) which is able to actively filter particles as small as 200 nm, although this is an estimate based upon the size/gap between the setulae. Feeding in this dimension is not surprising, since *D. magna* is often reported to feed on bacteria that fit within the size range of 200 nm and greater (Hartmann and Kunkel, 1991, U.S. Environmental Protection Agency (EPA), 2002a). For *D. pulicaria,* free dispersed bacteria are often ingested in the presence of mucus, although this mucus is normally absent when the bacteria are attached to bigger algae

(Hartmann and Kunkel, 1991). It was even suggested, that *Daphnia* not only feeds by mechanical sieving but also can gather food particles even in the nano range by direct interception (Bednarska, 2006, Gerritsen et al., 1988) or by drinking the surrounding media to replenish depleted sodium and to facilitate digestion (Bianchini and Wood, 2008, Gillis et al., 2005). The direct interception, drinking, as NP attached to mucus or as larger particulate matter are all possible uptake routes for particles in the nano size range. The appearance of ingested matter in the gut is reported to be very rapid after feeding. For example, under optimal conditions, the gut has been reported to fill within 30 min of exposure to a food source (Lotocka, 2001). Approximately 48 min is the estimated retention period of a food particle within the gut before defecation (McMahon and Rigler, 1965).

Once ingested, food particles are digested, and nutrients are transported across the epithelial lining of the digestive tract. The midgut region, which is lined with differentiated columnar epithelial cells, is especially responsible for both enzyme excretion and absorption of digested food (DeCoen and Janssen, 1997). The epithelium has a well-developed apical brush border and shows endocytotic activity at the base of the apical microvilli and heterophagic vacuoles (Bodar et al., 1990). Large spherical storage cells are scattered along the digestive tract with the largest concentration in the abdominal part of the animal near the posterior curve of the digestive tract (Bodar et al., 1990). These storage cells contain lipids such as triacylglycerol (Goulden and Hornig, 1980) and large amounts of glycogen (Bodar et al., 1990). The amount of lipid storage cells accumulated can be seen as a fitness parameter, since lipids are utilised in periods of low ambient food resources (Goulden and Hornig, 1980, Holm and Shapiro, 1984). The storage cells are also the site of synthesis of vitellogenin, which is then carried by the haemolymph to the ovaries where it forms yolk globules (Bodar et al., 1990). The storage cells are separated from the surrounding haemolymph by a folded plasma membrane.

Such storage droplets are not just found in *Daphnia,* but also in other invertebrates such as copepods (van Der Veen, 2005). An uptake of polystyrene fluorescent nanoparticle was observed into the oil droplets of eggs and the yolk area of medaka (*Oryzias latipes*), but also in various other organs,

most notably in the gills and intestines by Kashiwada (2006). This study aims to investigate such translocation in *D. magna*.

D. magna neonates, before hatching, also have a risk of being exposed to nanomaterials, if the adult happen to be in contaminated medium during the development of the embryo. To prevent low oxygen situations for the embryos, *D. magna* produces a flow of ambient media, diverted from the feeding flow by the abdomen of the animal. This water enters the brood chamber at the posterior end and flows to the anterior end before entering the ventral carapace chamber (Seidl et al., 2002). In this way nanomaterials could be present in the feeding current of the unhatched neonates and could expose them to elevated levels or even disturb their development.

A different way in which *D. magna* could be exposed to nanoparticles is via coating of the carapace. Although the name “Branchiopoda” suggests, that uptake of oxygen mainly happens through the branchial sacs on the thoraic limbs, gas exchange also takes place through the carapace surface (Colmorgen and Paul, 1995, Pirow et al., 1999). Nanoparticles might be small enough to enter into the haemolymph through the same pathway or disrupt the gas exchange by clogging up the exchange mechanism, although this mechanism has not yet been investigated.

In the present study the qualitative, as well as the quantitative, uptake and fate of nanoparticles and larger counterparts were studied using the aquatic invertebrate *D. magna* as a model organism. In the environment, organisms will be exposed to a wide range of different particle sizes. To assess size-dependent uptake, uniform sized particles were used to view uptake as a function of size. The particles studied were carboxylated fluorescent polystyrene beads of two different sizes; 20 and 1000 nm. These were used as model particles because they are well characterized (Colmorgen and Paul, 1995, Kolodny et al., 2001, Kulkarni et al., 2005, Pirow et al., 1999) and the nano-form has been shown to induce inflammation in animal models (Brown et al., 2001), and to produce pro-inflammatory signalling *in vitro* (Oberdörster et al., 2005). The carboxylic surface layer renders the particle negatively charged and relatively hydrophilic which, as a result, is less likely to adsorb proteins and

other biomolecules than the hydrophobic counterparts (Invitrogen, Paisley, UK, 2004). The pristine particle has a negative charge according to the supplier. This makes them ideal for the study of size dependent uptake.

The chosen method for assessing the qualitative uptake of fluorescent particles was confocal laser scanning microscopy (CLSM). This is a non-destructive method which allows the qualitative detection of ingestion in whole animals and the determination of the area of accumulation. Confocal microscopy also allows non-destructive optical sectioning (Chandler and Volz, 2004) due to the fact that illumination, specimen and detector have the same focus (Buttino et al., 2003). The final image corresponds to the point of focus in the specimen. This enables to locate fluorescence, for example, within cell organelles, reducing uncertainty of whether the fluorescence is inside or on the surface of the organelle. A similar method has been employed in ecotoxicology already, for example to assess viability of copepod embryos (Buttino et al., 2003) or quality and development of crustacean eggs (Chandler and Volz, 2004).

To quantify fluorescence a fluorimeter was also used. Quantification of fluorescence in several samples, simultaneously on a single 96 well plate, can thus be done, allowing comparisons between samples.

The particle concentration used in the present study (2 µg/L) was chosen to be low enough to minimize sublethal negative effects due to exposure to the animals that could interfere with the assessment and to be in a medium detectable fluorescence level that allows detection by our methods even when dilution or accumulation of particles takes place. The aim was also to use a low level concentration compared to other environmental studies (Lovern and Klaper, 2006, Lovern et al., 2008, Roberts et al., 2007, Smith et al., 2007) or predicted concentrations that could occur in the aquatic environment (Boxall et al., 2007).

4.1.1 Aims

The aims of this chapter were:

I. To assess and compare the uptake of nano or micro particles by *D. magna* during an exposure in an aquatic environment.
II. To qualitatively examine the total uptake of nano or micro particles and determine possible locations of accumulation.
III. To quantify uptake and depuration of nano or micro particles and compare the two sizes to each other.

4.2 Methods

4.2.1 Materials

The polystyrene beads (FluoSpheres® Molecular probes) were purchased from Invitrogen (Paisley, UK) and the chemicals (Analytical reagent grade) for the reconstituted water from Fisher Scientific (Loughborough, UK).

The reconstituted hard water used in the exposures and for culturing *Daphnia*, was prepared according to U.S. Environmental Protection Agency (U.S. Environmental Protection Agency (EPA), 2002a) guidelines for acute toxicity studies and is described in chapter 2.2.

4.2.2 Particles

Polystyrene carboxylated beads with diameters of 20 nm and 1000 nm were used in the present study. They were labelled with the fluorescent dye fluorescein isothiocyanate (FITC) with an excitation maximum at a wavelength of 505 nm and an emission maximum at 515 nm. The dye was encapsulated within the bead, rather than attached to the particle surface (Invitrogen, 2004).

4.2.3 Particle preparation

The polystyrene particles stock solution was sonicated in a water bath for 30 minutes and then dispersed in reconstituted U.S. EPA hard water (U.S. Environmental Protection Agency (EPA), 2002a) at a final concentration of 2 µg/L. The suspensions were generated immediately prior to use in each experiment.

4.2.4 Particle size Characterization

The size characterization of the polystyrene beads was conducted by photo correlation spectroscopy of quasielastically scattered light (PCS-QELS) (90Plus/BI-MAS, Brookhaven Instrument, New York, NY, USA) at the Institute of Occupational Medicine (IOM) in Edinburgh in collaboration with Dr. Roger Duffin. To measure the size distribution particles were suspended in reconstituted water at a concentration of 2 µg/L, as described above, and measurements were taken in a temperature controlled room at 20°C at 0h, 1h, 2h, 3h, and 4h. The light scattering was measured for 5 min. A pure, filtered sample of U.S. EPA reconstituted water was included as a control.

4.2.5 Source of *D. magna*

The animals used in these experiments were taken from an in house culture that was fed and maintained as described in chapter 2.

4.2.6 Qualitative and quantitative assessment of uptake

Adult and neonate (<24h old) *D. magna* were exposed to the 20 nm and 1000 nm carboxylated polystyrene beads (2 µg/L) in a climate chamber at 20°C and sampled for the qualitative assessment at 0min (control, no beads added), 30 min, 1 h, 2 h, 4 h, 6 h, 12 h and 24 h exposure time. For the quantitative assessment the time points chosen were: 0 h exposure (control, no beads added), 1 h, 2 h, 3 h and 4 h exposure. The animals were exposed in 100 ml beakers with 80 ml of reconstituted EPA hard water per beaker. Five animals were included in each treatment beaker, and each treatment was replicated three times.

For the qualitative assessment of uptake, following exposure, animals were washed twice with deionised water for approximately 2 min and preserved in 10% formalin. The uptake and fate of fluorescent polystyrene beads within the body of the invertebrates was then observed using laser scanning confocal

microscopy (LSM 510 Meta, Carl Zeiss, Welwyn Garden City, UK). The magnification used for adults was 25 times, while for neonates the magnification was 100 times and 200 times. The gain and offset were held constant at 700 and 0 respectively.

For the quantitative assessment of uptake only adults were used (6 weeks old). After sampling, the five animals per replicate were washed once with Trypan blue, to quench fluorescence from the surface of the animals, and twice with deionised water. Afterwards they were sacrificed and preserved in 10% formalin. The water and formalin were removed and replaced with 40 µl of fresh reconstituted water before the *D. magna* were homogenised with a Kontes Pellet Pestle (Fisher Sciences Loughborough, UK). This step was necessary, since the five homogenized animals do not give enough homogenate to cover the bottom of a well in a 96 well plate. Five homogenized six week old *D. magna* delivered approximately 10 µl of homogenate. It was determined that 50 µl were needed to cover the bottom of a well in a 96 well plate reliably which was reached by the 10 µl of homogenate and the 40 µl of reconstituted water added to the sample before homogenation. The homogenised samples were then transferred into a 96 well plate (96 Well Krystal 2000 white, Porvair Sciences, Shepperton, UK) and the fluorescence quantified using a fluorimeter (Fluo Star Optima, BMG Labtech, Aylesbury, UK) at an excitation wavelength of 485 nm and emission wavelength of 520 nm. The concentrations of the fluorescent beads were calculated by linear regression of a standard curve prepared by suspension of the particles, at different concentrations, in EPA reconstituted hard water. This standard curve for the 1000 nm particles ranged from 53 µg/L to 422 µg/L final concentration, while the curve for the 20 nm particles ranged from 1 µg/L to 21 µg/L final concentration. Each standard curve had two more intermediate concentrations. New standard curves were prepared for each of the three repetitions of the experiment. For the calculation of the uptake concentration the values were multiplied by five to take the previous dilution of the *D. magna* homogenate into consideration. Shading or interference of the fluorescence measurement by inclusion of *D. magna* fragments within the homogenate were ruled out by adding polystyrene beads to *D. magna* homogenate and comparing their fluorescence to an identical concentration of pure polystyrene beads (data not shown).

In addition to measuring uptake at the specific time points of 0 h to 4 h, animals were also exposed for 4 h to the polystyrene beads as described above and then transferred to 80 ml of fresh U.S. EPA reconstituted hard water in order to investigate depuration, that is, what proportion might be excreted and/or retained within the organism over time. At the end of the 4 h incubation with particles, the medium was changed hourly to minimize re-ingestion of excreted material. Five time points were also chosen for sampling, as follows: 0 hr, 1 h, 2 h, 3 h and 4 h post exposure. The fluorescence of the exposed organisms was assessed by fluorimetry as described above.

The uptake as well as the depuration experiments were repeated three times, with five animals per replicate and three replicates per time point at each repetition. No mortality was observed in any of the experiments.

For transmission electron microscopy (TEM), the samples were fixed in 3% glutaraldehyde prepared in 0.1 M sodium cacodylate buffer at pH 7.3, for 2 h. The fixed samples were then washed three times, for 10 min per wash, in 0.1 M sodium cacodylate. Specimens were subsequently post-fixed in 1% osmium tetroxide prepared in 0.1 M sodium cacodylate for 45 min, before washing three times, for 10 min per wash, in 0.1 M sodium cacodylate buffer. These sections were dehydrated in 50%, 70%, 90% and 100% normal grade acetone for 10 min each, followed by further two 10 min incubations in acetone (Analar grade). Samples were then embedded in Agar 100 resin, before cutting 1 µm sections using a Reichert OMU4 ultramicrotome (Leica Microsystems,, Milton Keynes, UK). The resultant sections were stained with Toluidine blue and viewed by light microscopy in order to select suitable areas for investigation. Ultrathin sections, 60 nm thick were cut from selected areas, stained in uranyl acetate and lead citrate and viewed in a Phillips CM120 TEM (FEI UK, Cambridge, UK), and images were collected via a Gatan Orius CCD camera (Gatan, Oxon, UK). The fixing, dehydration, embedding, cutting and imaging of the samples were done at the University of Edinburgh by Mr. Stephen Mitchell.

4.2.7 Statistical methods

Statistical tests were carried out with Minitab release 13.1 or release 14 (Minitab, Coventry, UK). Data were checked for normality and homogeneity of variances and upon compliance parametric methods were used. One-way analysis of variance (ANOVA) was used to assess any effects of each particle size and two-way ANOVA was used to compare the effects of the two particle sizes against each other. A Tukey post hoc test was carried out for multiple comparisons of means if the variances of the residuals were homogenous, otherwise a Games-Howell post hoc test was used for the same purpose. Significance was assumed with $p \leq 0.05$. Given that two independent hypotheses were tested using the same data, the Bonferroni correction was applied and a significance level of 0.025 was considered.

4.3 Results

4.3.1 Size distribution of polystyrene beads in water

Dynamic light scattering with PCS-QELS revealed that for the 20nm polystyrene beads the most frequently observed sized particles found in the reconstituted water were approximately 20 nm or 40 nm in diameter, resulting in an overall average of approximately 30 nm (24.6 nm ±1.9 nm standard error for 0 h, 34.2 nm ±1.6 nm standard error for 4 h). The particle size was monitored over 4 h in three replicates with a sample taken from each every hour (Figs. 4.1 - 4.5), and a one way ANOVA of the averages at the different time points showed that there was no significant difference in average size between different time points ($p = 0.776$) (Fig. 4.6). It was observed, that replicates, especially at the time points of 2 h and 3 h, had different distribution of particles from each other, with the distributions being either around 20 nm or around 40 nm. It was never observed that a single replicate had a particle distribution with one peak at 20 nm (single particles) and a second peak at 40 nm (particles as duplets). Few agglomerates above 100 nm were detected, and in all cases they stayed below 0.5% of overall occurrence, showing that the 20 nm polystyrene beads are well dispersed in the reconstituted water over the exposure period. An assessment of the 1000 nm polystyrene beads by PCS-QELS proved to be impossible since they were outside the detection limit of the equipment.

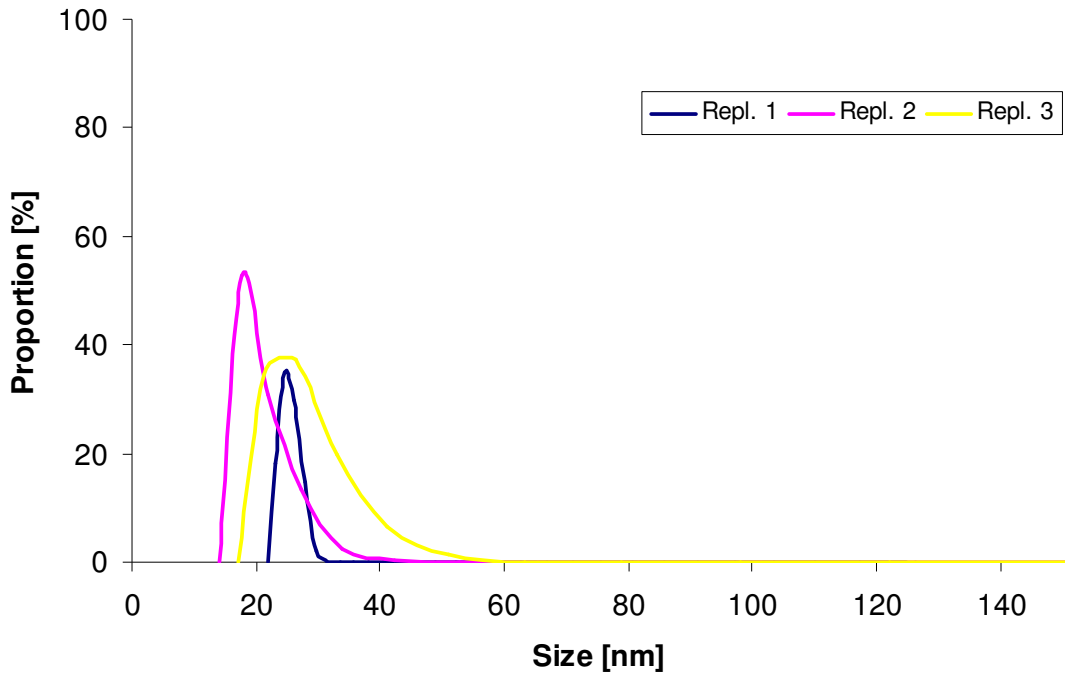


Figure 4.1: Size distribution of 20 nm polystyrene beads in water (2 µg/L) after 0 h. Shown are the three different replicates that were measured independently at each time point

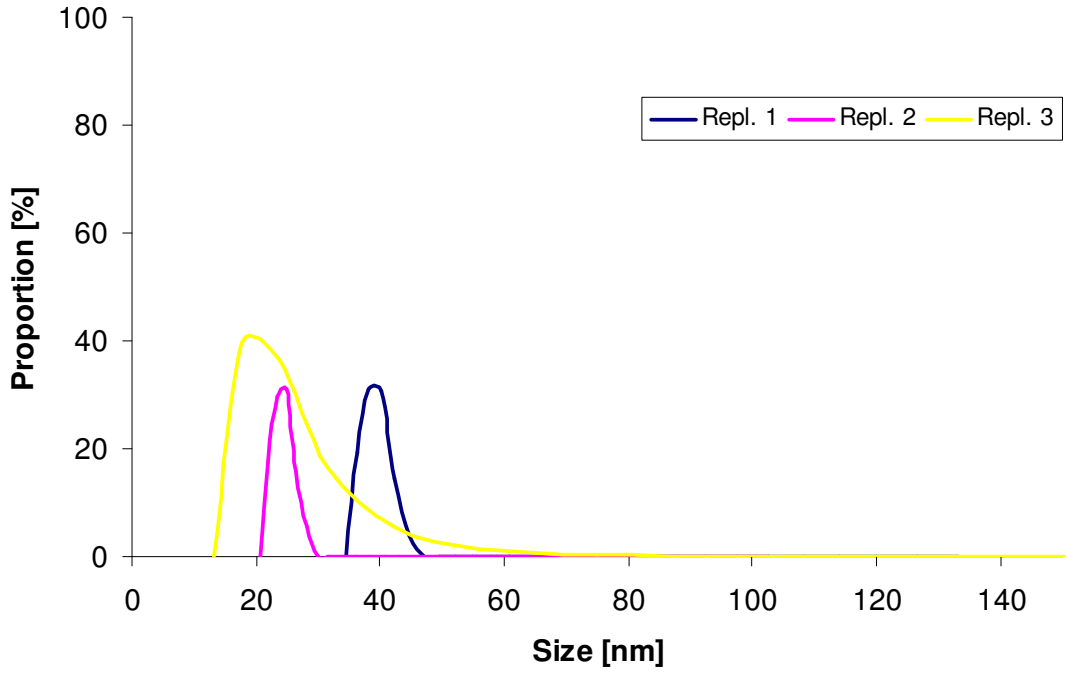


Figure 4.2: Size distribution of 20 nm polystyrene beads in water (2 µg/L) after 1 h. Shown are the three different replicates that were measured independently at each time point.

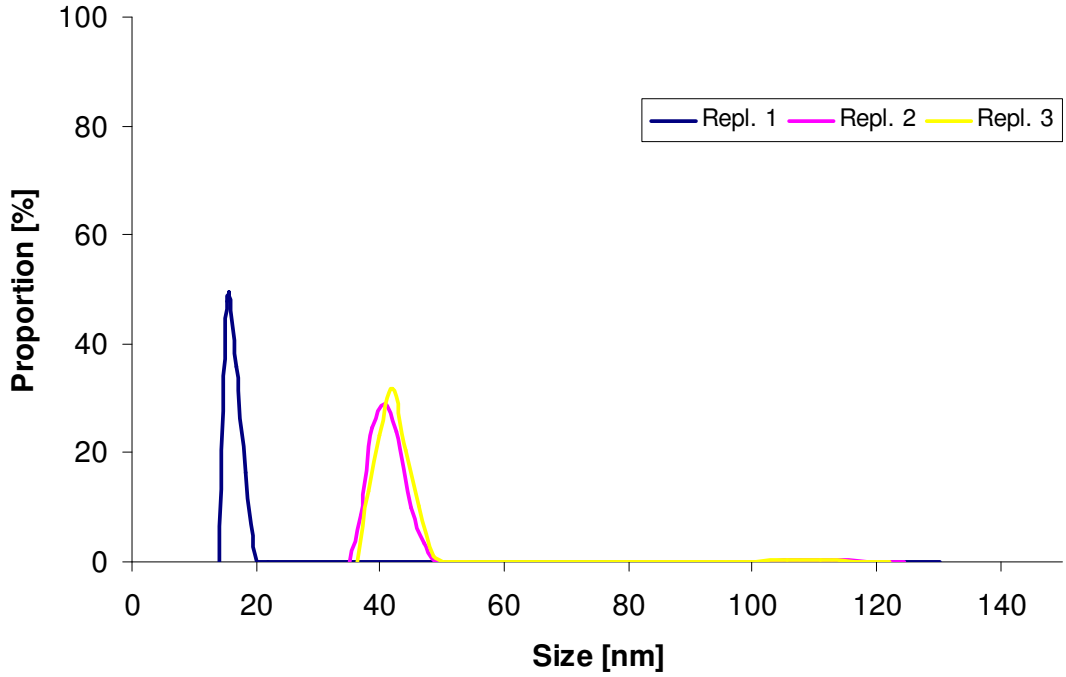


Figure 4.3: Size distribution of 20 nm polystyrene beads in water (2 µg/L) after 2 h. Shown are the three different replicates that were measured independently at each time point.

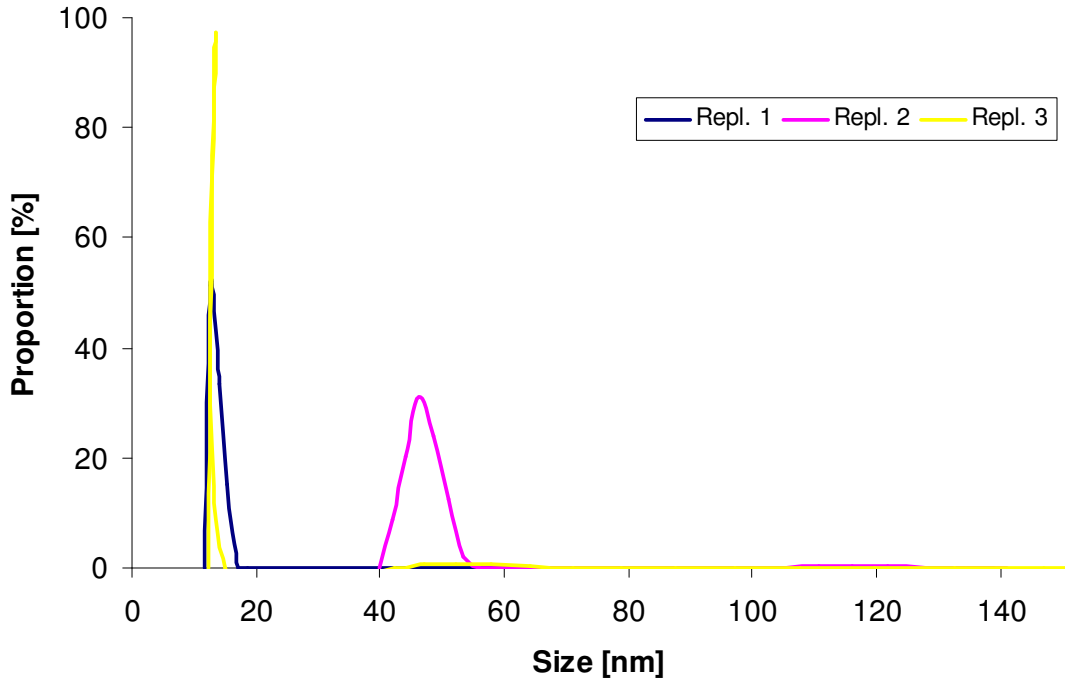


Figure 4.4: Size distribution of 20 nm polystyrene beads in water (2 µg/L) after 3 h. Shown are the three different replicates that were measured independently at each time point.

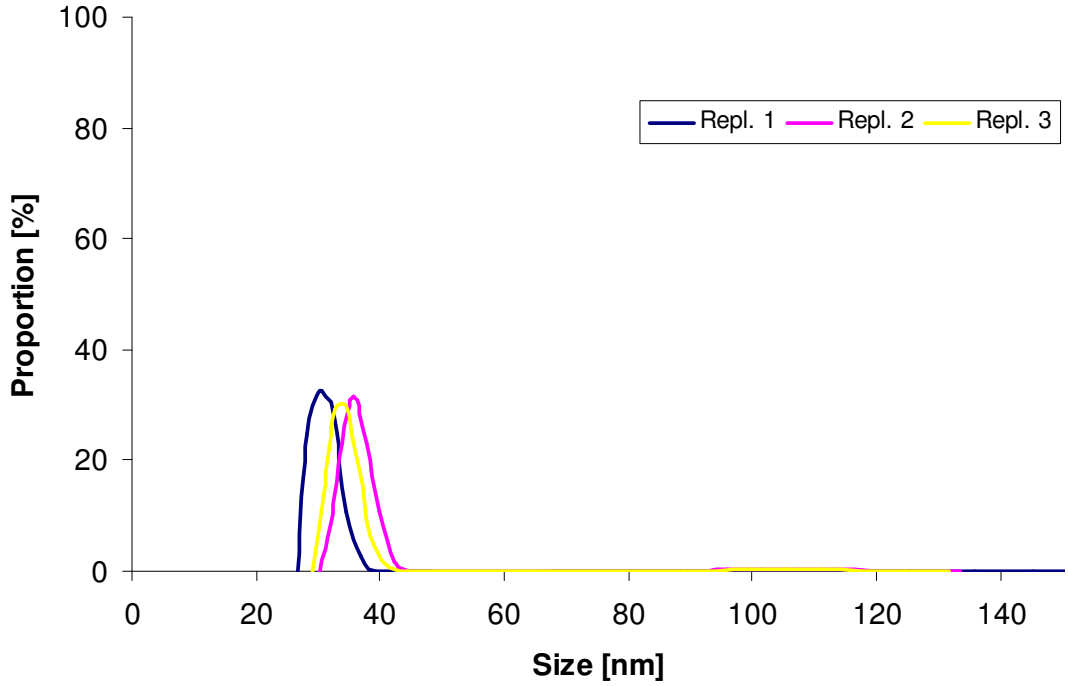


Figure 4.5: Size distribution of 20 nm polystyrene beads in water (2 µg/L) after 4 h. Shown are the three different replicates that were measured independently at each time point.

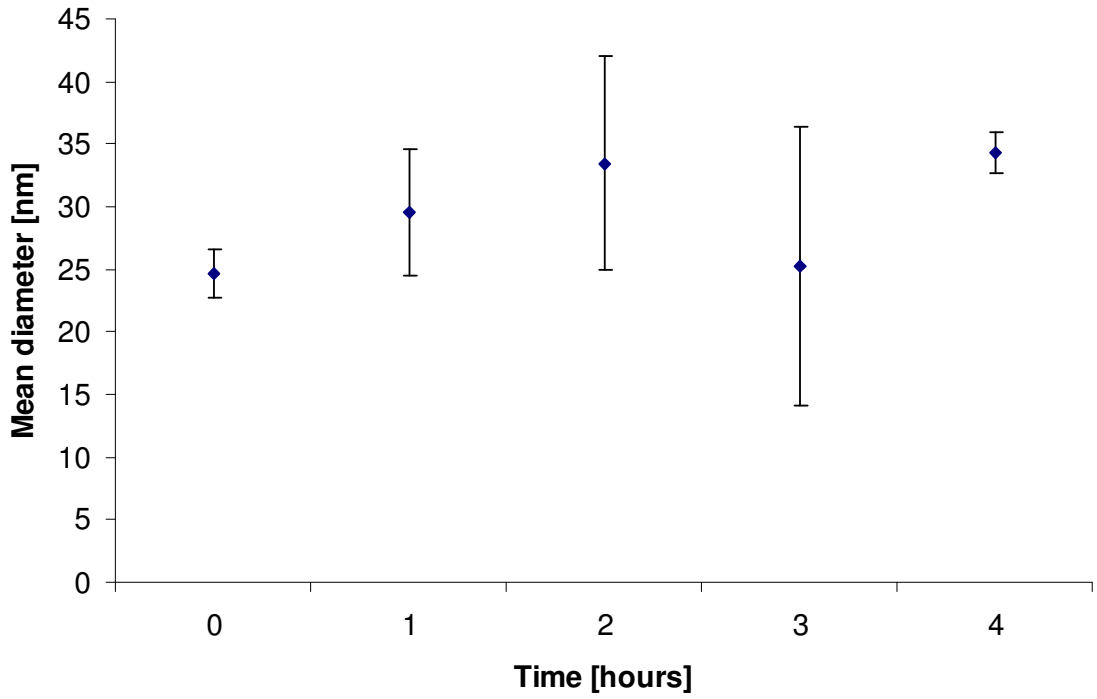
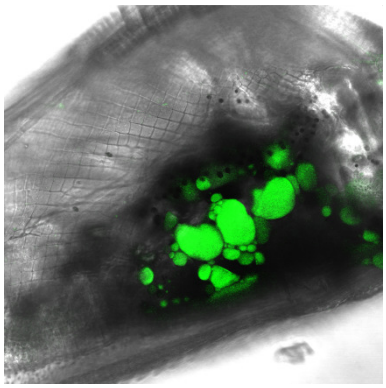
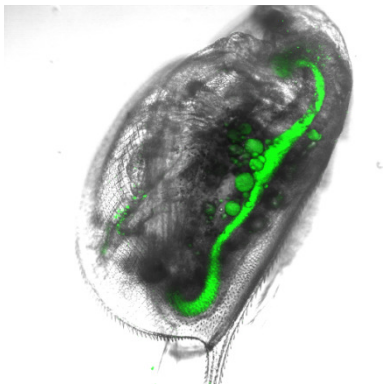
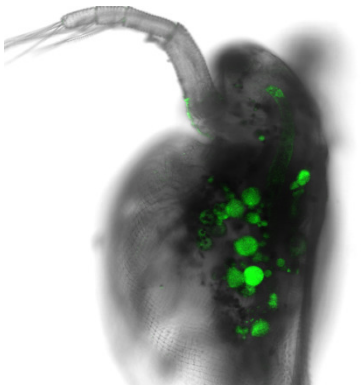
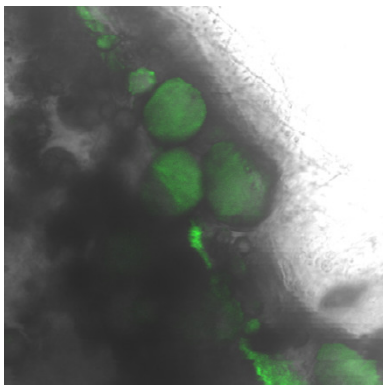


Figure 4.6: Mean diameter of 20 nm polystyrene beads in water (2 µg/L) over a time period of 4 h (values are means +/- standard error).

4.3.2 Qualitative assessment of fluorescent polystyrene bead uptake by *D. magna*.

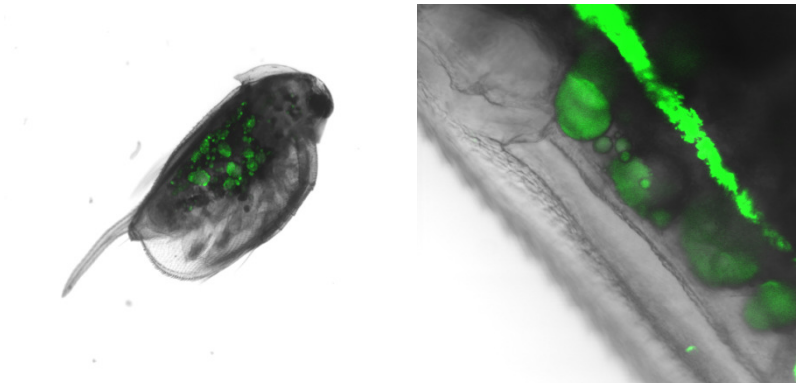
To assess the pattern of uptake of 20 nm and 1000 nm fluorescent polystyrene beads, three adults and three neonates were observed for each time point of 0 min (control, no beads added), 30 min, 1 h, 2 h, 4 h, 6 h, 12 h and 24 h, and confocal images, superimposed on bright field images were captured (Fig. 4.7 and Fig. 4.8). Uptake was observed at the first time point of 30 min for both 20 nm and 1000 nm particles. In all observations of the *D. magna* exposed to 1000 nm the gastrointestinal tract clearly contained sufficient fluorescent particles to allow observation of the entire tract, while the same observation was not always as clear with the 20 nm particle treatments. In the presence of both particle sizes, accumulation of fluorescence was observed in structures distinct from the gastrointestinal tract, hypothesised to be the oil storage droplets.

Neonates	**20 nm**	**1000 nm**
Duration of exposure		
<u>30 min</u> 20 nm 200X magnification 1000 nm 100X magnification		
<u>60 min</u> 20 nm 100X magnification 1000 nm 400X magnification		

120 min

20 nm 100X magnification

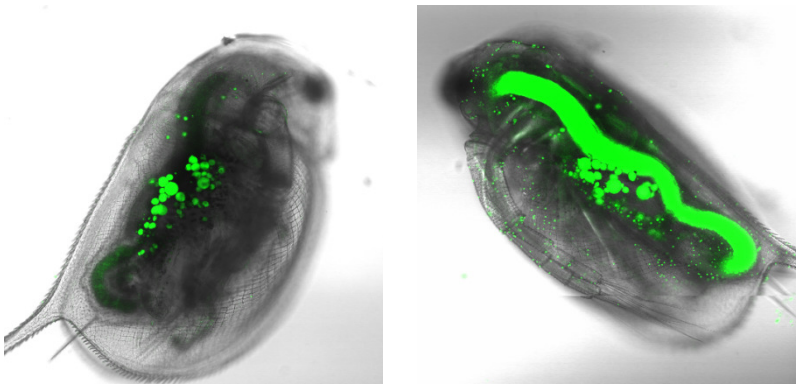
1000 nm 400X magnification



240 min

20 nm 100X magnification

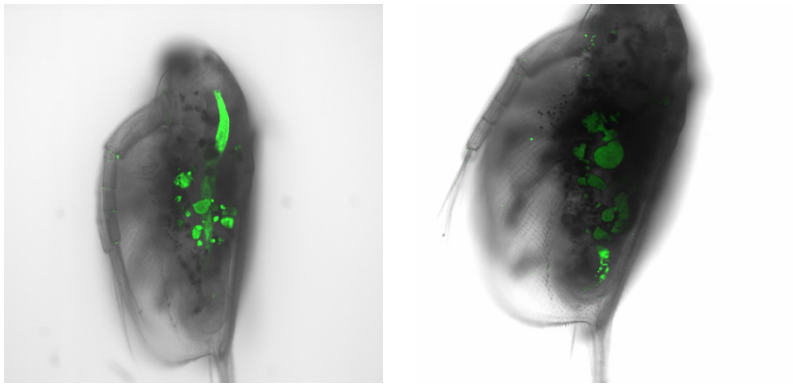
1000 nm 100X magnification



360 min

20 nm 100X magnification

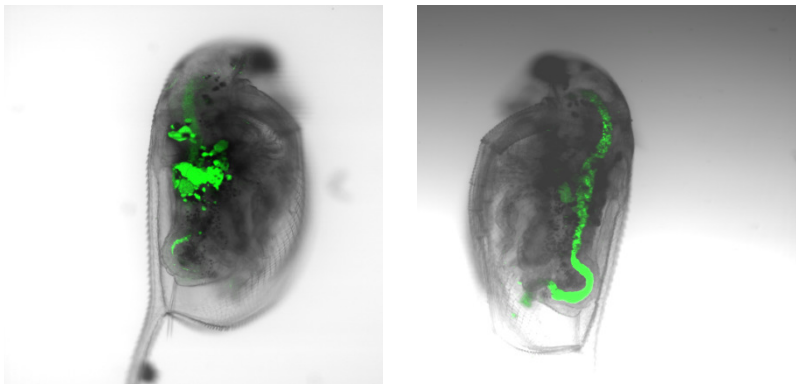
1000 nm 100X magnification



720 min

20 nm 100X magnification

1000 nm 100X magnification



1440 min

20 nm 200X magnification

1000 nm 100X magnification

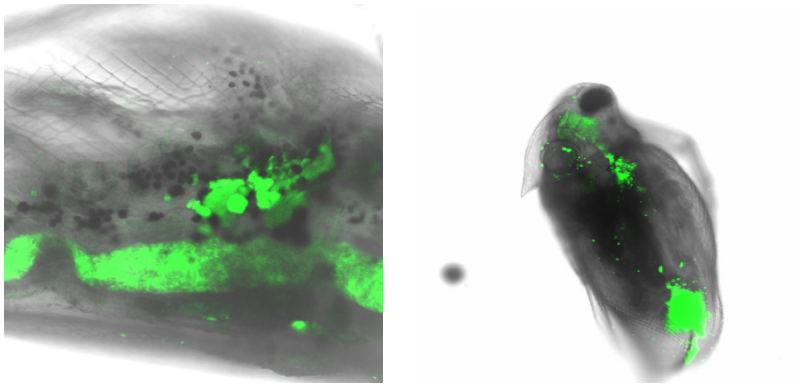
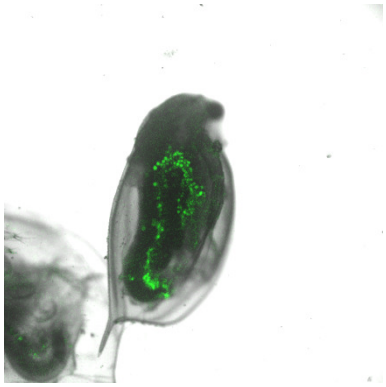
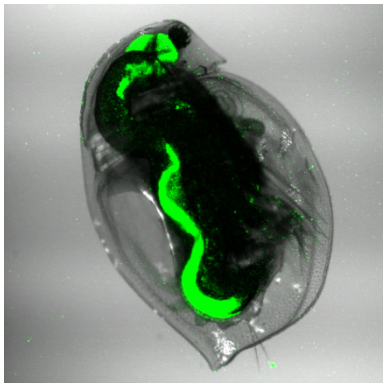
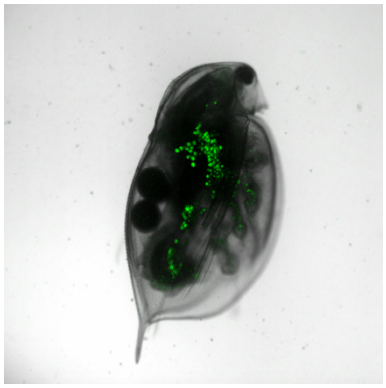
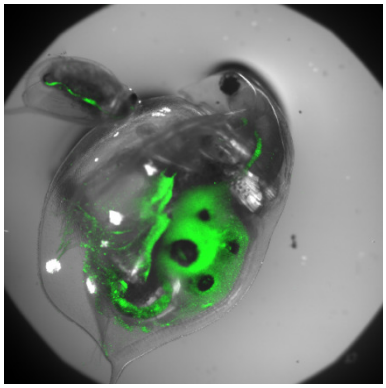


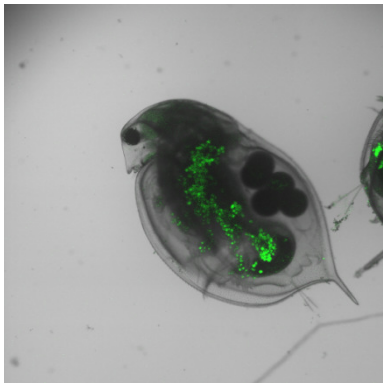
Figure 4.7: Uptake of 20 nm and 1000 nm polystyrene beads in neonate *D. magna* (< 24 hours old at start of experiment) over 24 h

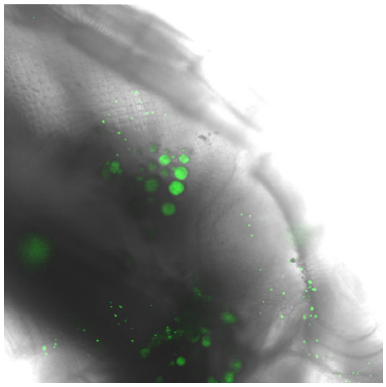
Adults	**20 nm**	**1000 nm**
Duration of exposure		
30 min 20 nm 25X magnification 1000 nm 25X magnification		
60 min 20 nm 25X magnification 1000 nm 25X magnification		

120 min

20 nm 25X magnification

1000 nm 200X magnification

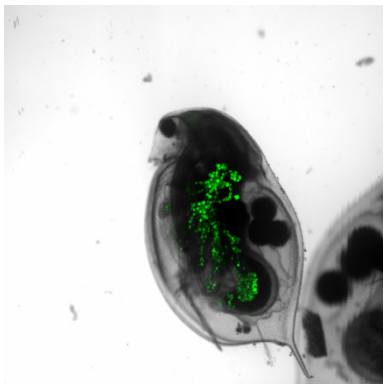


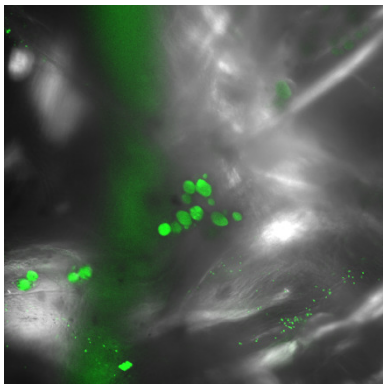


240 min

20 nm 25X magnification

1000 nm 200X magnification

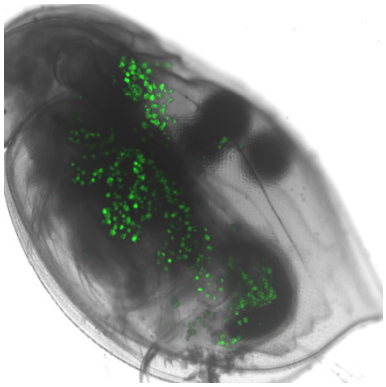


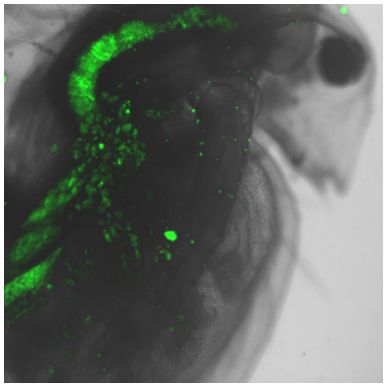


360 min

20 nm 25X magnification

1000 nm 100X magnification

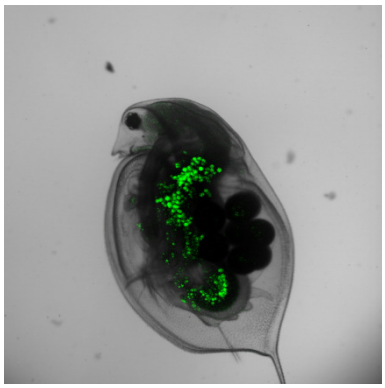


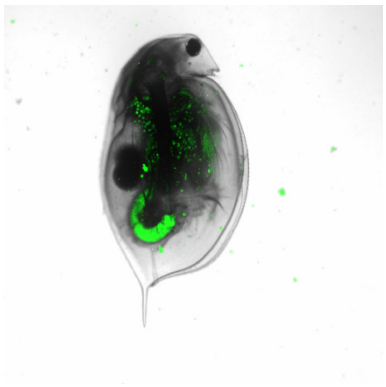


720 min

20 nm 25X magnification

1000 nm 25X magnification





1440 min

20 nm 25X magnification

1000 nm 100X magnification

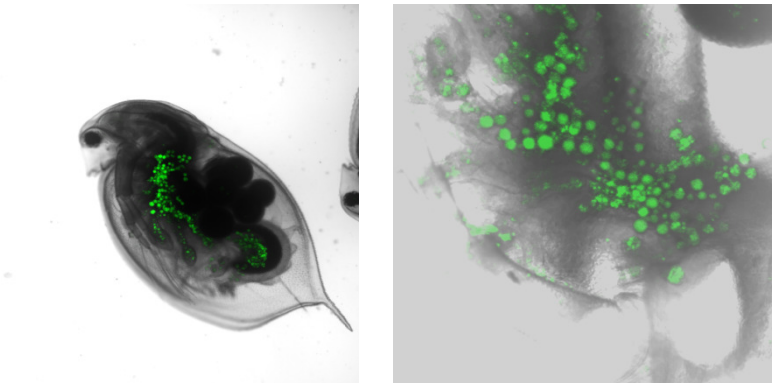


Figure 4.8: Uptake of 20 nm and 1000 nm polystyrene beads in adult *D. magna* over 24 h

4.3.3 Quantitative assessment of fluorescent polystyrene bead uptake by *D. magna*.

The accumulation of fluorescent polystyrene beads in *D. magna* was quantified by fluorimetry and expressed in terms of the mass of particles per organism (Fig. 4.9 and Fig. 4.10). Assuming that all of the fluorescence remained trapped within the polystyrene bead, fluorescence associated with both particle sizes accumulated in the organisms within 60 min generating a particle burden that was significantly greater than the control treatment ($p < 0.001$) when tested by a one way ANOVA, but they were not significantly different from each other ($p > 0.05$).

Animals treated with 1000 nm took up approximately 1.45 ng per animal after 60 min and reaching 1.67 ng per animal at 240 min (Fig. 4.9). In contrast, animals treated with 20 nm took up much less particulate, reaching 0.036 ng at 60 min and rising to 0.055 ng per animal at 240 min (Fig. 4.10). In fact, the uptake in terms of mass of 1000 nm beads was found to be 40 times higher at 60 min and 30 times higher at 240 min than the uptake of 20 nm beads when the means were compared to each other by one way ANOVA ($p < 0.001$) (Fig. 4.11).

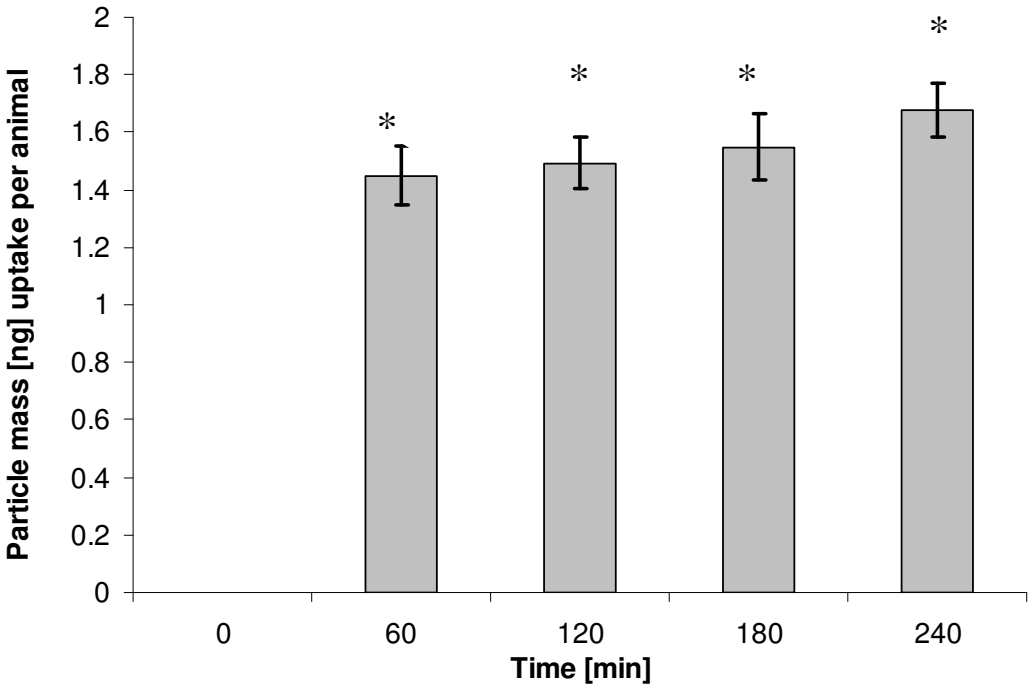


Figure 4.9: Uptake of 1000 nm polystyrene beads by *Daphnia* over 4 h. The data represents the mass of particles measured by fluorimetry per animal (values are means +/- standard error n= 9, * indicates a result with $p \leq 0.05$).

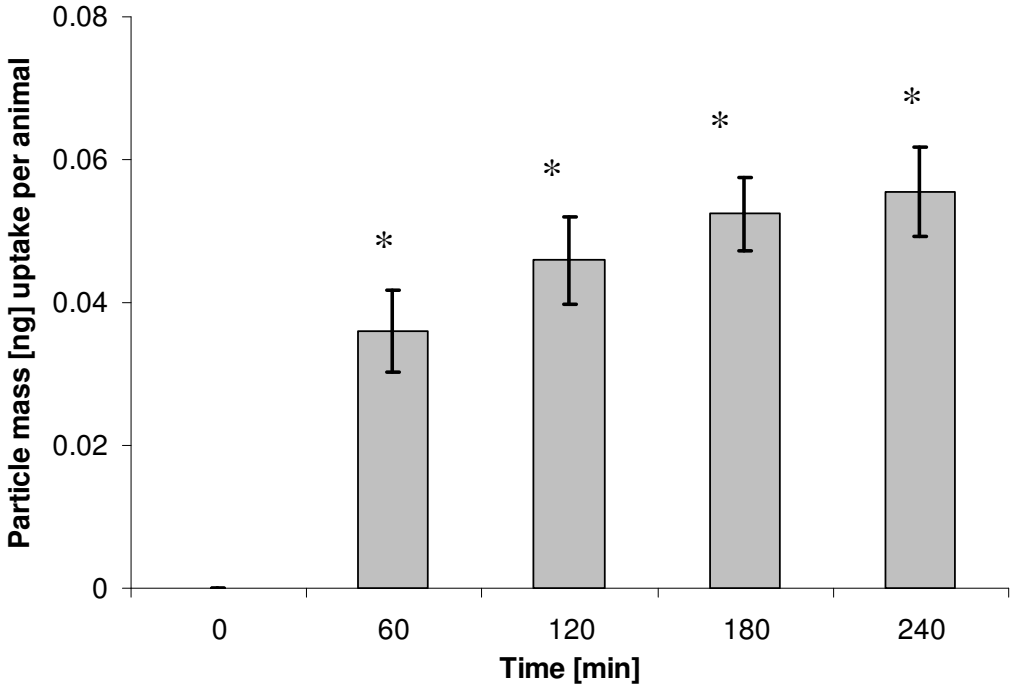


Figure 4.10: Uptake of 20 nm polystyrene beads by *Daphnia* over 4 h. The data represents the mass of particles measured by fluorimetry per animal (values are means +/- standard error n= 9, * indicates a result with $p \leq 0.05$).

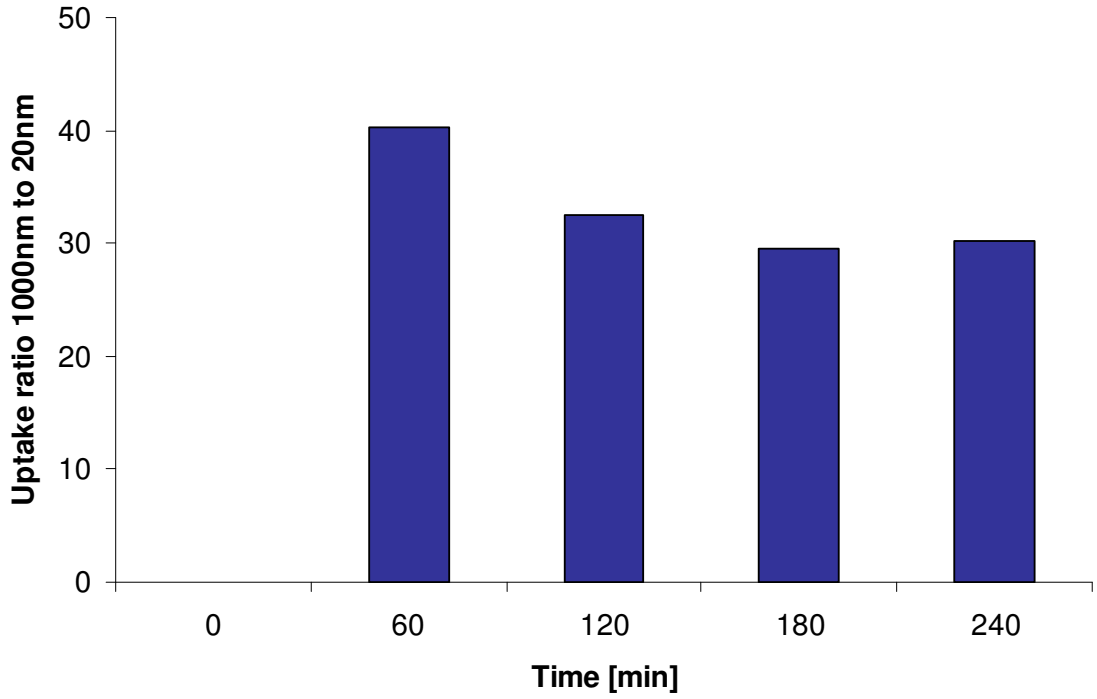


Figure 4.11: Comparison of uptake of 1000 nm polystyrene beads against the uptake of 20 nm polystyrene beads over a period of 4 h. Compared are the means of uptake at the various time points.

After exposure to the particles for 240 min, the organisms were transferred to clear water, and depuration determined. These experiments revealed a relatively rapid clearance of the 1000 nm particles from the *D. magna*. The particle content dropped from 1.45 ng per animal to 0.12 ng per animal, a decrease of more than 90% over 240 min (Fig. 4.12). The 0 min and 60 min time points were significantly different to each other and all other time points ($p < 0.001$) when tested by a one way ANOVA. The time point at 120 min was significantly different to the time point at 240 min ($p = 0.011$). No more significant differences were found.

A statistically significant decrease in particle burden after a depuration period of 240 min in organisms treated with the 20 nm particles (Fig. 4.13), was not observed when tested by a one way ANOVA followed by a Tukey post hoc test.

After 4 h of depuration the mean mass dose of 1000 nm particles still remaining within the animal was just 3.8 times higher than in the 20 nm treatment, in contrast to 26 times higher at the beginning of the depuration phase (Fig. 4.14),

but this difference at the 4 h time point lacked statistical significance when tested by a one way ANOVA followed by a Tukey post hoc test (p= 0.187).

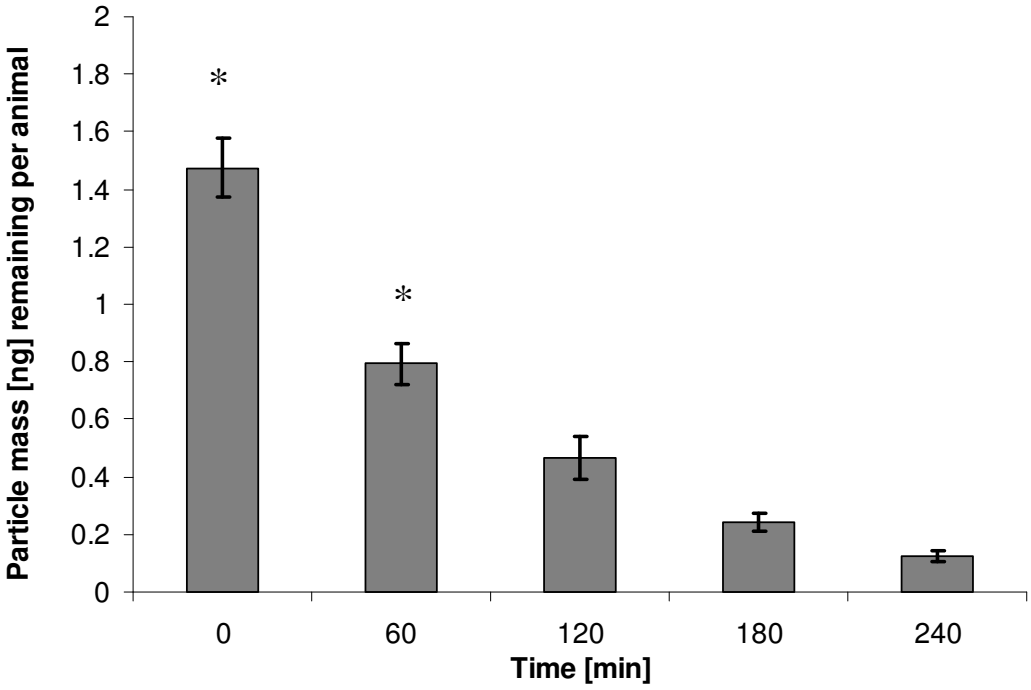


Figure: 4.12: *D. magna* were treated with 1000 nm particles for 4 h before transfer to clean water. The graph represents the mass of particulate remaining per animal over 4 h in clean water (values are means +/- standard error n= 9, * indicates a result with p ≤ 0.05).

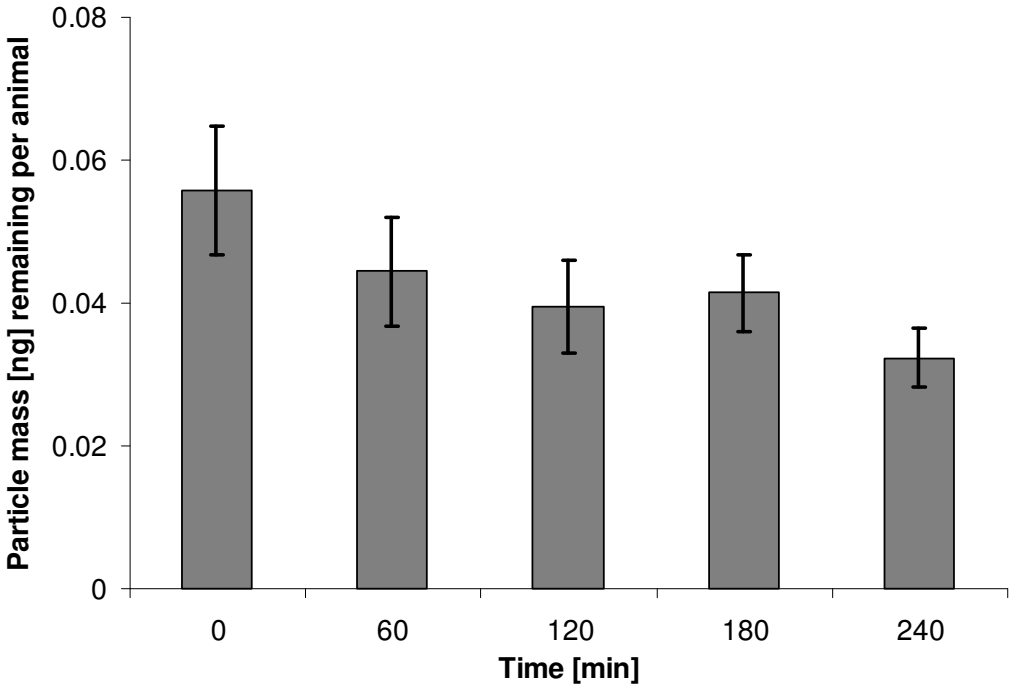


Figure 4.13: *D. magna* were treated with 20 nm particles for 4 h before transfer to clean water. The graph represents the mass of particulate remaining per animal over 4 h in clean water (values are means +/- standard error n= 9).

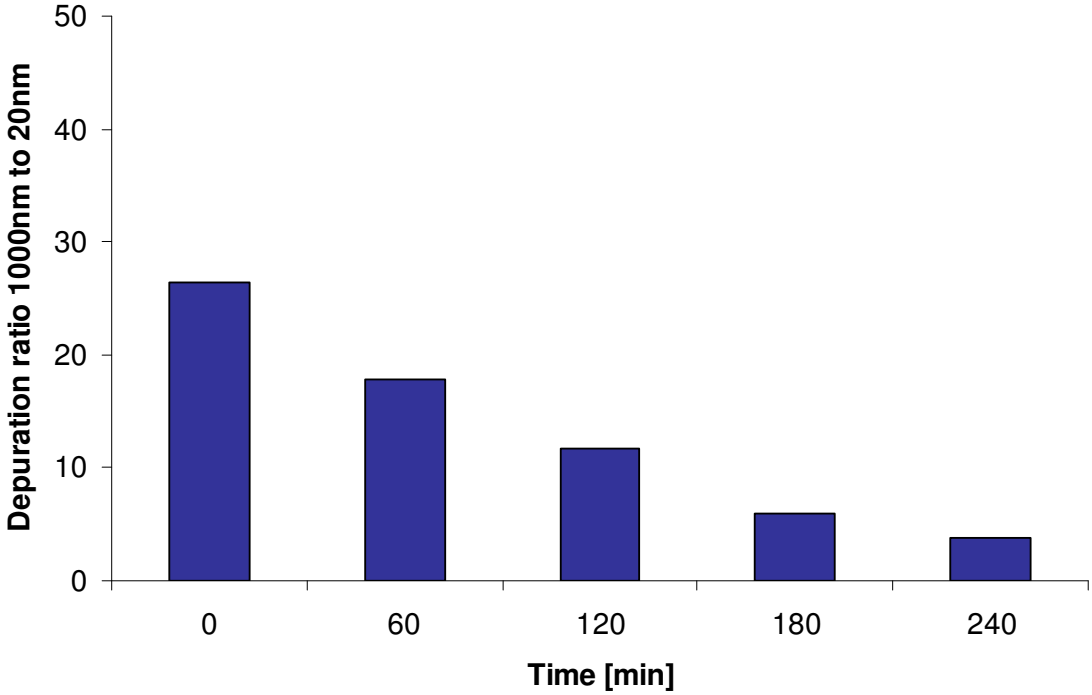


Figure 4.14: Comparison of depuration of 1000 nm polystyrene beads against the depuration of 20 nm polystyrene beads over a period of 4 h. Compared are the means of uptake at the various time points.

Since one of the characteristics of nanoparticles is that they have a higher surface area per mass dose than their larger parent particle, the same data were reanalyzed and expressed as surface area per organism, rather than particle mass per organism. This recalculation revealed that the final surface area dose reached 13332 μm^2 per animal for the 20 nm particles compared to just 8711 μm^2 per animal for the 1000 nm polystyrene beads (figures 4.15 and 4.16). When comparing the different sized particles at the different time points in the uptake phase, uptake was significantly higher at 2 h ($p = 0.005$), 3 h and 4 h ($p < 0.001$) for the 20 nm particles than the 1000 nm particles when compared by a one way ANOVA. Due to the more effective excretion of the 1000 nm particles, the surface area particle burden decreased within 4 h of depuration to 635 μm^2 per animal, compared to 7758 μm^2 per animal for the organisms exposed to 20 nm particles. In the depuration phase, the 20 nm particle load was in all time points significantly higher ($p < 0.001$) than the 1000 nm particle load when compared by a one way ANOVA.

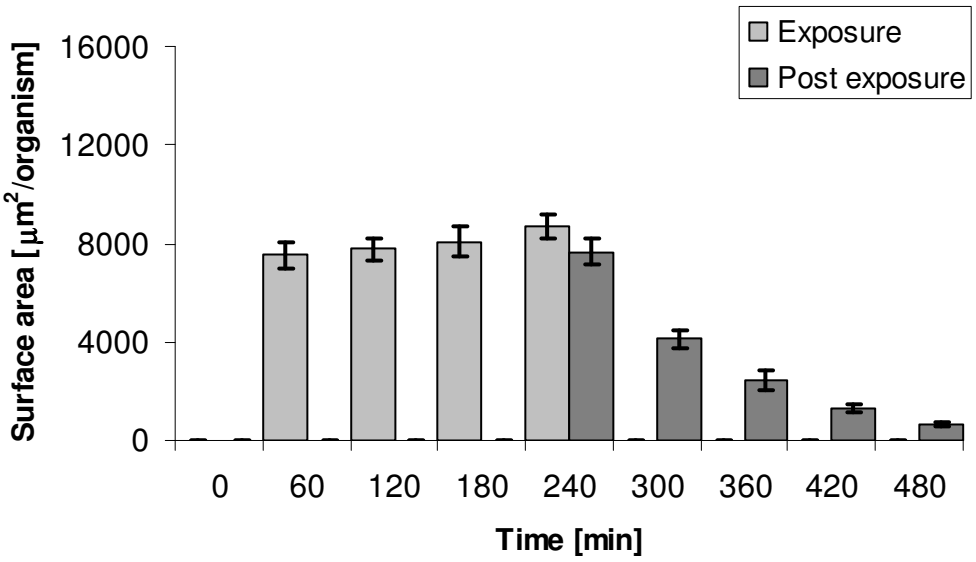


Figure 4.15: Uptake and clearance rate of 1000 nm polystyrene beads by *D. magna* over 8 h expressed in surface area taken up/remaining. Calculated as dose per animal (values are means +/- standard error n= 9)

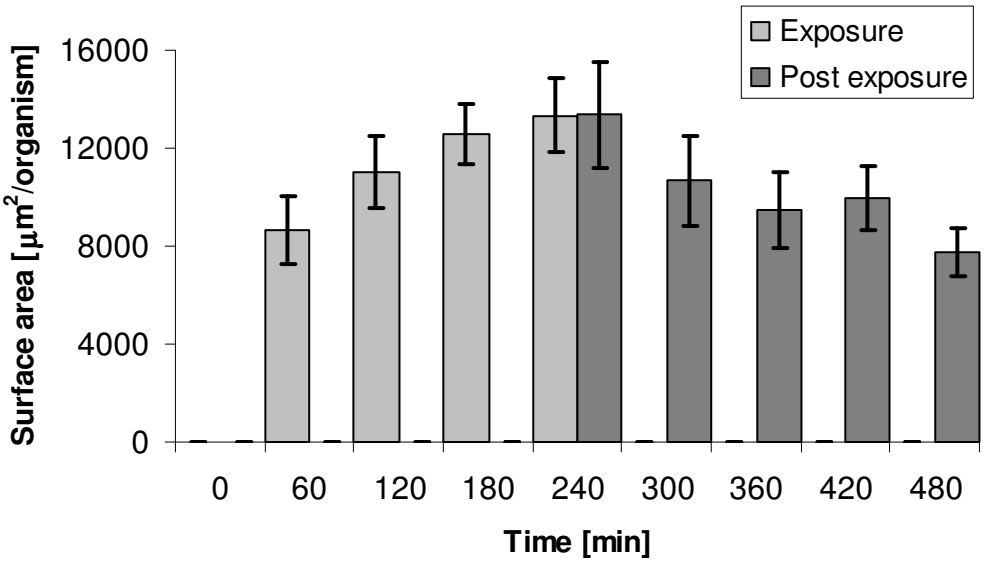


Figure 4.16: Uptake and clearance rate of 20 nm polystyrene beads by *D. magna* over 8 h expressed in surface area taken up/remaining. Calculated as dose per animal (values are means +/- standard error n= 9).

If the data were expressed as particle number per organism (Figures 4.17 and 4.18), than the uptake of 20 nm particles exceeds after 4 h of exposure the uptake of 1000 nm particles by a factor of 3300 and after a 4 h depuration the 20 nm particles remaining in the organism exceed the 1000 nm by a factor of 25000. The 20 nm treatment proved to be significantly higher than the 1000 nm treatment in the uptake phase as well as in the depuration phase at every time point when compared by a one way ANOVA ($p < 0.001$).

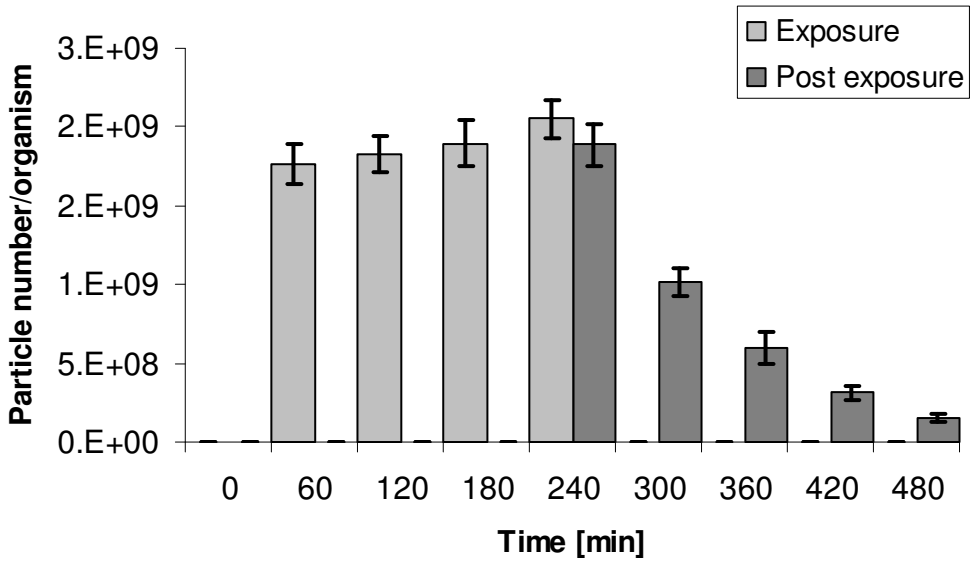


Figure 4.17: Uptake and clearance rate of 1000 nm polystyrene beads by *D. magna* over 8 h expressed in particle number taken up/remaining. Calculated as dose per animal (values are means +/- standard error n= 9).

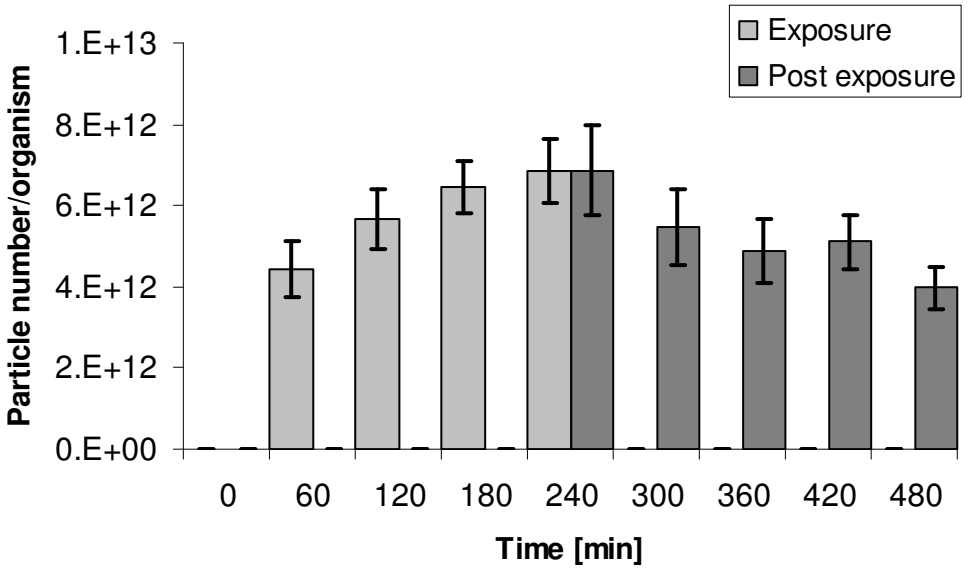


Figure 4.18: Uptake and clearance rate of 20 nm polystyrene beads by *D. magna* over 8 h expressed in particle number taken up/remaining. Calculated as dose per animal (values are means +/- standard error n= 9).

4.3.4 Electron microscopy

The polystyrene particles were imaged by applying approximately 1 µl of each onto a TEM grid and drying them until no liquid was detected. Afterwards the grids were imaged by TEM (Fig. 4.19). Pictures of sections of exposed *D. magna* were also taken by TEM and confirmed the translocation of 1000 nm particles into the oil storage droplets (Fig. 4.20) as previously suggested by the confocal microscopy images. Images taken from samples prepared after exposures to 20 nm particles showed also particles of the right size within the oil storage droplets but similar inclusions were discovered in control treatments without the possibility of making a clear distinction.

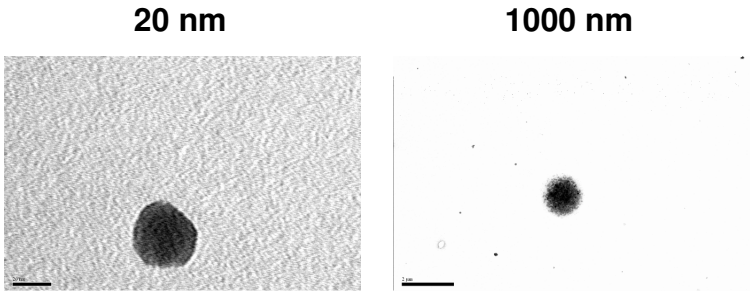


Figure 4.19: Transmission electron microscopy pictures of a 20 nm polystyrene bead (left) and a 1000 nm polystyrene bead (right). The black bar represents on the left hand side 20 nm and on the right hand side 2 µm.

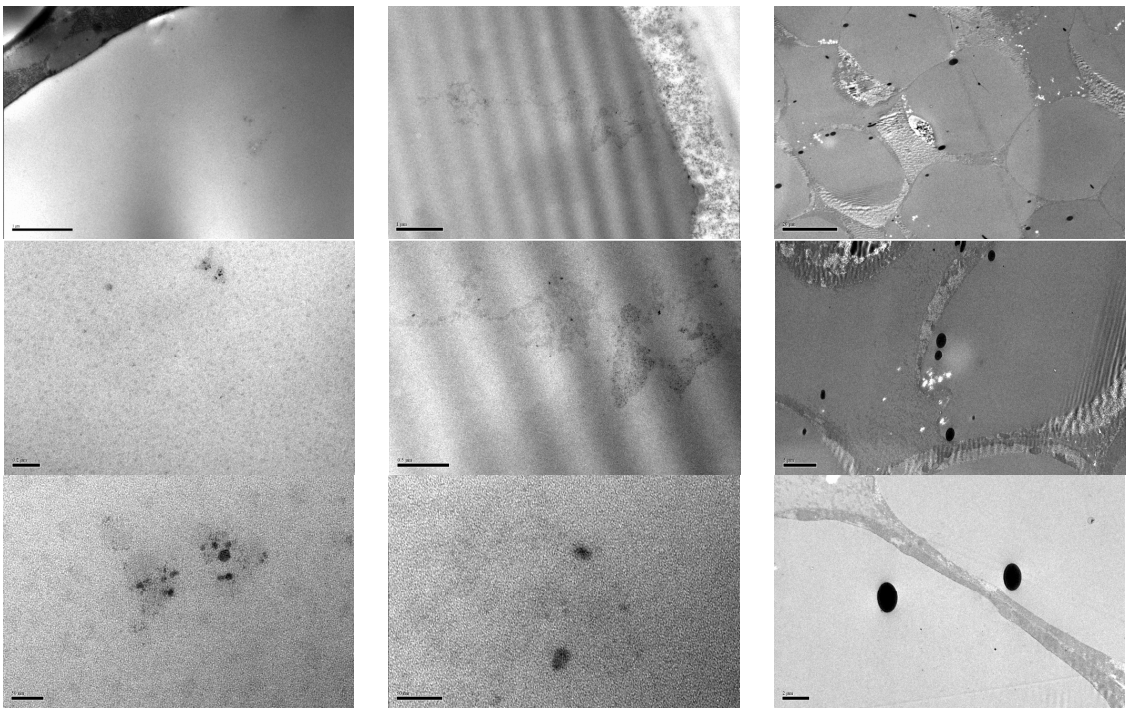


Figure 4.20: Transmission electron microscopy pictures of oil storage droplets in *D. magna* that were either unexposed (left), exposed for 1 h to 20 nm polystyrene beads (middle) or 1000 nm polystyrene beads (right). The black bar represents in the control and 20 nm treatments 1 μm (top), 0.2 μm (middle control), 0.5 μm (middle 20 nm) and 50 nm (bottom). In the 1000 nm treatment, the black bar represents 20 μm (top), 5 μm (middle) and 2 μm (bottom).

4.4 Discussion

Due to the expansion of nanotechnology, the potential for release of nanoparticles into the environment is considered significant in many reports (Boxall et al., 2007, Department for Environment Food and Rural Affairs (DEFRA), 2007, Joner et al., 2008). Although it can be assumed that nanoparticles in an aquatic environment will have a wide distribution of different sizes, little information is available at this time to indicate how nanoparticles will behave in the environment and what their impact might be on different species. This study aimed to investigate the potential for particles of different sizes to be taken up by *D. magna*, and then to determine their potential for translocation within the organism. It has been observed in the experiments described in chapter 3 that uptake of various nanoparticles took place already at short exposure durations, with the particles observed in the gastrointestinal tract (Fig. 3.2 and 3.3)

For this purpose fluorescent, carboxylated polystyrene beads were employed due to the ability to be imaged by confocal microscopy and detected by fluorimetry. *D. magna* were chosen because they are a test species used in many standard ecotoxicity tests, for which much data are available for other environmental contaminants. Feeding of the animals during the experiment was avoided, because this could have interfered in several ways: Uptake rates could have been greatly enhanced by aggregation or agglomeration of particles to algae. This would without a doubt happen in a natural environment but would add the additional component of taking food concentration into consideration in the assessment. The autofluorescence of algae cells could have provided false positive results for uptake via ingestion. It is also known that the gut passage time is inversely related to food concentration (Gillis et al., 2005) with clearance rates reported of less than an hour at high food concentrations. Since these clearance rates are quite rapid and uptake as well as depuration would have been strongly dependent on food concentration in the media as just being dependent on particle concentration it was deemed better to exclude food in the present study.

In terms of characterising the particles to which the organisms were exposed, the size distribution of 20 nm polystyrene beads shows a size range mostly at around 17-20 nm or 35-45 nm. This would mean that the dispersal of the particles is mostly as single beads or as doublets. There does not seem to be a stable distribution between both states but rather a flipping back and forth between the two. It could also be that this is an artefact created by the methodology. Agglomerations of larger particles was also observed but of negligible concentration and not at all time points, suggesting that they probably broke up in the course of the experiment. With the data shown it can be assumed that the 20 nm polystyrene beads stayed within the nano-size range in the course of the 4 h experiment.

As seen in the confocal images, both the 20 and 1000 nm polystyrene beads were taken up into the organism within just 30 min of exposure. Relatively high concentrations of the beads were easily visible by confocal microscopy, within the gastrointestinal tract (GIT), indicating that ingestion is a major route of uptake for both particle sizes. The intensity of fluorescence within the GIT appeared greater for the 1000 nm polystyrene particles than for the 20 nm particles. *D. magna* are filter feeders, enabling them to ingest particles between the sizes of 70 μm (Burns, 1968) and down to around 200 nm (Geller and Muller, 1981). Therefore the 1000 nm polystyrene particles are within the lower size range of the food particles ingested by *D. magna* allowing their active and intended uptake leading to accumulation within the GIT.

The ingestion of the 20 nm particles was more surprising as these particles should be too small for the feeding apparatus of *D. magna* (Geller and Muller, 1981), therefore suggesting that the uptake was passive and unintentional. As stated previously the confocal pictures illustrated that the GIT of animals exposed to 1000 nm particles was brightly fluorescent. The same observation was not always true when the animals were exposed to 20 nm particles, indicating a lower uptake via ingestion. The relatively higher efficiency of 1000 nm particle uptake versus the 20 nm particle uptake was verified by fluorimetry when the data were considered on a mass per organism basis. The ingestion of the 20 nm particles could happen by various mechanisms, for example they could be taken up randomly by accident, entering the GIT by means of being

washed in along with water which is taken up in considerable amounts in a drinking-like process (Gillis et al., 2005). Alternatively, they could be taken up actively alongside or adsorbed onto larger particles such as algae, bacteria, carapace fragments from previous moults or faeces in the exposure water. A third possibility could be that they are transformed into larger agglomerates by handling and incorporation into mucus used by *D. magna* to enhance the uptake of bacteria (Hartmann and Kunkel, 1991) or by direct interception (Gerritsen et al., 1988).

The confocal images indicated that the fluorescence had translocated into lipid storage droplets. This was positively confirmed for the 1000 nm particles by Transmission Electron microscopy. Images taken from animals treated with 20 nm particles are less conclusive. The images showed particles in the 20 nm range within the oil storage droplets, but similar inclusions can be observed in control pictures. A differentiation between those particles proved impossible due to the resolution of the method. Since 1000 nm particles were found in the storage droplets, having crossed the GIT barrier, a mere leaching of the fluorescent dye instead of the whole particle seems unlikely for both sized particles.

An accumulation of nanoparticles in storage compartments could have serious consequences for *D. magna*. Polystyrene nanoparticles have been shown to generate reactive oxygen species *in vitro*, to a greater extent than larger particles (Brown et al., 2001). If the nanoparticles are reactive they could interact with vital food compounds and degrade them, rendering them potentially less useful for the organism. Since many commercially used nanoparticles such as fullerenes are lipophilic (Moore, 2006, Oberdörster, 2004) it will be important to determine their potential to accumulate in such storage compartments and even cause toxicity. A similar study looked at qualitative and quantitative uptake of gold nanoparticles in *Daphnia* (Lovern et al., 2008). This article also recognizes the possibility of uptake of nanoparticles in the GIT of *Daphnia.* However a translocation of gold nanoparticles was not observed and no uptake in the midgut region after one hour of exposure was detected via TEM images. As stated earlier, uptake observed by confocal microscopy was clearly higher with 1000 nm particles in the gut than with the nano sized

particles but translocation took place nevertheless in neonate and adult *D. magna* as early as the first time point of 30min. The difference of the observed effects might be due to different surface characteristics of the used particles. Also during the intake phase in the gold uptake study, only samples of the midgut, not the upper part of the digestive tract were taken into consideration stating that an uptake earlier in these regions might be possible.

Given the quick uptake and relocation within the body, and the fact that a quantity of oil droplets are transferred from the mother to newly generated eggs (Goulden and Hornig, 1980, Tessier and Goulden, 1982), even a short term exposure could lead to residues of nanoparticles remaining in the planktonic community, although this is likely just for an intermediate time period, since energy reserves are continually used for maintaining metabolism and support processes like moulting (O'Connor and Gilbert, 1968). Also, in the environment, populations of *Daphnia* oscillate between stages of good food supply and phases of starvation (Goulden and Hornig, 1980) where energy reserves would be mobilized and accumulated particles might therefore become available to induce toxicity.

An accumulation of the 20 or 1000 nm nanoparticles in unhatched eggs could not be observed in any organisms, although fluorescence from 20 nm particles appeared to accumulate around the shell of a permanent egg (ephippia) with consequences unknown. An exposure of the eggs seems likely, since the brood pouch is connected directly to the ambient media and a steady flow of water through this chamber is maintained by the adult to provide oxygen to the developing embryos. The developing embryos, in contrast to eggs, were observed to experience an accumulation of fluorescent particles of both sizes in their storage droplets as soon as they showed filter activity in the brood pouch, but prior to hatching.

The quantitative uptake as detected by fluorimetry shows a similar pattern of particle uptake as the qualitative confocal studies. The particles were taken up within 60 minutes with a relatively small subsequent increase over time until 240 minutes, suggesting that uptake and excretion rates are almost balanced at these times. The *D. magna* accumulated 1000 nm particles into their bodies

reaching a concentration approximately 700 times greater than the concentration of the surrounding media, with most of the fluorescence located in the well filled GIT. Exposure to 20 nm beads shows an accumulation of approximately 20 times the concentration in the surrounding medium. It has to be taken into consideration, that the fluorimetry measurements involved the use of whole animals, while most of the fluorescence was actually located in the GIT and adjacent oil droplets, making it likely that the concentration in those specific locations was even higher.

D. magna were also exposed to particles for 4 h before transferring to fresh water and studying elimination of the particles from the organisms over time. The 1000 nm particles cleared relatively quickly from the organisms, reaching just 12.5 % of the original particle burden within 240 min, indicating that most of the particles had been located in the GIT as observed in the confocal pictures and were subsequently excreted with faeces. In contrast, the 20 nm particles reached 67 % of their maximum particle burden, suggesting a lower clearance than the 1000 nm particles. At the end of the 4 h incubation in clean water, the mass concentration detected in the 1000 nm treatments were just double the concentrations detected in the 20 nm treated organisms, compared to the 38 fold difference observed at the end of the 4 h particle exposure. However, it should also be noted that the particle burden, in terms of mass was still lower in the 20 nm particle exposed organisms than the 1000 nm exposed *D. magna* at the end of the clean water exposure period.

Recalculation of the data to express the particle burden as surface area dose revealed that the maximum surface area taken up by the *D. magna* was 1.65 times greater for the 20 nm particles than the 1000 nm particles. In addition, the surface area dose remaining at the end of the clean water incubation was approximately 40 times greater for the 20 nm polystyrene beads than the 1000 nm particles. Surface area has been linked to the ability of nanoparticles to induce inflammation in the lung of animals (Duffin et al., 2002, Stoeger et al., 2006). It has been hypothesised that this is due to the fact that it is the surface over which interactions and reactions with biological molecules occur (Stone and Kinloch, 2007). Similar numbers can be expected if the particle burden would be calculated as particle number instead of mass dose.

Once the gut is cleared, a better estimate can be made with respect to what actually was taken up and relocated from the gut and has to be considered bioaccumulation. A short term exposure of females and a tracking of fluorescence in neonates produced after the exposure should provide information as to whether deposited nanoparticles have the potential to be passed on to the next generation.

This study clearly demonstrates the ability of *D. magna* to take up nanoparticles and micron sized particles from water by ingestion. The study demonstrates that the mass uptake of nanoparticles is less than for micron sized particles, but that the uptake is easily detected and results in translocation from the GIT of the nanoparticles into other compartments of the adult and neonate organisms. Both the nanoparticles and the micron sized polystyrene beads were excreted to some extent, although a greater proportion of the 20 nm particle dose was retained within the organism. This study also demonstrates the impact on data interpretation of considering the exposure dose in terms of mass, versus surface area, versus particle number. Finally, this study also indicates the potential for uptake of nanoparticles by eggs and the developing foetus, probably via direct exposure to water circulating within the brood pouch. In the future it will be essential to relate toxicity and biomarker endpoints to all 3 dose metrics in order to identify which is most relevant in terms of hazard assessment.

The study presented here is in press in *Environmental toxicology & chemistry* (Rosenkranz et al., 2009).

5. Bioindicators for measuring oxidative stress in *Daphnia magna* due to nanoparticle exposure

5.1 Introduction

Many toxicological studies concerning nanomaterials have focussed on oxidative stress as an endpoint (Brown et al., 2001, Maynard et al., 2004, Oberdörster, 2004, Stone et al., 1998). Due to the ability of some of the nanomaterials to produce free radicals and/or reactive oxygen species, this is a logical approach. Since oxygen is required for all aerobic prokaryote and eukaryote cells for energy production through the electron transport chain, reactive oxygen species (ROS) and derivates are produced naturally (Kohen and Nyska, 2002). Among these oxygen compounds produced in high concentrations in the living cell are hypochlorous acid (HClO), hydrogen peroxide (H_2O_2), the superoxide anion radical, the hydroxyl radical, organic peroxides, aldehydes, ozone (O_3), and O_2 (Kohen and Nyska, 2002). The most vulnerable targets to oxidative damage within a cell are (Kohen and Nyska, 2002):

(i) proteins, leading to loss of structure and function,

(ii) lipids, leading to lipid peroxidation and a weakening of cell membranes,

(iii) DNA, leading to base modification and single and double strand breaks

To detoxify endogenous, but also with exogenous sources of free radicals and ROS, the different cells, and the whole organism have a wide array of defence mechanisms to deal with reactive metabolites. Principally the cell tries to cope with oxidative stress by either preventing the endogenous production of ROS by regulating enzymes that indirectly produce ROS, by detoxifying ROS with the aid of antioxidant enzymes and antioxidant molecules, so called scavengers, or repairing damage caused by oxidative stress. Physical defence is also possible, for example, tocopherol (vitamin E) is an antioxidant that promotes the stability

of membranes but also prevents the ROS from approaching the target through steric hindrance (Kohen and Nyska, 2002). Steric hindrance occurs when the size of functional groups within a molecule prevent a chemical reaction due to interfering with the reactant by the three dimensional arrangement of the atoms.

Biological effects related to oxidative stress that are suitable as biomarkers include either cellular responses, such as increased activities of antioxidant enzymes and concentrations of non-enzymatic antioxidant compounds, or the results of oxidative stress toxicity such as oxidation of proteins, lipids and nucleic acids (van der Oost et al., 2003).
Defence systems that play a role in the detoxification of ROS include the antioxidant enzymes such as;

- superoxide dismutase (SOD),
- catalase (CAT) and
- glutathione-dependent peroxidase (GPO_X)

(van der Oost et al., 2003).
Superoxide dismutase (SOD) is a group of metalloenzymes that catalyze the conversion of reactive superoxide anions (O_2^-) to hydrogen peroxide (H_2O_2), which in itself is an important ROS as well (Kohen and Nyska, 2002). H_2O_2 is subsequently detoxified by two types of enzymes: CAT and GPO_X (van der Oost et al., 2003).
CAT is a hematin-containing enzyme that facilitates the removal of hydrogen peroxide (H_2O_2), by metabolizing it to molecular oxygen (O_2) and water (Kohen and Nyska, 2002). While GPO_X also catalyses the metabolism of H_2O_2 to water, involving a concomitant oxidation of reduced GSH to its oxidized form (GSSG) (van der Oost et al., 2003).

There are also a number of low-molecular-weight antioxidants, such as GSH (glutathione in reduced form), β-carotene (vitamin B), ascorbate (vitamin C), α-tocopherol (vitamin E) and ubiquinol. Ascorbate and α-tocopherol are not synthesized by animal cells and therefore have to be taken up by the diet (van der Oost et al., 2003).

GSH (γ-glutamylcysteinylglycine) is the major cytosolic low molecular weight antioxidant (Kohen and Nyska, 2002, van der Oost et al., 2003, Zhang et al.,

2004). It acts as a reducing agent and is often the first line of defence against oxidative stress (Zhang et al., 2004). When a cell is faced by an oxidizing agent, two GSH molecules are oxidized to one GSSG molecule, thus reducing the oxidant:

$$2\ GSH + H_2O_2 \longrightarrow GSSG + 2\ H_2O$$

GSSG can either be converted back to GSH or secreted from the cell (Barata et al., 2005, Zhang et al., 2004). In order to convert GSSG back to its reduced form, another electron donor like NADPH is needed, as well as the enzyme glutathione reductase. A mild oxidative stress results in an initial GSH depletion, followed by an activation of the enzymes responsible for intracellular synthesis of GSH (Stone et al., 1998), leading to an overall increase. The activation of those enzymes is time dependent (within 6- 8 h, (Stone et al., 1998)). Therefore a time dependent response of GSH to mild oxidative stress can be observed. When the oxidative stress exceeds the capacity of recovery of GSH, like in excessive oxidative stress, it leads to toxicity without any recovery or compensation.

To measure oxidative stress via GSH depletion it is advisable to measure the total glutathione concentration alongside, since an elevated glutathione level gives a good indication of increased synthesis of GSH or a shifting in the reduced glutathione -total glutathione balance due to oxidative stress (Zhang et al., 2004). Oxidized and reduced glutathione can be measured by fluorimetry through reaction with the fluorophore *o*-phtalaldehyde (OPT) (Hissin and Hilf, 1976). However, in a study by Senft et al. (2000) it was shown, that assay relying on the binding of GSH to OPT at high pH, were overestimating the GSSG levels due to other components in the sample either reacting with OPT or quenching fluorescence when measuring GSSG. Another problem in the assay was that after measurement of GSH, N –ethylmaleimide (NEM) was added to the sample so GSH would not interfere with measurements of GSSG. NEM alkylates GSH, rendering it inactive towards OPT. As Senft et al. (2000) points out, NEM that did not react can remain in the sample and interfere with measurements as soon as GSSG was reduced to GSH. In the method used in this chapter, which is a modified version of the method employed by Senft et al. (2000) to allow the use of a 96 well plate, reduced GSH and total glutathione were measured. The GSH measurement still relies on the reaction of GSH with

OPT, although a lower pH is used. The reduction of GSSG in the sample was achieved by sodium hydrosulfite (dithionite) and measured alongside GSH. NEM was merely used to confirm the absence of background fluorescence in the sample.

Another method to estimate oxidative stress is to measure the total antioxidant capacity. In the method applied here, a radical cation is generated in the presence or absence of an antioxidant (Re et al., 1999). The radical (ABTS [2,29-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid)]), a blue/green chromophore, and metmyoglobin (Met Mb) act as reagents and H_2O_2 as the oxidant. When H_2O_2 oxidizes the ABTS Met Mb complex, a blue colour appears. The antioxidant Trolox (6-hydroxy-2,5,7,8-tetramethychroman-2-carboxylic acid) prevents oxidation if it is present in high enough concentration. The intensity of the blue colour is directly proportional to the presence of oxidized ABTS Met Mb complex and inversely proportional to the antioxidant capacity. That means the higher the absorbance, the lower the antioxidant capacity. The samples, in this case homogenized *D. magna,* are compared to the Trolox standards to define their Trolox equivalent antioxidant capacity (TEAC).

5.1.1 Aims

The aims of this chapter were to assess oxidative stress induced by exposure to nanoparticles and to see if a size dependent relationship of this stress exists. To achieve this, two methods were used. One measuring the total antioxidant capacity and a second one that looks at a specific low-molecular-weight antioxidant.

5.2 Methods

5.2.1 TEAC assay

5.2.1.1 Exposure of Daphnia magna

The solutions were prepared as described in section 3.2.1.1. Animals (4-6 weeks old) were exposed in 100ml beakers with nanoparticles suspended in 80 ml of reconstituted EPA hard water. Four replicates per treatment and one control were used with 10 animals per replicate. The animals were exposed to NP carbon black (Degussa Printex 90, average size 14 nm) and to fine sized carbon black (Degussa Huber 990, average size 260 nm) at the concentrations of 10 mg/L, 1 mg/L, 0,1 mg/L and a control. Ten animals were sampled each after 4 h and 24 h and transferred into eppendorf vials.

5.2.1.2 Preparation of Metmyoglobin

The dialysis tubing was prepared by cutting it into a 20 cm length and soaking it in tap water for 30 min with four or five changes of water. It was then rinsed with distilled water and heated for 3 min in a 5 mM EDTA (Sigma-Aldrich Company Ltd., Gillingham, UK) solution (1.25 ml of a 3 g/20 ml solution in 100 ml of distilled water) at 60-70°C. Afterwards it was rinsed with distilled water 2-3 times.

The stock solution of potassium ferricyanide (Sigma-Aldrich Company Ltd., Gillingham, UK) at a concentration of 740 µM was made by dissolving 2.4 mg of potassium ferricyanide in 10 ml of PBS pH 7.4 (Invitrogen Ltd Paisley, UK). To the stock solution of potassium ferricyanide, 7.5 mg/ml myoglobin (Sigma-Aldrich Company Ltd., Gillingham, UK) was added. It was mixed and allowed to stand for 5 min at room temperature. The solution was then transferred into the dialysis bag and dialysed against 400 ml of PBS pH 7.4 for 30 min. Then the

buffer was changed and the solution was again dialysed for 15 min. The solution was then decanted into a test tube with a screw top and kept on ice.

An amount of 0.1 ml of the metmyoglobin (Met Mb) solution was diluted with 0.9 ml of phosphate buffered saline (PBS) and the absorbance was read in a spectrophotometer (Optima Fluo Star) at 490 nm, 560 nm, 580 nm and 700 nm against PBS as a blank.

5.2.1.3 Trolox Solution

To prepare the stock solution, 6.2 mg of trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) (Sigma-Aldrich Company Ltd., Gillingham, UK) was weighed and added to 10 ml of PBS. The solution was then sonicated for 15-30 min and kept on ice.
The working solution was 1 ml of the stock solution added to 9 ml of PBS.

5.2.1.4 ABTS

To prepare the stock solution, 27.4 mg of ABTS (2,29-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid)) (Sigma-Aldrich Company Ltd., Gillingham, UK) were weighed and added to 10 ml of PBS. The working solution was 1 ml of the stock solution added to 9 ml of PBS and kept at room temperature.

5.2.1.5 Hydrogen peroxide

To prepare the stock solution, 100 µl of a 30 % H_2O_2 (Sigma-Aldrich Company Ltd., Gillingham, UK) solution were added to 9.9 ml of PBS. The working solution was 50 µl of the stock solution added to 10.95 ml of PBS and kept at room temperature.

5.2.1.6 Sample preparation

The exposed *D. magna* were homogenized in an eppendorf vial, using a homogenizer (Kontes Pellet Pestle, Fisher Sciences Loughborough, UK).

5.2.1.7 Preparation of measurement

Prior to the experiment 1 ml eppendorf tubes were prepared in duplicates and the required reagents were added into each, in the order shown in table 5.1 from top to bottom. The reaction was started with the addition of H_2O_2.

Table 5.1: Solution composition for TEAC assay: All volumes are in µl. Ten µl of Trolox standard is equal to 2.5 mM Trolox equivalent antioxidant capacity (TEAC)

	Standards						**Sample**
	Blank	2.5 mM	5 mM	7.5 mM	10 mM	12.5 mM	
ABTS	300	300	300	300	300	300	300
Trolox (Stds) or sample	0	10	20	30	40	50	10
Met Mb	36	36	36	36	36	36	36
PBS	497	487	477	467	457	447	487
H_2O_2	167	167	167	167	167	167	167

The tubes were inverted and the reaction carried out for 6 min. One hundred microliter each were added into a 96 well plate (96 Well Krystal 2000 white, Porvair Sciences Ltd., Shepperton, UK) and the absorbance was read immediately at a wavelength of 734 nm. The antioxidant capacity was calculated by comparing the results to different concentrations of Trolox standards by using linear regression (Re et al., 1999).

Furthermore, to measure the background absorbance of homogenized *D. magna* and nanoparticles, samples were measured by leaving H_2O_2 out of the sample or measuring the sample in pure PBS.

5.2.2 Reduced Glutathione/ total Glutathione assay

5.2.2.1 Exposure of *Daphnia magna*

The solutions were prepared as described in section 3.2.1.1. Animals (4-6 weeks old) were exposed in 100 ml beakers with nanoparticles suspended in 80 ml of reconstituted EPA hard water. Three replicates per treatment and one control were used with 10 animals per replicate. The animals were exposed to carbon black, cerium dioxide and silver in both micro and nano size (particle description in chapter 3.2.1) at concentrations of 10 mg/L for cerium dioxide and carbon black and 0.1 mg/L for silver, concentrations effects have been observed in acute studies beforehand (see chapter 3). One treatment of unexposed animals was included as a control. The animals were sampled after 3h.

5.2.2.2 Reagents needed for assay

All reagents were purchased from Sigma-Aldrich, Gillingham, UK)

5.2.2.3 Redox quenching buffer

To prepare the redox quenching buffer (RQB buffer) 490 ml of distilled water were measured in a glass cylinder and 10 ml of 1 M HCl were added inside a fume cupboard. To this acid, 0.4 g of ethylendiamine tetra acetate (EDTA) and 0.88 g of ascorbic acid were added to give a buffer with a final concentration of 20 mM HCl, 2.15 mM EDTA and 10 mM ascorbic acid. The buffer was then stored until use at 4°C.

5.2.2.4 Extraction buffer

To prepare the extraction buffer (5% Trichloracetic acid in RQB) 5 g of Trichloracetic acid were weighed and dissolved in 100 ml of RQB buffer. The buffer was then stored until use at 4°C.

5.2.2.5 Phosphate buffer saline solution

From a 1 L bottle of distilled water, 100 ml of water were removed, and then 100 ml of PBS 10x solution were added. The solution was mixed by gentle agitation. The buffer was then stored until use at 4°C.

5.2.2.6 Potassium phosphate (pH 7.0 1 M)

In a bijou tube 6.8 mg of potassium phosphate were weighed and dissolved in 30 ml of distilled water and the pH adjusted to pH 7.0 with 10N NaOH. Afterwards the volume was filled to 50 ml with distilled water. The solution was then stored until use at 4°C.

5.2.2.7 Potassium phosphate (pH 6.9 0.1 M)

In a bijou tube 6.8 mg of potassium phosphate were weighed and dissolved in 200 ml of distilled water and the pH adjusted to 6.9 with 10N NaOH. Afterwards the volume was filled up to 500 ml with distilled water. The solution was then stored until use at 4°C.

5.2.2.8 N-ethylmaleimide (NEM) in RQB (7.5 mM)

In a bijou tube .7 mg of NEM were weighed and dissolved in 5 ml of RQB buffer. The NEM was prepared fresh on each day of experiment and stored on ice.

5.2.2.9 *O*-phtalaldehyde (OPT) in methanol (5 mg/mL)

In a bijou tube 10 mg of OPT were weighed and dissolved in the fume cupboard in 2 mL of methanol.
The OPT was prepared fresh on each day of experiment and stored on ice, protected from light in tin foil**.**

5.2.2.10 Dithionite (sodium hydrosulfite) in RQB (10 mM)

In a bijou tube 17.4 mg of dithionite were weighed (avoid any contact with water as it reacts violently).
In the fume cupboard, the dithionite was dissolved in 1 mL of RQB (in contact with an acid like RQB, the dithionite produces a toxic gas, so the process had to be kept in the fume cupboard).

5.2.2.11 Preparation of the sample for measurement

D. magna were removed from the exposure vessel and transferred to a 1.5 ml eppendorf vial. All exposure media was removed and the *D. magna* were homogenized using a homogenizer (Kontes Pellet Pestle, Fisher Sciences Loughborough, UK). Then 100 µl of cold RQB-TCA buffer was added and the vial vortexed and incubated on ice for 5 min. Afterwards, the eppendorf vials were centrifuged for 5 min at 15 000 g. and replaced on ice until the sample was applied to the 96 well plate.

5.2.2.12 GSH standards

A first stock solution of GSH at 0.05 M (15 mg/mL) was prepared by dissolving 15 mg of reduced GSH into 1 mL of extraction buffer (5% TCA in RQB). This 0.05 M stock solution of GSH was stored on ice.

The first stock solution was diluted 1 in 100 (990ul 5% TCA in RQB and 10ul of 0.05 M GSH) to give a 0.5 mM GSH second stock solution. Both stock solutions were prepared fresh and kept on ice.

The standards used for comparing against a sample were prepared by diluting standard 2 as described in table 5.2.

Table 4.2: Dilution volumes used for making up standards for comparing against measurement of reduced glutathione

GSH Concentration (µM)	Dilution
100	800 µl 5% TCA in RQB : 200 µl 0.5 mM GSH
50	900 µl 5% TCA in RQB : 100 µl 0.5 mM GSH
25	500 µl 5% TCA in RQB : 500 µl 50 µM GSH
12.5	500 µl 5% TCA in RQB : 500 µl 25 µM GSH
6.25	500 µl 5% TCA in RQB : 500 µl 12.5 µM GSH
3.125	500 µl 5% TCA in RQB : 500 µl 6.25 µM GSH
0	1 ml 5% TCA in RQB

5.2.2.13 GSSG standards

As negative control for the reduced GSH measurements and positive control for total GSH measurements GSSG standards were used. To achieve this, 15 mg of GSSG were weighed and dissolved in 1 ml of 5% TCA in RQB (25 mM solution of GSSG). The 25 mM stock solution was diluted afterwards 1 in 100 (990 ul 5% TCA in RQB and 10 µl of GSSG solution) to give a second stock solution of 0.25 mM GSSG. Both stock solutions were prepared fresh and kept on ice.

The standards used for comparison were made out of the second stock solution as described in table 5.3.

Table 5.3: Dilution volumes used for making up standards for comparing against measurement of total glutathione.

GSSG Concentration (µM)	Dilution
100	400 µl 5% TCA in RQB : 600 µl 0.25 mM GSSG
50	700 µl 5% TCA in RQB : 300 µl 0.25 mM GSSG
25	500 µl 5% TCA in RQB : 500 µl 50 µM GSSG
12.5	500 µl 5% TCA in RQB : 500 µl 25 µM GSSG
6.25	500 µl 5% TCA in RQB : 500 µl 12.5 µM GSSG
3.125	500 µl 5% TCA in RQB : 500 µl 6.25 µM GSSG
0	1 ml 5% TCA in RQB

5.2.2.14 Preparing the 96 well plate

The 96 well plate was prepared following the order described in table 5.4.

A negative control for background fluorescence was used to determine that no other fluorescence source was present besides the fluorophore OPT. NEM alkylates GSH, rendering it inactive to a reaction with OPT and thus would show only fluorescence with an origin different from GSH (Senft et al., 2000).

The GSH standards were used to quantify the GSH concentrations while the GSSG standards were used to verify that the reduction of GSSG by dithionite was quantitative. This was confirmed when readings were equal to the GSH standards.

Additionally two more negative controls were added to the plate:

The first was a sample that included GSSG at a concentration of 100 µM. It was used to verify that no background fluorescence would arise from the molecule itself.
The second was GSH at a concentration of 100 µM, which was prepared like the negative control for background fluorescence. Its function was to verify that NEM was used in adequate concentration to block all GSH.

Samples were prepared in triplicates while standards or controls were prepared in duplicates or triplicates according to the amount available.

5.2.2.15 Reading the plate

The plate was read in a fluorescent plate reader (Fluostar Optima, BMG Labtech, Aylesbury, UK) with Excitation at 350 nm and emission at 420 nm.

Table 5.4: Preparing a 96 well plate for measurement of reduced and total glutathione. The plate was loaded, following the order from top to bottom

	red. GSH measurement	Total GSH measurement	Neg. control for background fluorescence	GSH standards	GSSG standards
19 µL 5% TCA in RQB	✓	✓	✓	✓	✓
10 µL of sample	✓	✓	✓		
10 µL of GSH standard				✓	
10 µL of GSSG standard					✓
4 µL per well of RQB	✓			✓	
4 µL per well of 7.5 mM NEM			✓		
48 µL of 1 M potassium phosphate pH 7	✓	✓	✓	✓	✓
Incubate 5 min	✓	✓	✓	✓	✓
7 µL of 10 mM dithionite		✓			✓
incubate 1 h		✓			✓
200 µL of 0.1 M potassium phosphate pH 6.9	✓	✓	✓	✓	✓
29 µL OPT 5 mg/mL	✓	✓	✓	✓	✓
Incubate 30 min in the dark	✓	✓	✓	✓	✓

5.3 Results

5.3.1 TEAC assay

The result description of the TEAC assay includes some logical discussion in order to allow the reader to understand the protocol modifications and development incorporated.

A comparison of antioxidant capacities of untreated *D. magna*, *D. magna* treated with t-BHP (tert. butyl hydroperoxide) as positive control and *D. magna* exposed to 14 nm carbon black (10 mg/L for 1 h) showed a strong negative value for the trolox equivalent in the case of *D. magna* exposed to carbon black (Fig. 5.1). The animals in the control treatment showed as well as the positive control a positive value for trolox equivalents that did not differ significantly from each other ($p = 0.5$) when compared by a one way ANOVA. This indicated, besides the negative value for carbon black, that t-BHP was not functioning at the tested concentration as a positive control, since the value should have been significantly below the control.

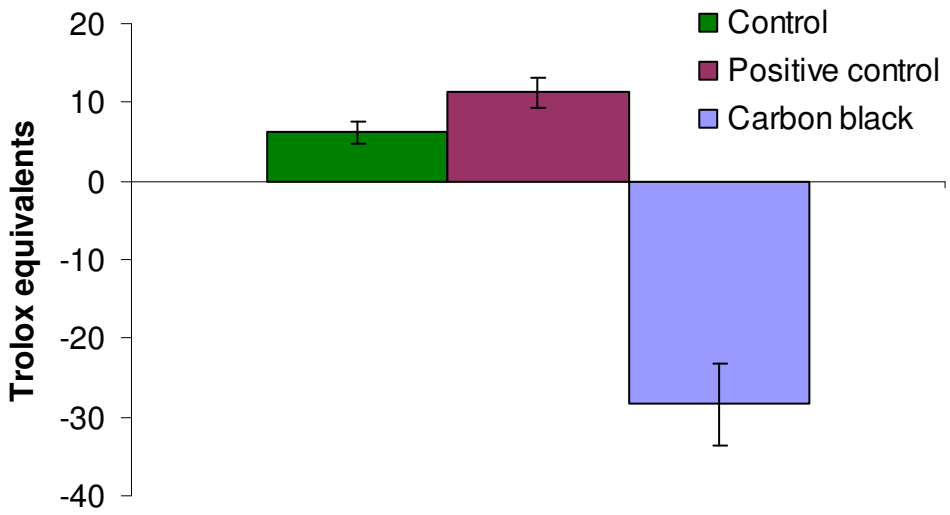


Figure 5.1: Antioxidant capacities of untreated *D. magna*, *D. magna* treated with t-BHP (tert. Butyl hydroperoxide) as positive control and a carbon black treatment (10 mg/L for 1 h). Ten trolox equivalents are equal to 2.5 mM trolox.

In the case of the *D. magna* exposed to carbon black, within the assay a negative trolox equivalent value is without sense and a lower trolox equivalent value is synonymous to a higher absorbance, it was hypothesized that the carbon black particle might interfere with the absorbance measurements.

To test this hypothesis, standard curves where 10 µl PBS were exchanged with a suspension of 100 mg/L carbon black in water media. These were compared to standard curves with 10 µl water media instead of carbon black (Fig. 5.2).

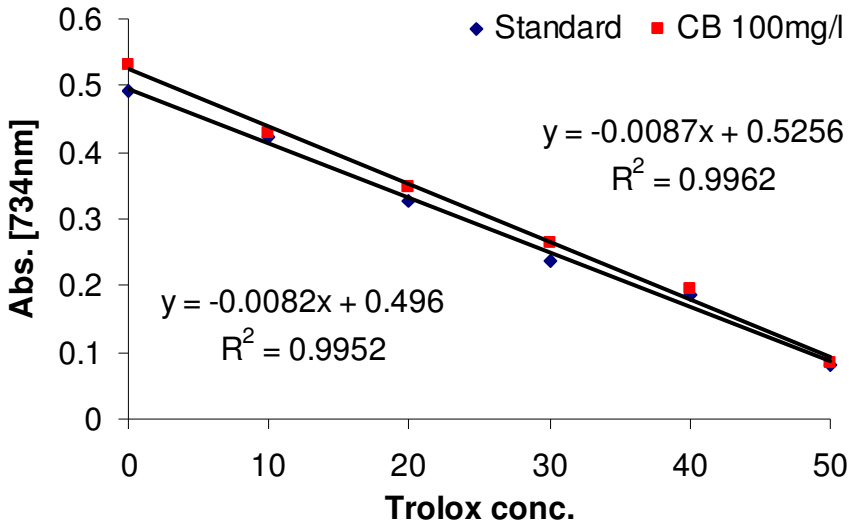


Figure 5.2: Comparison of a normal standard curve with a standard curve containing 100 mg/L carbon black. The equation of the normal standards and the R^2 value are below, while for the carbon black they are on top

These results suggest that carbon black at the measured concentration might just have a minor effect, if any, on absorbance measurements. These results were further confirmed by measuring several concentrations of carbon black (0 mg/L; 10 mg/L, 100 mg/L and 1000 mg/L) prepared according to sample preparation but without adding H_2O_2 (expected was no colour change) (Fig. 5.3).

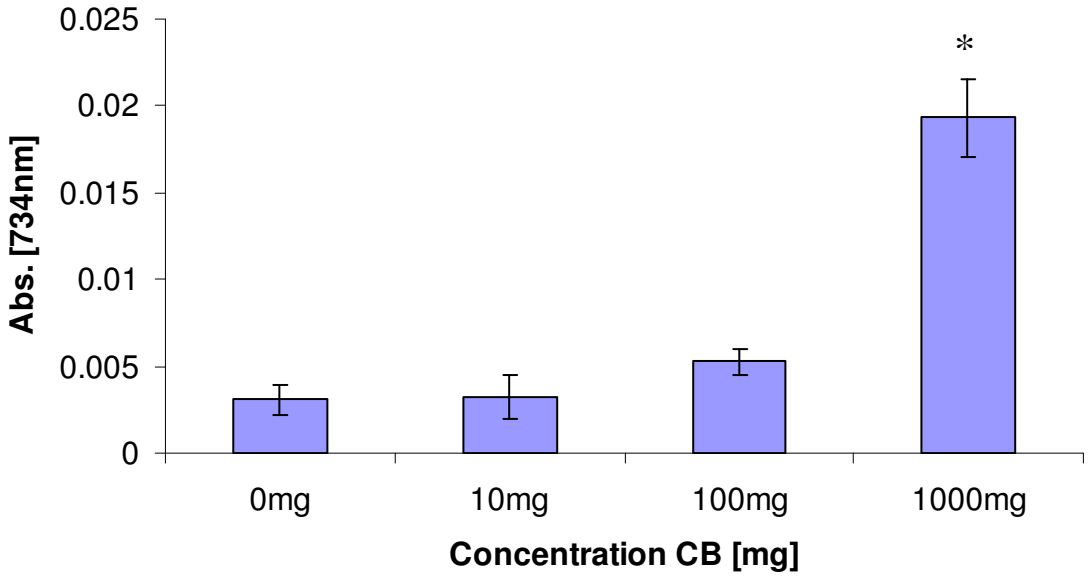


Figure 5.3: Absorbance comparison of several carbon black concentrations (n =3 per treatment, values are means +/- standard error, * indicates a result with $p \leq 0.05$).

These results indicated, that a slight, but not significant, increase in absorbance was observed for the treatment containing 100 mg/L carbon black. A significant increase in absorbance was observed for the treatment containing 1000 mg/L carbon black ($p < 0.001$) when compared by a one way ANOVA to the other treatments.

5.3.1.1 Measuring the antioxidant capacity of unexposed *D. magna*

For this study, ten *D. magna* per replicate were homogenized and 10 µl of this homogenate were added to the test as described in table 5.1. An average absorbance of 0.252 at 734 nm was measured for the three replicates but it was observed that no colour change at all took place in the set time of 6 min indicating that the measured absorbance was probably background absorbance instead of absorbance produced by the reaction of ABTS, Met Mb and H_2O_2. To verify this, a measurement of homogenized *D. magna* with all the reagents, but without H_2O_2 (no colour change), confirmed that the measured absorbance was background absorbance (0.237 at 734 nm). The reading should have been close to 0 if the sample has no background absorbance.

A dilution of 10 µl *D. magna* homogenate in 90 µl PBS showed to be sufficient to reduce the background absorbance to 0.022, allowing the antioxidant capacity to be detectable within the standard curve.

The TEAC measured for untreated *D. magna* was 3.25 mM trolox equivalents in the dilution. Taking the dilution into consideration, a trolox equivalent of 32.5 mM trolox for 10 µl of *D. magna* homogenate was calculated.

5.3.1.2 Antioxidant capacity of *D. magna* exposed to carbon black

D. magna were exposed to two different concentrations (1 mg/L and 10 mg/L) of carbon black in EPA media for 1 h and, along with untreated *D. magna,* prepared for measurement according to the TEAC assay. The homogenised *D. magna* were measured with all reagents added, but before H_2O_2 was added (no colour change) (Fig. 5.4) and 6 min after H_2O_2 was added (colour change) (Fig. 5.5 blue bars)

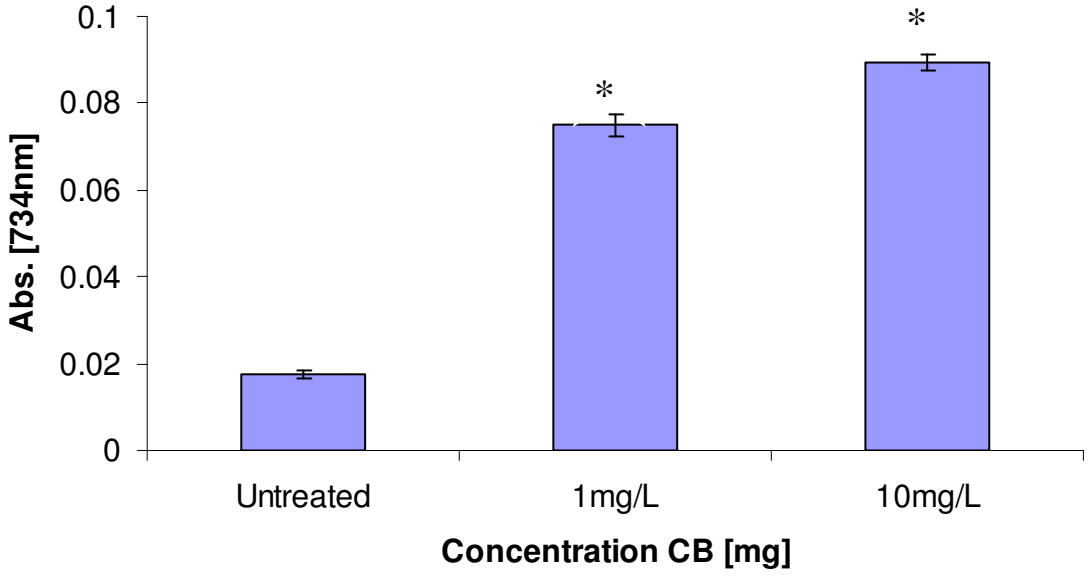


Figure 5.4: Absorbance of *D. magna* untreated, treated for one hour with 1 mg/L and 10 mg/L carbon black, before H_2O_2 was added (n= 3 per treatment, values are means +/- standard error, * indicates a result with $p \leq 0.05$).

Fig. 5.4 shows that there was already inherent absorbance present in the samples before H_2O_2 was added, since all treatments were significantly different from each other ($p \leq 0.005$) when tested by a one way ANOVA This background absorbance has to be taken into consideration when calculating the antioxidant capacity. If this background absorbance was not taken into consideration no difference of antioxidant capacity could be observed after 1 h of exposure to different concentrations of carbon black (blue bars Uncorrected in Fig. 5.5). However, if the background absorbance was taken into consideration by subtracting the background from the measurements in the assay, an increase in antioxidant capacity could be observed (red bars Corrected in Fig. 5.5). The controls as well as the uncorrected values differed not significantly from each other, while the corrected values for 1 mg/L and 10 mg/L had a significantly increased trolox equivalent in relation to the controls or uncorrected values ($p< 0.004$ for the uncorrected values, $p< 0.027$ for the corrected control). The 1 mg/L and 10 mg/L value did not differ significantly from each other.

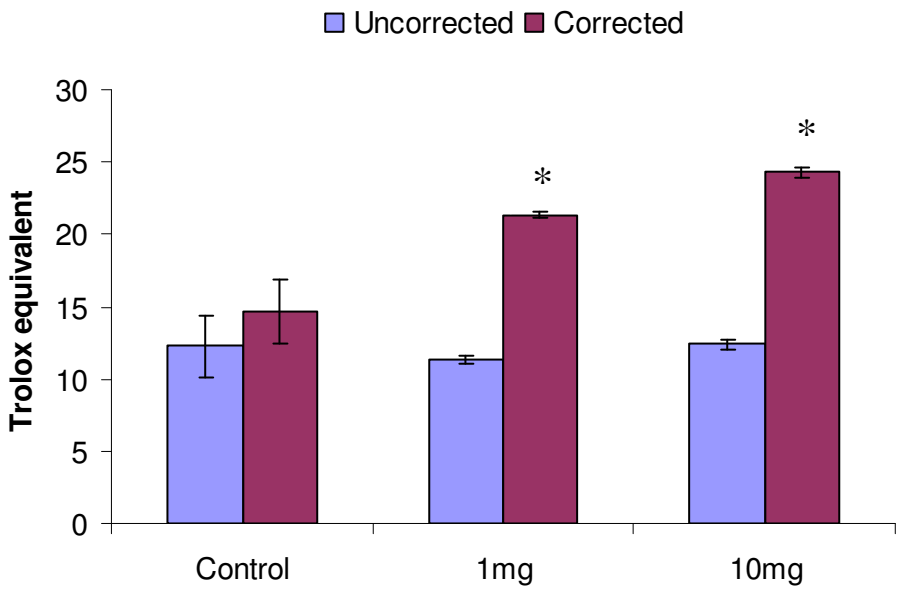


Figure 5.5: Antioxidant capacities of five untreated control *D. magna* and carbon black treatments (1 mg/L and 10 mg/L for 1 h). Ten Trolox equivalents are equal to 2.5 mM Trolox. (n = 3 per treatment, values are means +/- standard error, * indicates a result with $p \leq 0.05$).

To ensure that these absorbance's are really a background absorbance and not an involuntarily offset of reaction caused by the presence of carbon black as radical producer, the absorbance of homogenised *D. magna* untreated, with

carbon black added to the *D. magna* homogenate subsequently, as well as two additional nanoparticles in two further treatments, TiO_2 and silver, were measured in pure PBS (Fig. 5.6).

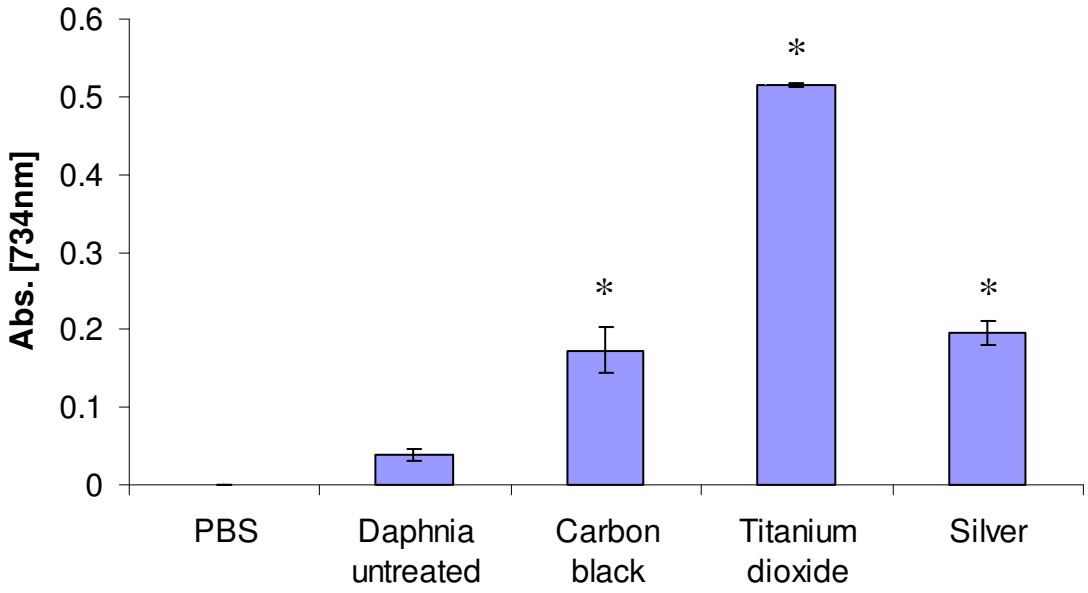


Figure 5.6: Absorbance of PBS, homogenized *D. magna* in PBS and three nanoparticles added to homogenized *D. magna* in PBS (*D. magna* untreated n= 6, otherwise n = 3 per treatment, values are means +/- standard error, * indicates a result with $p \leq 0.05$).

While a sample of *D. magna* homogenate showed no significantly increased absorbance when compared to the PBS control, the three nanoparticle treatments showed significantly increased background absorbance ($p < 0.001$) when compared to the PBS control (Fig. 5.6) when tested by a one way ANOVA.

5.3.2 Reduced Glutathione/ total Glutathione assay

The measurement of reduced and total glutathione was repeated three times. The negative control for checking background fluorescence (see table 5.4) delivered a slight fluorescence in all samples, analogous of a glutathione concentration between 6 and 8 µM. Since GSH 100 µM with addition of NEM (GSH should be blocked and all fluorescence should be quenched) showed that

NEM was not able to quench all fluorescence of GSH reliably and the detected background fluorescence was homogeneously spread over all samples independent from the treatment. Therefore they did not interfere in the assessment of differences between reduced or total glutathione, particle or size of particle.

The standard curves of reduced GSH at the concentrations described in table 5.2 delivered in all three triplicates of the experiment standard curves with a R^2 of 0.98 and above. To assess the concentration of reduced and total glutathione and the ratio of reduced glutathione against total glutathione, the averages of the replicates in each single experiment were determined first, before the data of each treatment were pooled.

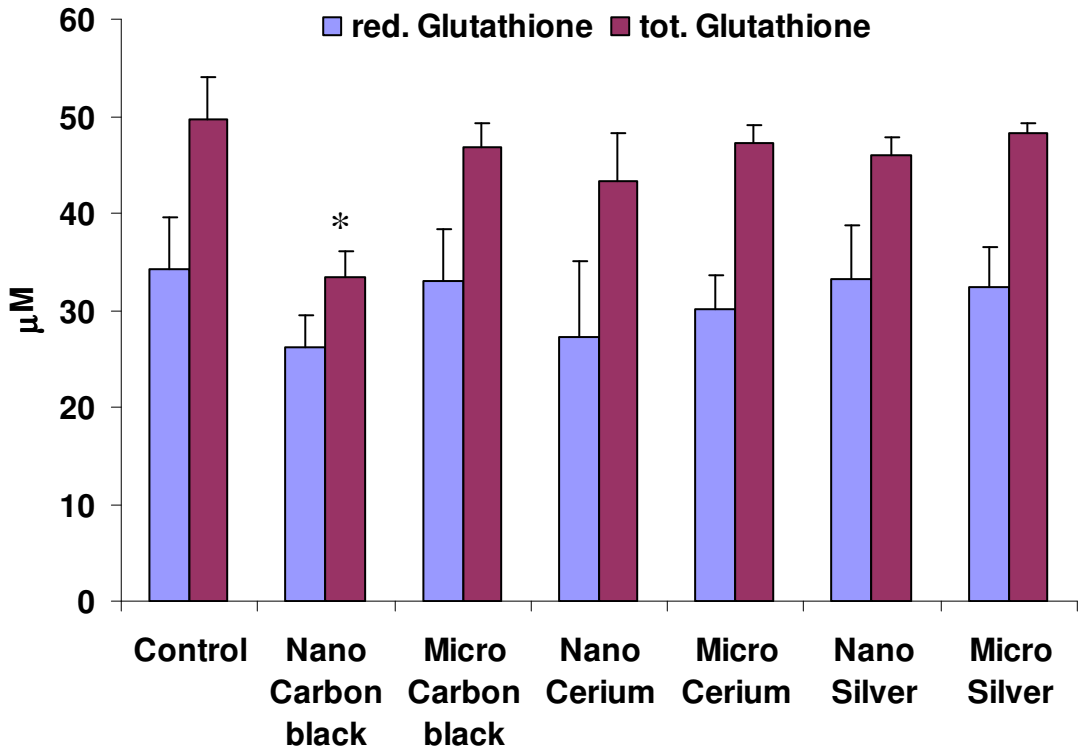


Figure 5.7: Levels of reduced and total glutathione in *D. magna* exposed for 3 h to different sized particles (n= 49 for reduced glutathione and n= 45 for total glutathione, values are means +/- standard error, * indicates a result with $p \leq 0.05$).

While the average total amount of reduced glutathione was lower in all treatments than in the control, this difference failed to be statistically significant (Fig. 5.7). However, the total glutathione concentration was significantly

decreased (p= 0.042) in the nano sized carbon black treatment when compared to the control (Fig. 5.7) when tested by a one way ANOVA. The other treatments showed no significant difference when compared to the control.

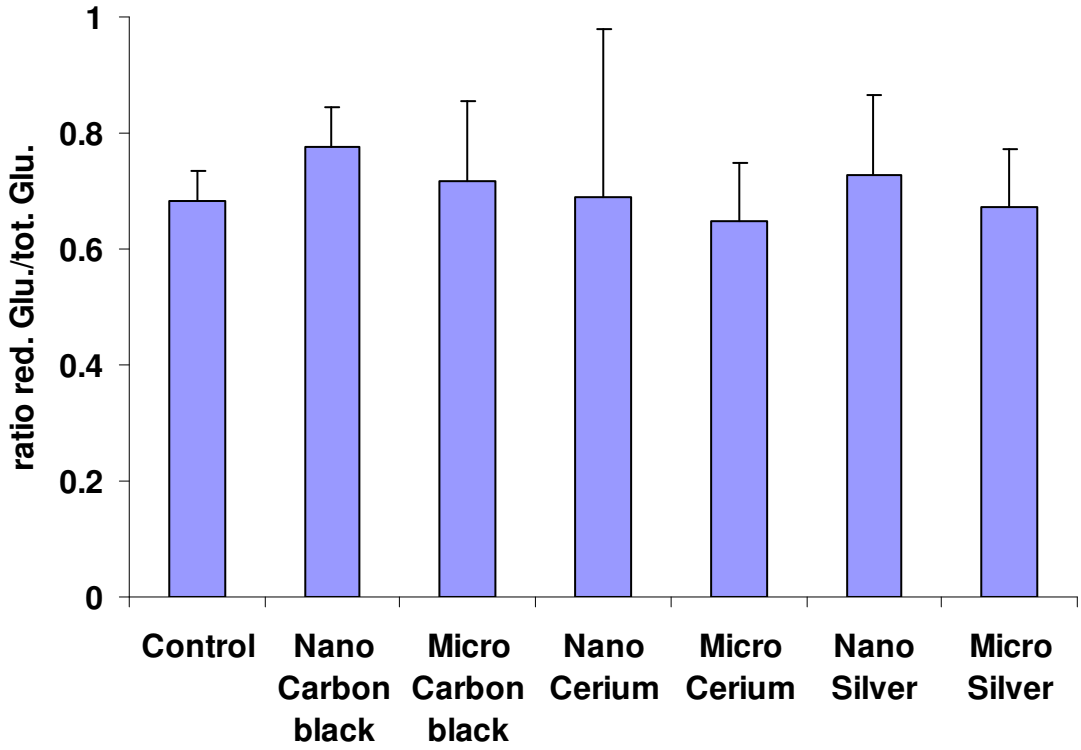


Figure 5.8: Ratios of reduced to total glutathione in *D. magna* exposed for 3 h to different types and sized particles (n= 21 for reduced glutathione and total glutathione, values are means +/- standard error).

Comparing the ratio of reduced glutathione to total glutathione, it was observed, that the ratio was independent of particle or size and it was nearly constant at two third when the ratio reduced glutathione to the total glutathione concentration was calculated (Fig. 5.8).

5.4 Discussion

The results of the TEAC assay show clearly that such absorbance methods are difficult to employ when measuring biomarkers on the exposure to particulate matter. Although the particles initially did not seem to interfere substantially with the assay (see Fig. 5.2) when used at concentrations of up to 100 mg/L, the situation changed when the animals were homogenized after exposure to particles. It is possible that the particles become better dispersed in the *D. magna* homogenate, allowing them to interfere strongly with spectrophotometrical readings and masking the required colour change indicating the build-up of the blue/green chromophore ABTS. This masking leads to a higher absorbance which in turn leads to an underestimation of trolox equivalent antioxidant capacity. The good dispersion of particles might be partly due to lipids and carotinoids used as energy storage in *D. magna* (Holm and Shapiro, 1984, van Der Veen, 2005). It is likely that these lipids mix with the particles during the homogenising process and this mixture of finely dispersed particles becomes the source of the background absorbance observed. In addition, this background absorbance is dependent on concentration of nanoparticles used in the exposure or more precisely it is dependent on uptake of particles by *D. magna* during the exposure time. This would make the background absorbance that has to be taken into consideration dependent on concentration as well as time of exposure. An animal exposed to 1 mg/L of carbon black for 3 h would have a different background absorbance from an animal exposed to 10 mg/L for the same duration due to a higher uptake.

The result of not taking background absorbance into consideration can be seen in Fig. 5.5. The uncorrected values would lead to the conclusion that the animals would have an equal antioxidant capacity over a range of concentrations they are exposed to. With the background absorbance taken into consideration, it can be seen that the antioxidant capacity actually rises as soon as animals are exposed to nanoparticles for 1 h.

Although it is possible to estimate the background absorbance as shown by measuring the absorbance before adding the H_2O_2 that starts the reaction and

subtracting those results from readings gathered after the reaction is finished, this method quickly becomes unreliable if differences in the concentration of antioxidants are small between the treatments and there is a significant background absorbance. Also, uptake over time makes it difficult to compare treatments with different time points or concentrations. Lastly, Fig. 5.6 shows that those problems are not limited only to carbon black, but can be expected for other particles as well.

In the light of these results it has to be concluded that the TEAC assay is unsuitable in its current state for assessing the antioxidant capacity of *D. magna* due to nanoparticles.

Since the glutathione assay measures levels of antioxidants via fluorescence instead of absorbance, fewer problems might be expected. The control for checking on background fluorescence shows that there is a level of fluorescence from the samples but, this background is relatively less than in the previous TEAC assay. Also, the methodology of this assay includes centrifugation which helps remove most of the particle burden. The TEAC assay is very unspecific, since it assesses total antioxidant capacity and gives information on the overall status of antioxidants within a biological sample. That means centrifugation that would remove from the sample particles that interfere with the assay could also remove components that act as antioxidants and so alter the results. In the GSH assay on the other hand, the homogenized sample is treated with RQB-TCA buffer. Trichloracetic acid (TCA) is used to precipitate high MW proteins and nucleic acids, leaving just analytes of interest in the supernatant after centrifugation. This step also removes interfering particles and carapace fragments.

The results of the GSH assay show that the only treatment effected by exposure to particles is the carbon black treatment to nanoparticles. Results from the exposure indicate that the reduced glutathione and the total glutathione are lower than in the other treatments although only the total glutathione concentration was significantly lower than the control. This result was unexpected. A significantly lower level of total glutathione compared to a reduced, but not significantly lower level of reduced GSH, would lead to the

conclusion that the concentration of GSSG must be severely reduced. It would also mean that the concentration of glutathione that can be recycled to GSH, and therefore would be available to reduce further oxidative stress, would be reduced. Normally, when mild oxidative stress is observed, an increase in GSH levels can be observed (Zhang et al., 2004), since GSSG levels rise at the expense of GSH levels, once oxidative stress gets more severe. GSSG is recycled by the enzyme glutathione reductase and the oxidation of NADPH to $NADP^+$ (Zhang et al., 2004). If accumulation of GSSG is too high, it is transported outside of the cell to prevent NADPH depletion.

In this study the ratio of reduced glutathione to total glutathione was not significantly elevated in any treatment, indicating that higher production of GSH did not occur, as would be expected with mild oxidative stress. Additionally, no accumulation of GSSG was observed, which could be detected by a higher amount of total glutathione and a lower ratio of reduced glutathione to total glutathione and what would point to more severe oxidative stress.

While it can be concluded that the GSH assay succeeded in highlighting changes due to exposure to nanoparticles, the changes cannot be explained by the normal mechanism of glutathione homeostasis. An assessment of the enzyme glutathione reductase, which might have been inhibited due to exposure, or an assessment of NADPH levels, which, if they would be too low, would lead to excretion of GSSG, would be good leads for follow up experiments.

6. Final conclusions and future work

The present work clearly shows that nanoparticles have the potential for negative effects on *D. magna* upon exposure. It was further demonstrated that nanoparticles are readily taken up by *D. magna* and can be subsequently relocated within the body of exposed individuals *D. magna*.

The aim of chapter 3 was to assess the effects of different particles on *D. magna* by exposing them to different sized particles and measuring lethal and sublethal endpoints in acute, short term exposures and chronic, long term exposures at different mass doses. The results thus obtained should enable an estimate not only of the toxicity of the different particles but also if the size of the particles is an important factor of toxicity.

Results obtained indicate that, when an effect was observed, it was always more pronounced in treatments to nano sized particles than micro sized particles. The main causing factor(s) underlying these observations could include parameters such as larger surface area or higher particle number in the nano treatments, however due to lack of evidence the actual factors remain unknown. Although exposures to carbon black suggest that for this material surface area is likely to be responsible for some effects on *D. magna* such as mortality and moulting, results obtained on exposures to silver indicate a different mode of toxicity, with free silver ions being one of the most obvious factors. The endpoints chosen for the acute and chronic tests were found to be suitable to detect effects due to exposure.

Regarding the results from chapter 3, further experiments would seem promising. Exposures to carbon black and silver, with different sized particles on a surface area dose or particle number dose would allow further investigation of the relationships between surface area and toxicity. Exposures to cerium dioxide resulted in no evident toxicity in the concentrations tested and would require probably concentrations that would not be environmentally relevant to detect effects at equal surface area dose of micro and nano sized particles. However, since *D. magna* exposed to cerium

dioxide showed a significant reduction in growth after 4 day exposures to 10 mg/L of nano sized particles it would be interesting to investigate if this size reduction is due to reduced nutrient uptake. This could be done by exposures to micro and nano sized cerium dioxide at different food levels.

With silver micro and nano particles, the moulting frequency and mortality seemed to be not exclusively surface area related. Exposures to micro and nano sized particles in the presence of cysteine, which acts as a ligand to Ag ions and reduces their free concentration in the media (Navarro et al., 2008), would reveal if the toxic effects observed with silver are partly due to free ions in the media.

Another promising field of future research would be studying the association between manufactured nanoparticles and naturally occurring colloids and organic substances such as humic and fulvic acids. Manufactured nanoparticles most likely will encounter those substances in aquatic systems. It is important therefore to address how this could affect the bioavailability of manufactured nanoparticles and their uptake into cells and organisms as well as how it could alter their toxicity.

The most important drawback of the studies chapter 3 is describing was that no characterization of the particles in the exposure medium could be done. Dynamic light scattering and Zeta potential measurements of the particles in media would have allowed assessing better the actual particle size and the stability of aggregates or agglomerates initially and over the whole exposure duration. This would have allowed getting a better approximation of the exposure conditions and relating the mass dose more efficiently to a surface area dose. These two methods, among others, (Joner et al., 2008, Klaine et al., 2008, Powers et al., 2006) seem to become standard in the laboratory when assessing the toxicity of nanomaterials and further experiments with a similar aim would benefit from data acquired through these methods.

Nevertheless, dispersion data gathered in laboratory experiments as here described have to be taken with caution when applied to the environment or even other studies using the very same materials. Since dispersion, shape of

occurring aggregates and surface charge can depend on the dispersion protocol used as well as the media the particles are suspended in (Royal Commission on Environmental Pollution, 2008). In this study an artificial EPA media, which is widely used in D. magna toxicity testing, was chosen. Using this artificial media as well as a constant dispersion protocol assures that one toxicity test using this protocol can be compared to another toxicity test using the same protocol and has the same duration. Even comparing laboratory data of experiments using the same protocol but different exposure durations are difficult to compare, since nanoparticle aggregates might not be stable over time (Royal Commission on Environmental Pollution, 2008) or excretory products of the test animals might shift the pH in the exposure media and change the dispersion as well. Excretory products might even interact with the nanomaterials directly with unknown consequences.

However, it is a good approach to assess toxicity initially with a widely used media like the OECD or EPA artificial water since it allows comparisons between results of different publications that use the same media as well. The impacts of other environmental factors like the pH or particulate organic matter in the water, which can both influence the behaviour of NP´s in water as already mentioned elsewhere, would have expanded the scope of this thesis too much. In fact, both environmental factors, influence of the pH and influence of particulate organic matter on the toxicity of NP´s, would require own research projects to do credit to these important factors.

A further point that should be included in future research would be the use of positive controls alongside particle exposures. This would add an additional quality control of the animals used. In the here presented study, the test results were accepted if 90% of the control animals survived. Standard reference toxicants could be SDS (Sodium dodecyl (lauryl) sulfate), NAPCP (Sodium pentachlorophenate) or cadmium chloride as proposed by EPA (U.S. Environmental Protection Agency (EPA), 2002a). Using those reference toxicants would have ensured a better evaluation of the health and sensitivity of the test organisms over time. Even salts of the particles tested, like silver nitrate as done in Navarro et al. (2008) or cerium hydroxide ($Ce(OH)_3$) could be used and give additional information about the toxicity of dissolved ions.

The aims of chapter 4 were to assess and compare the uptake of nano or micro particles by *D. magna*, to examine qualitatively the total uptake of those particles and determine possible locations of accumulation as well as to quantify uptake and depuration and compare the two particle sizes.

Results obtained in chapter 4 showed that uptake of micro sized, as well as nano sized, particles took place. Although uptake of nano sized particles was considerably lower than micro sized particles, when compared on a mass dose basis, when compared on a surface area dose or particle number dose basis, the uptake of the nano sized particles exceeded the uptake of the micro sized particles. Additionally, depuration was quicker in the micro sized particles than in the nano sized particles, resulting in mass doses for both sized particles after a depuration time of 4 h that were not significantly different from each other anymore. In addition, translocation was observed by confocal microscopy for both sized particles as soon as after 30 min of exposure, with the particles crossing the barrier between the gastrointestinal tract and the body cavity and accumulating in oil storage droplets. From these results it can be concluded that it is possible for nanoparticles to be ingested and accumulated readily in the gut in significant concentrations and that a potential exists for these ingested nanoparticles for translocation with possible toxic effects. The accumulation of nanoparticles in the gut could lead to transfer of considerable amounts to higher trophic levels in the food chain of the aquatic environment with effects yet not investigated for *D. magna* and rarely for other invertebrates (Holbrook et al., 2008). Also, the lipid containing storage droplets are normally partially transferred to the offspring (Goulden and Hornig, 1980, Tessier and Goulden, 1982). An uptake of nanoparticles by embryos via maternal translocation could mean higher mortality of neonates due to toxic effects or reduced nutrient uptake due to a lesser nutrient content in the transferred storage droplets.

These last two points mentioned, accumulation of nanoparticles via a food chain, as well as maternal transfer of nanoparticles to embryos, would be good approaches to further research. In addition, expanding the time line of depuration would give more helpful information on the long term fate of

nanoparticles in *D. magna.* Lastly, exposures at different mass doses would give further information of concentration dependent uptake and depuration.

Just as interesting as the uptake and translocation of nano sized particles, is the uptake and translocation of their micro sized counterparts. A similar experiment as presented here was done by Browne et al. (2008) with the mussel *Mytilus edulis*. Here, even bigger polystyrene beads, 3 µm and 9.6 µm, were used. As in this study, a translocation across the epithelial barrier was observed and particles were detected in the hemolymph (Browne et al., 2008). In contrast to the study presented in chapter 4, translocation took much longer and the polystyrene beads were detected in the hemolymph at 3 days with the maximum at 12 days after an initial 3 h exposure. Nevertheless, the first time point of examination for translocation was not stated in the study, making a quicker translocation possible. The study also points out that the particle number encountered in the hemolymph was at all times higher for the smaller sized particles than for the larger sized particles. This was also true for the here presented study when the results were calculated as particle number. In contrast to the here presented study the particles were not detected in storage droplets but in haemocytes, a cell type found in invertebrates that fulfils the function of phagocytes in vertebrates. The mechanism of translocation across the gut epithelial could not be determined but it was assumed that phagocytosis played a role (Browne et al., 2008). The aim of Browne et al. (2008) was to study possible uptake and translocation of microplastic particles in the marine environment by the mussel *Mytilus edulis.* The accumulation and fragmentation of plastics in the terrestrial and marine environment are becoming more and more a reason of concern (Barnes et al., 2009). Although no reference was found that stated that plastic particles are a major concern in freshwater habitats, this might mean the problem did not get any attention yet. On the other hand, the subclass cladocera has severall members that are part of the marine planctonic community (Rupert et al., 2004) and could take up and translocate plastic particle in a way similar as described in chapter 4.

The aims of chapter 5 were to study oxidative stress induced by exposure to nanoparticles and to assess if a size dependent relationship of this stress could be detected. To achieve this, two methods were used: One measuring the total

antioxidant capacity and a second one that focuses on a specific low-molecular-weight antioxidant.

In the case of the measurement of total antioxidant capacity by the TEAC assay, it has to be concluded that the TEAC assay is unsuitable in its current state for assessing the antioxidant capacity of *D. magna* due to nanoparticles. The reason for this conclusion was the fact that particles were found to interfere with the measurement of absorbance. The dispersion of particles in the presence of homogenized *D. magna* was enhanced, most likely by lipids in the homogenate. An adapted method, also measuring absorbance in homogenates of exposed *D. magna,* could be used for quick estimates of uptake of nanoparticles.

While it can be concluded that the GSH assay succeeded in highlighting changes due to exposure to nanoparticles, the changes cannot be explained by the normal mechanism of glutathione homeostasis with one time point measured. To get a clearer picture of the processes involved, several time points should be chosen as well as multiple concentrations. Lastly, as previously mentioned in chapter 5, an assessment of the enzyme glutathione reductase, or of NADPH levels, would be good leads for follow up experiments.

The results presented in chapter 5 should also make cautious about using commonly employed biomarkers in combination with nanoparticles without proper control treatments to verify that the nanoparticles do not interfere with the method. Concerns have already been raised (Jones and Grainger, 2009) that many standard assays might not be suitable. For example, a widely used cytotoxicity assay, the MTT assay that measures the ezymatic reduction of a tetrazole to a formazan in active mitochondria, was found to give false results when used in assays with carbon nanotubes (Wörle-Knirsch et al., 2006) or quantum dots (Hoshino et al., 2004). In one case, the formazan was found to attach to the carbon nanotubes and could therefore not be detected by the assay (Wörle-Knirsch et al., 2006), in the other case the tetrazole was reduced to formazan without an enzymatic reaction, giving false positive results (Hoshino et al., 2004). It is also known that proteins can bind to nanoparticles (Aggarwal et al., 2009, Cedervall et al., 2007) making the use of assays

involving proteins, like the LDH assay or the glutathione reductase assay mentioned before, problematic. A good approach to prevent errors is, including control treatments that test for interferences of nanoparticles as shown in (Clift et al., 2008).

Finally, it has to be discussed, how the findings of this thesis can help with assessing the risk that nanomaterials can pose to the environment. As already mentioned, this study concentrated on hazard assessment under controlled laboratory conditions. To test if exposures in the environment will happen with the nanoparticles tested in chapter 3, was not an aim of this thesis, since this would have been far too complex to accomplish in the given time. Nevertheless, the data point to a potential hazard of at least the silver particles and carbon black particles and possible exposure pathways should be researched to perform a risk assessment. There have been already several publications discussing approaches to manage environmental risk assessment of nanomaterials (Crane et al., 2008, Owen et al., 2009, Owen and Handy, 2007). The problem with nanoparticles is that it is not certain in what form an exposure will take place for each nanoparticle since from the spill side to the exposure side they may undergo complex changes (Owen et al., 2009). Furthermore, surface coating or surface alteration of a nanoparticle might change his behaviour and toxicity drastically. According to severall websites there are already thousands of consumer products in circulation and one report states that three to four new products containing nanoparticles are added each week (National Institute for Occupational Safety and Health (NIOSH), 2009). So what is the right approach to assess their risk to the environment? Owen et al. (2009) suggested two different approaches to managing the risk: A hazard driven approach and an exposure driven approach. The first approach would involve the assumption that an exposure route for any nanomaterial cannot be ruled out and therefore extensive hazard testing should take place. The second approach would involve a first step in assessing the environmental behaviour of nanomaterials to find out if an exposure might take place. Hazard assessment would only take place if an exposure would be possible. The author also points out, that both approaches would take a large amount of time and the risk assessment would be lagging behind due to the quick appearance of novel nanomaterials (Owen et al., 2009). The danger of lagging behind makes a

hazard assessment similar as presented here a reasonable approach, using nanomaterials that are widely used in consumer products. For future hazard assessments, similar approaches as proposed from EU REACH "Implementation Project 3.3 for aquatic toxicity" (Crane et al., 2008) seems sensible. Here, short term assessments are first undertaken with either *Daphnia* or algae species, if the need for further investigations arise, chronic testing is employed (Crane et al., 2008). Since even acute *Daphnia* 96 h exposures are quite labour intensive and therefore costly and considering the number of nano sized particles as well as the different environmental parameters that need to be considered, testing *in vivo* test systems for their suitability as screening tools might be a good idea.

The testable **Null Hypothesis** in this project that NPs tested will have no different negative effects than larger particles of the same composition in the same mass dose on *D. magna,* can be rejected in the light of the results presented in this thesis.

The author hopes that the results, suggestions and ideas presented in this thesis will contribute to future research in the area of ecotoxicology of nanoparticles. As with all research, the results lead to as many additional questions as they answer and more research is needed for each section described here.

7. References

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