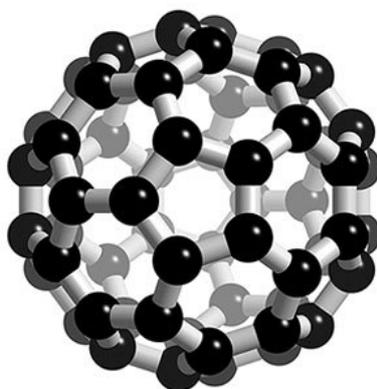


Risk assessment of engineered carbonaceous nanoparticles in the aquatic environment



Master Thesis MSc program 'Toxicology & Environmental Health'

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Abstract

The emerging nano-industry and increasing use of manufactured nanoparticle-containing (consumer) products worldwide will ultimately lead to growing nanoparticle emissions into the environment. Since most nanoparticles have different properties than their larger counterparts and therefore, obviously, behave differently, these new materials require risk assessment to understand their impact on the environment. So far there has been minimal research on nanoparticle mobility, reactivity and ecotoxicity.

This review presents literature which demonstrates that carbon nanoparticles could have effects on (aquatic) organisms under environmental conditions, though mostly at high exposure levels. Also, it is found that the behavior of carbon nanoparticles in aqueous media is very complex, depending on a lot of particle and medium conditions. All together, these elements make risk assessment of (carbon) nanoparticles a real challenge in the future.

The objectives of this thesis are to introduce key aspects of the behavior and fate of carbonaceous nanoparticles in the aquatic environment and (future) risk assessment. Summarized are ecotoxicity testing results up until this moment, challenges in toxicity testing and recommendations concerning the needs in future testing and improving universal nanoparticle risk assessment in general.

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1. INTRODUCTION

1.1. Nanotechnology: a particular promising domain

Recently the focus on nanotechnology has grown tremendously due to the increasing application of nanomaterials in the global consumer, industrial and technological sectors. Nanotechnology is a relatively new area still in its infancy, but the field of nanomaterials is in full progress and rapidly developing as we speak. Nanotechnology is, up until this moment on, defined as the development and application of materials and structures with nanoscale dimensions, usually in the range of 1-100 nm (Borm et al., 2006; Moore, 2006; Crane et al., 2008). Compared to larger substances of the same material nanomaterials offer new properties, for example mechanical, optical, electrical, and magnetic attributes, due to their specific size and shape. This therefore makes these small materials exceptionally suitable and effective for all kinds of purposes. Nanotechnology, which is more often seen as the new industrial revolution or the next logical step in science, thus makes it possible to design and engineer materials at the molecular level to give it specific, new properties.

Apart from their high surface area to volume ratio the biocompatibility surface properties of nanomaterials depend on the electrical charges carried by the particle and its chemical reactivity (Moore, 2006). The interaction of these particles is thus determined by their surface chemistry and reactivity. For example, this means that nanoparticles can be more reactive and, in this way, create more catalysts or more efficient materials. This could lead to weight reduction in composite materials with higher surface energies, increasing stronger interactions (Yoo, 1998; Borm et al., 2006). The idea of building materials and devices from the bottom up, molecule by molecule, has long been a goal within this technology (Maynard, 2006b). Considering the diverse and cross-disciplinary nature of the technology it may even be more precise to use the term 'nanotechnologies' in stead of 'nanotechnology'. Roughly the same goes for 'nanoscience' and 'nanotechnologies' (Maynard 2006a; The Royal Society, 2004), clearly stating this as follows:

“Nanoscience is the study of phenomena and manipulation of materials at atomic, molecular and macromolecular scales, where properties differ significantly from those at a larger scale.”

“Nanotechnologies is the design, characterization, production and application of structures, devices and systems by controlling shape and size at the nanometer scale.”

In its very early stage nanotechnology was a field of study for research and improving (laboratory) technologies only. But not long after that its great potential opened new doors in the commercial consumer products business (e.g., sunscreens, sport goods, cosmetics, resins) and the medical/pharmaceutical (e.g., targeted cancer therapies, drug-delivery systems) and environmental (e.g., purify water, treating wastes) areas (Bell, 2007). Due to rapid progress nanotechnology made a big developmental flight, especially in these sectors, not only replacing or improving the conventional materials with these new nanomaterials, but also creating new applications (Borm et al., 2006).

1.2. Nanotechnology: a new industrial revolution?

Since nanoparticles have been present on earth for millions of years many organisms, and amongst them mankind, have been exposed to these particles throughout their evolution (Dowling, 2004). Volcanic eruptions, one of the largest sources of ambient nanoparticles, occurred throughout the history of earth. Natural occurring nanoparticles (e.g., fullerenes) coming from, for example, natural combustion processes and biologically based (e.g., biogenic magnetites like a ferromagnetic iron oxide (Fe_3O_4)) have been found in organisms living since the beginning of life on this planet.

It is since the past industrial revolution in the late 18th century that such exposures have increased dramatically because of anthropogenic sources (Oberdörster G. et al., 2005a). The rapidly developing and expanding field of nanotechnology can thus be seen as a new industrial revolution and a new exposure source to (engineered) nanoparticles. It has been predicted by the American National Science Foundation that the global marketplace for products and services using nanotechnologies will grow from €25 billion (in 2004) to €0.7 trillion by 2015 (Borm et al., 2006) (amounts shown in Euros to simplify comparisons). The United States only invests approximately €2.1 billion annually in nanotechnology research and development, which accounts for approximately one-third of total public and private sector nanotechnology investments worldwide (Maynard, 2006a). This future projection indicates a massive growth of the nano-industry, which will be approximately an output of 10% of the whole chemical sector (Borm et al., 2006). Because it produces the basis materials for products across many sectors the chemical industry plays a key role in exploiting nanomaterials.

1.3. Nano-industry: potential and novel risks?

With the strong increase of engineered nanomaterials production, and the well-known consequences of the earlier industrial revolution on human and environmental health in mind, the underlying and growing concern surrounding an increase in the production and application of many types of nanomaterials is that, inevitably, they will end up in the environment eventually, either intentionally or unintentionally (Nowack & Bucheli, 2007; Hassellöv, 2008). Similar, in the past years the focus has been on ultrafine particles (UFPs; smaller than 0.1 μm), which have been extensively monitored in the air in epidemiological studies, especially in the domestic and occupational sector. Ultrafine particles are believed to contribute to (human) morbidity, cardiovascular and cardio-respiratory diseases (Pope et al., 2008; Simkhovic et al., 2008). Hypothesized is that this is because of their high number concentration, high surface area and inflammatory potential (Oberdörster G., 1995). Since ultrafine particles, sometimes defined by 'ambient and laboratory-generated nanosized particles' (Oberdörster G. et al., 2005a), are in the same size range as (engineered) nanoparticles these latter particles are believed to have also the potential to induce negative health effects on humans and other organisms. So how safe are these new materials?

The impact of ultrafine particles only, solely looking at particle size, on the aquatic environment and its wildlife has not been reported in literature yet. The past years the focus has extensively been on exposure to ultrafine particles (UFPs) from traffic emission (source of 60% of total atmospheric UFPs in the UK) and industrial and commercial combustion processes (source of 23% of UFPs in the UK) and inhalation by humans (Borm et al., 2006).

Studies have been performed with, for instance, powdered black carbon materials like soot or activated carbon, which in potential could contain particles in the ultrafine particle size range (20-300 nm) and cause adverse health effects on organisms (Nowack & Bucheli, 2007). The focus was mainly on black carbon behavior, fate and effects in the aqueous environment, including biota (Jonker et al., 2004; Millward et al., 2005; Jonker & Suijkerbuijk, 2009 (not yet published at this moment)). These papers report negative health effects on various organisms due to black carbon exposure. Since the precise particle size range of the activated carbon materials used was not that relevant and the focus was not on the ultrafine aspect, the particle size range has never been determined for the carbon materials which had unknown size-specific specifications, especially for the lowest size boundaries. So therefore it not yet known if there were any ultrafine particles present in these exposure test set-ups and if these particles were responsible for the effects found.

Despite that ultrafine particles present in the air have been linked to (human) negative health effects the subject of ultrafine particles in the aquatic environment has never been a real issue or has never caught the full attention of researchers and policy makers. The most probable explanation is that there have never been very clear observations of environmental impacts related and these materials have not been detected *in situ*. Looking at its carbonaceous, inert character it was very assumable that these types of material can hardly pose a threat to the aquatic environment. Also, besides this it is hard to distinguish natural occurring particles from the anthropogenic ones, not only in the air but also in aquatic media, making risk assessment more challenging.

It is since 2004 that the topic about engineered nanoparticles (sometimes referred to as manufactured nanoparticles) is receiving the attention from environmental managers and policy makers. In the same year the 'Royal Society and Royal Academy of Engineering', founded in 2003 in the United Kingdom to advise the government on this issue, published a comprehensive report about the opportunities and challenges of the upcoming nano-industry (The Royal Society, 2004). After this publication the scientific scope on this theme grew tremendously, with academic and even government reports about the balance of the benefits and novel risks (Oberdörster G. et al., 2005b; Tsuji et al., 2005; Maynard, 2006). It may be the same nanotechnological products that put risks to human health playing a role in possible toxicological and pathological risks to the environment (Nel et al., 2006). However, the knowledge on harmful effects of nanoparticles is very limited and almost non-existent in aquatic animals (Moore, 2006). Besides that it is inevitable that these particles eventually end up in the environment (Moore, 2006; Tiede et al., 2008). Specific numbers of nanoparticle production, their fate and occurrence in the environment are still sparse (Behra & Krug, 2008). It was just recently that, for instance, the European Commission started inventorying the production of nanoparticles per member country (Schmid & Riediker, 2008).

1.4. Assessing nanoparticle risk

The rapidly expanding nano-industry and product use, the expected increase of nanoparticles ending up in the environment and the unexplored area of the potential and hypothesized novel risks these relatively new materials could have on the (aquatic) environment, all together make this topic an interesting and necessary field of study. Despite all the turmoil it is still the question if nanoparticles will or do already play a distinct part in environmental toxicity, since no serious concentrations have been reported yet and the fate and effects of these particles is still largely unclear.

This thesis (generally) focuses on the risk assessment of carbonaceous nanoparticles, estimated as one of the most naturally occurring and industrially produced nanoparticles, in the aquatic environment. It will not only give more understanding of the issues involving potential risks of carbon nanomaterials, their fate and behavior in aquatic media, but also defines the problems and challenges relating the assessment of nanomaterials in general. At last, recommendations are given aiming on the needs in future characterization and assessments of these materials concerning the aquatic ecosystem.

2. CARBON NANOPARTICLES

2.1. *Introducing: carbonaceous nanoparticles*

Besides metals, like gold (Au) and silver (Ag) and metal oxides, like titanium dioxide (TiO₂) and silicon (SiO₂), nanoparticles made of carbon form a group of produced and applied engineered nanomaterials. The physical, chemical, and electronic properties of carbonaceous nanomaterials are strongly coupled to carbon's structural conformation and, thus, its hybridization state (Ajayam, 1999). Different mutable hybridization states, depending on bonding relationships with neighboring atoms, account for the differences among carbon's bulk configurations. To put it boldly, at high heat or pressure carbon takes the diamond configuration, at lower energies the carbon molecules adopt a planar conformation and forms monolayer sheets (Mauter & Elimelech, 2008). Besides their super adsorbing capacity, outstanding thermal, chemical stability, hydrophobicity and porous structure, carbon nanomaterials are especially used to reinforce other materials (compositions), making them stronger, less heavy and electrically active at the same time (Lu & Chiu, 2008). Fig. 1 shows a broad range of different carbonaceous nanoparticles, natural occurring as well as engineered ones, which can be divided into different categories. All have a graphene-like structure as a basis, but have other properties due to changes at the nanoscale level of configuration. In the next section only the most familiar and produced carbon nanoparticles appearances are elucidated.

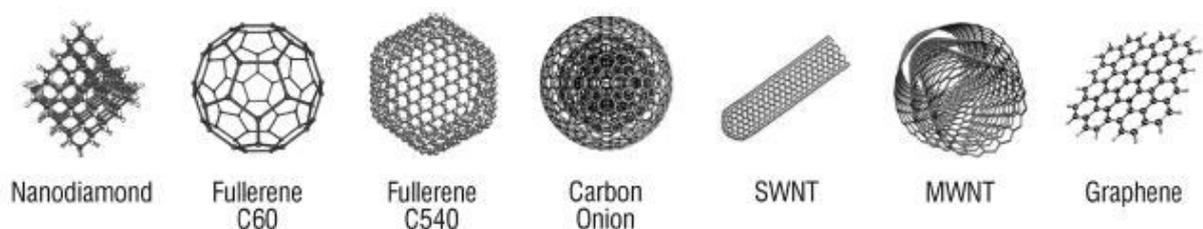


Figure 1. Hybridization states of carbon-based nanomaterials (Mauter & Elimelech, 2008). SWNT: single-walled nanotubes, MWNT: multi-walled nanotubes.

2.2.1. *Fullerenes (C₆₀)*

C₆₀ fullerene, a very hydrophobic, stable nanoparticle composed of 60 carbon molecules configured in a cage-like structure comprised of twelve 5-member rings and an unspecified number of 6-member rings, has generated a great deal of interest because of its structural properties and chemical behavior (Blickley & McClellan-Green, 2008). Fullerenes are naturally non-ionogenic but, under select conditions, have the ability to acquire a charge (Brant et al., 2005b). Fullerene molecules are strong electron acceptors that can interact with electron donors such as water to cause the formation of a hydrated shell (Brant et al., 2006). It is known, due to its hydrophobicity, to aggregate (or cluster) in water-like media.

Thanks to its discoverer Richard Buckminster Fuller and its shape the C_{60} molecule was given the name 'buckminsterfullerene' or simply 'buckyball'. The diameter in the nano-range of a fullerene particle influences its optical, mechanical, elastic and thermal properties. The smallest fullerene in a stable pentagon shape has a diameter of 0.7 nm (Mauter & Elimelech, 2008).

Fullerenes, which are already produced in large quantities (even up to 1,500 tons a year), are mainly used in polymer-fullerene combinations as thin films, in electro-optical devices and biological applications (Bosi et al., 2003; Klaine et al., 2008). It is possible to entrap metal atoms into the fullerene cage obtaining 'endohedrals metallofullerenes', which can be useful as radiotracers in magnetic resonance and X-ray imaging (Bosi et al., 2003) and as anti-tumor growth application in cancer therapy (Nielsen et al., 2008). The sphere of the fullerene molecule can be modified (functionalized) by adding carboxyl groups (e.g., -OH, -COOH and -NH₂) creating new fullerene derivatives possessing not only different physical and chemical properties, but also highly dissolvable (Fig. 2) (Bosi et al., 2003). The lipophilicity of the fullerene sphere can be helpful for interactions with the active site of various enzymes, HIV protease (HIVP) which they can enter (Friedman et al., 1993), and makes C_{60} able to move into biological membranes, destabilizing them. These actions could have a role in the antibacterial activity found for several derivatives (Bosi et al., 2003), which is discussed in chapter 4.

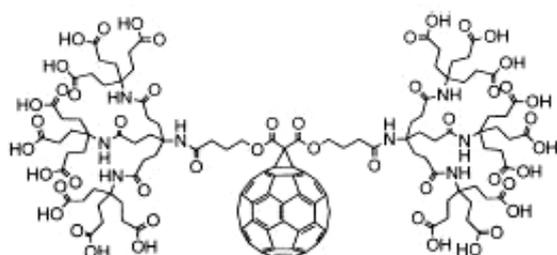


Figure 2. A fullerene derivative. This figure shows a dendrimeric functionalized fullerene derivative bearing 18 carboxylic groups, reaching a high level of solubility in water (Bosi et al., 2003).

Natural occurring fullerenes can, for example, be found in volcano dust and ashes from wildfires. Some part might even be brought to earth by comets or asteroids. But the majority present on earth is believed to have been derived from algae matter (Becker et al., 2001). This implies that life on earth has been exposed to low concentrations of fullerenes for hundred of millions of years.

2.2.2. Carbon nanotubes

Carbon nanotubes, which were discovered in 1991, are the one-dimensional analogues of non-dimensional fullerene molecules and could be appointed to the same group. Basically, a nanotube is a micrometer-scale graphene (a planar fullerene sheet with carbon atoms arranged in a hexagon pattern) sheet rolled into a cylinder of approximately 1.5 nm in diameter and capped with a spherical fullerene (Maynard, 2006a; Mauter & Elimelech, 2008). Normal bulk graphite, consisting of various graphene sheets, is a semiconductor and the orientation of the rolled graphene sheet determines its electronic properties. Nanotubes can have single or multiple dimensions and walls.

Isolated single-walled carbon nanotubes (SWCNTs) (1 graphene sheet) of small diameter exhibit metallic, semi-metallic, or semiconducting behavior (Mauter & Elimelech, 2008). Just as for the fullerene the smallest stable diameter of SWCNTs is 0.7 nm (Mauter & Elimelech, 2008). Around 2011, the annual worldwide production of SWCNT is estimated to exceed 1,000 tons (Klaine et al., 2008). Generally, carbon nanotubes are, just like fullerenes, produced in quantities up to 1,500 tons a year (Klaine et al., 2008).

Double-walled carbon nanotubes (DWCNTs) and multi-walled carbon nanotubes (MWCNTs) are one-dimensional analogues of carbon onions (Fig 1.). Multi-walled nanotubes do not have the conducting properties of the single-walled nanotubes, but own the semiconducting characteristics of bulk graphite (Mauter & Elimelech, 2008).

Nanowires, which can be embedded in fullerenes as well as nanotubes, are one-dimensional strings of carbon atoms threaded through a carbon nanotube. Carbon nanowires, as quasi-one-dimensional nanomaterials, have a similarly attractive property compared to carbon nanotubes, such as high aspect ratio, high conductivity, and high elastic modulus (Cheng et al., 2006).

Nanosheets or nanofilms are layers of graphene, usually about one to seven layers thick (Zhao et al., 2006).

Unlike fullerenes there is too little evidence to assume that carbon nanotubes occur naturally. Researchers have claimed to have found MWCNTs in a Greenland ice core (Esquivel & Murr, 2004) and hollow carbon fibers in samples from an oil well (Velasco-Santos et al., 2003), but there remain serious questions about the validity of these pieces of evidence, since images made by electron microscopes lack a clear high resolution. Plus there is still no evidence that proves that SWCNTs can be synthesized by nature (Velasco-Santos et al., 2003).

2.2.3. (Ultra) Fine particles

There are very fine carbonaceous bulk materials which could contain very small percentages of nanoscaled particles. One of these barely mentioned types of materials are the black carbons, like soot and activated carbon. Soot, as a (fossil fuel) combustion product, is abundantly present in the air near freeways and industry. Wildfires, also a source of black carbon, are usually not intentionally produced by humans. Also the incomplete combustion of biomass resides in carbonized residues in the nanoscale. Activated carbon on the other hand is manufactured at high temperatures in special ovens. Since the powdered versions of activated carbon consist of very small particles, some manufacturers only set an upper boundary, which leaves the suggestion that the smallest particles could be in the nano-range. For example, 90% of Desotec's *Organosorb 200-1* activated carbon particles is smaller than 0.074 mm. Since it has been shown that the addition of activated carbon to contaminated soils could be a promising remediation technique to sequester pollutants (Cho et al., 2007) the environment and its biota could be exposed to nanosized activated carbon particles. Chapter 4 explains that some nanoparticle aggregates could have sizes in the ultrafine-particle range.

2.3. Carbon nanoparticle risk assessment

For most engineered nanoparticles no toxicity, emission and environmental measurement data are available. There has been some toxicity testing performed using carbon nanomaterials, which for the most part has consisted of research on fullerenes and nanotubes. The behavior and fate of carbon nanoparticles, which are considered persistent, in the aquatic environment is also just recently being investigated. The next chapter will give more insight in this matter.

3. CARBON NANOPARTICLES: BEHAVIOR & FATE

3.1. Carbon nanoparticles: understanding emission effects

It is inevitable that engineered nanoparticle products and by-products eventually enter the aquatic environment, especially in view of the emerging large-scale nanomaterial production worldwide. The most likely causes are spilling during shipping and handling (The Royal Society, 2004; Moore, 2006), mostly unintentional or by accident. Besides industrial related spilling and wastes the use of personal-care products, like cosmetics and the washing of nanoparticle containing clothes, will be a growing continual source of nanoparticles entering waterways (Daughton, 2004). Therefore it is assumed that there are already many nanoparticle species present in the environment. Also, for many (consumer) products, such as electronics and fuel cells, is it still unknown whether some of these materials may leak out nanoparticles or be worn off over the period of use (Oberdörster G. et al., 2005a). The concerns about adverse effects should therefore not only aim at the health of humans or large wildlife, but also at the basis of many food chains. Benthic flora and fauna could seriously be affected by exposure to nanoparticles, despite that these are basically inert carbon materials from the start of their lifecycle. So the expected increase of widespread carbon nanoparticle use and accompanying emissions to the environment makes it important to understand their potential harmful effects and fate.

This third chapter attends to the behavior and the fate of carbon nanoparticles in aquatic systems. It will provide a more detailed look on various nanoparticle physiochemical processes which could take place in aquatic media and how other present natural and xenobiotic particles interact with these nanomaterials.

3.2. The nanoparticle way

Production processes have a great impact on the final properties of nanoparticles, like variations in shape, size and surface chemistry. It has been reported that property variations can have a great influence on the impact of a given nanoparticle (Christian et al., 2008). But not only these manufacturing-related factors play a role in the behavior and fate of (pristine or pure) nanoparticles ending up in the aquatic environment, also the (natural) materials present and environmental conditions are influencing the behavior of these nanomaterials out in the open. Because their widespread use has just begun, mechanistic studies involving carbonaceous nanoparticles in water are recently available, and therefore less is known about their routes to and fate in aquatic systems (Chen et al., 2008). For nanoparticles to be able to pose a risk there must be both a potential for exposure and a hazard that results after exposure. Fig. 3 shows the routes in which engineered nanoparticles most likely enter the environment from the anthroposphere, the part of the environment that is made or modified by humans for use in human activities and habitat.

To quantify the stability of (carbon) nanoparticles in the environment the stability of their suspension and their tendency to aggregate or interact with other particles has to be predicted (Mackay et al., 2006). To gain more insight in these processes and conditions the next section begins with the introduction of natural colloids first.

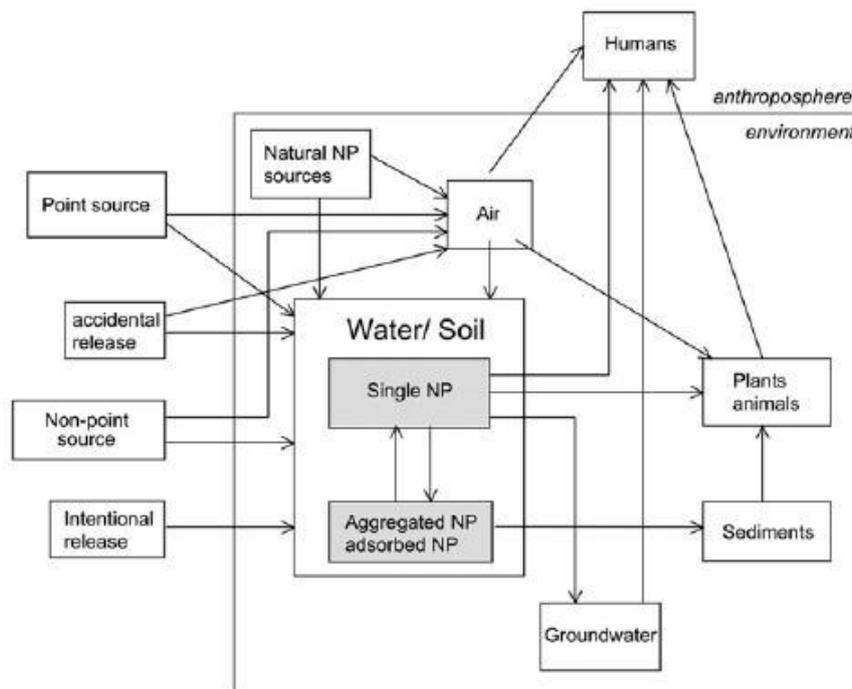


Figure 3. Nanoparticle pathways from the anthroposphere into the environment, reactions in the environment and exposure of humans and wildlife. In (soil)water carbon nanoparticles can occur in various forms (Nowack & Bucheli, 2007).

3.2.1. Natural colloids

As stated earlier, nanoparticles are nowadays still officially defined as particulate matter that is smaller than 100 nm. This puts them in a similar size group as that of the ultrafine particles and therefore makes them a part of the colloid particle domain (Fig. 4). In ecotoxicology colloids are solid phase materials with at least one dimension between 1 nm and 1 μm (Lead & Wilkinson, 2006; Baalousha & Lead, 2007). Colloids have polymeric, polyfunctional and polydisperse properties and include three major types of compounds: humic substances, large biopolymers and inorganic colloids, such as polysaccharides and peptidoglycans (Nowack & Bucheli, 2007). Although the knowledge of the structure and the environmental impact of natural occurring colloids have significantly increased in recent years their precise function and composition are still poorly defined (Nowack & Bucheli, 2007). Colloids have been present on earth for millions of years, produced by various natural processes. Most anthropogenic colloids find their origin mostly from (industrial) combustion processes and are widely present in the atmosphere. Once settled they will become a part of the terrestrial and aquatic environment, binding to soils and sediments (Christian et al., 2008).

Colloids play an important, often dominant, role in the aquatic environment in processes such as contaminant speciation, transport, and bioavailability (Baalousha & Lead, 2007) and therefore are important substances which partly determine the behavior and fate of (carbon) nanoparticles.

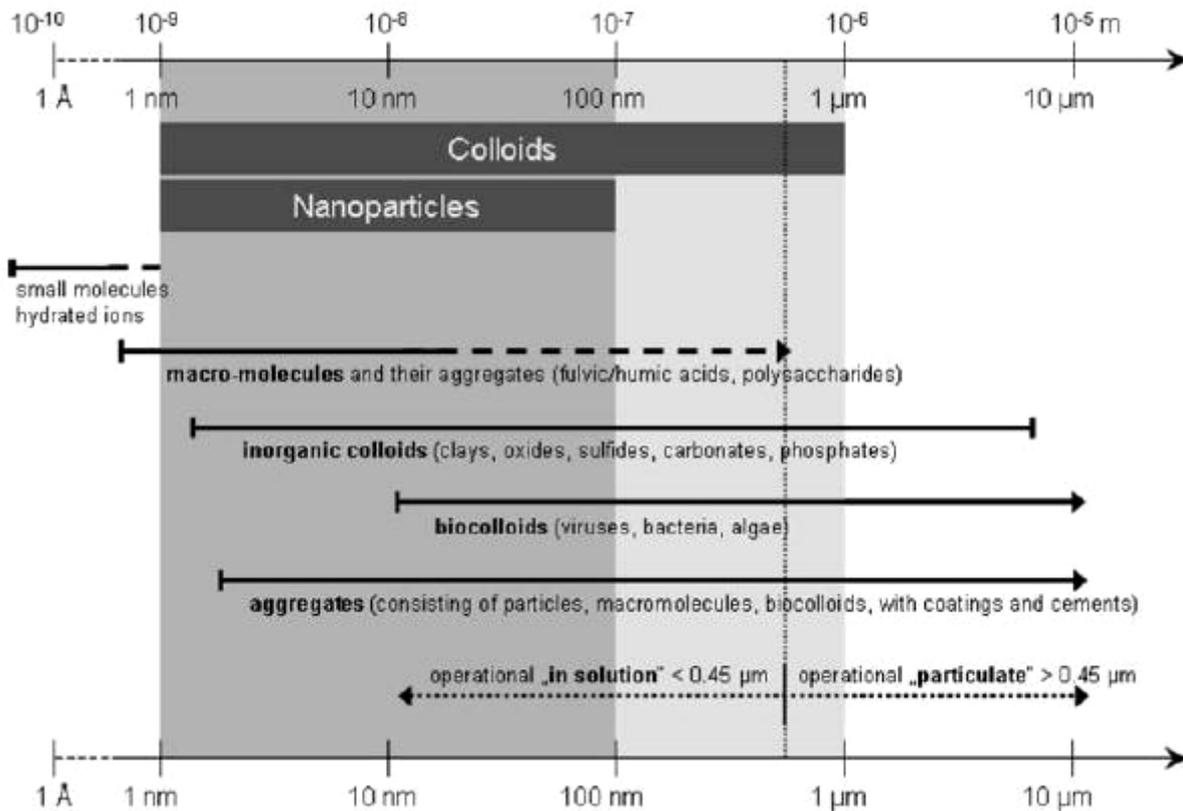


Figure 4. Size domains and typical representatives of natural colloids and nanoparticles (Christian et al., 2008).

3.2.2. Carbon nanoparticles: interactions in aquatic systems

Christian et al. state that nanoparticles could better be concerned complex mixtures than small particles: their actual behavior is often subtly different. The ability of molecules to attach to the surface of nanoparticles and exchange with other molecules already placed there indicates that careful consideration of the chemistry of nanoparticles and how this relates to their fate in surface waters and sediments is essential to predicting their final fate (Christian et al., 2008). Pristine fullerenes are insoluble in clean water, but can be solubilized in water by adding ionizable or hydrophilic groups, like short-chain peptides, other small molecules (by covalent-like bonds), metal ions, surfactants or polymers or by the formation of nanoparticle (C_{60} -fullerene) aggregates with about the size of 25 to 250 nm (Nowack & Bucheli, 2007).

Many engineered nanoparticles can lose their unique properties once they have aggregated and precipitated from a suspension (Christian et al., 2008), making their behavior quite hard to predict. C₆₀-fullerene particles may form stable colloidal aggregates through the rapid mixing with water of fullerene solutions in a variety of organic solvents or through long-duration stirring or sonication of C₆₀ in water (Brant et al., 2005a; Christian et al., 2008). Untreated carbon nanotubes are, as received from the manufacturers, not soluble in water, but can be solubilized by a similar way as the fullerenes by the interaction with organic molecules. Normally any collision between two particles will result in agglomeration and sometimes precipitation, but this process does not apply to carbon nanotubes under normal laboratory conditions (Christian et al., 2008). With the use of solvents, stirring for an extended period of time and sometimes sonication dispersion has been demonstrated. Also, for SWCNTs it has been found that dispersion is possible when certain proteins coat the nanotubes' surface (Maynard, 2006b).

The environmental impact of engineered nanoparticles is far less studied than the toxicological effects on humans. To get a better view on the behavior and fate of engineered nanoparticles in aquatic systems and to be able to assess their risks, one has to understand their interaction with components present in the aquatic environment under a variety of physical and chemical conditions (Fig. 5). And since nanoparticles end up in the environment in different forms the environmental factors can influence the fate of a particle of a certain material quite differently. These interactions could eventually result in chemical or biological modification (Nowack & Bucheli, 2007).

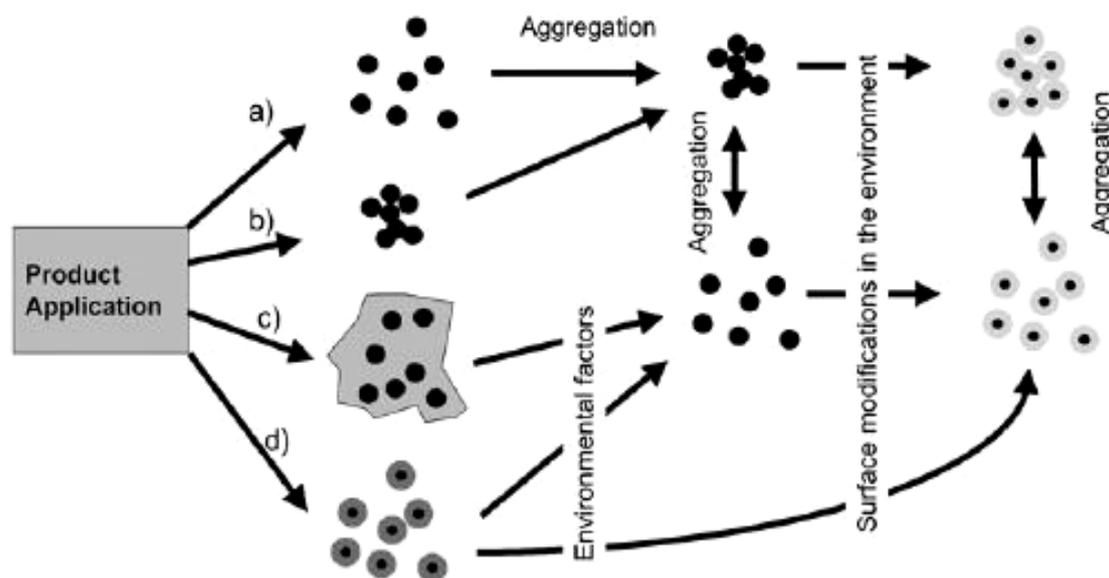


Figure 5. Release of nanoparticles (NP) from products and appearance in environment. (a) release of free NP, (b) release of aggregates of NP, (c) release of NP embedded in a matrix and (d) release of functionalized NP. Environmental factors (e.g. light, microorganisms) result in formation of free NP that can undergo aggregation reactions. Moreover, surface modifications (e.g. coating with natural compounds) can affect nanoparticle aggregation behavior (Nowack & Bucheli, 2007).

The gathered knowledge about colloids could, in potential, be of help to predict the behavior and fate of related nanoparticles in aquatic media. But since most natural aquatic environments differ strongly due to many (a)biotic factors, it is therefore not really possible to simulate a commonly used, standard aquatic environment in laboratory tests. Therefore it is very difficult to predict the precise behavior and fate of a given nanoparticle in a given complex, natural environment at this moment, based on current knowledge. Nanoparticles in the aquatic environment will, besides the exposure to many environmental conditions, meet colloids, natural organic matter (NOM), cations and possibly contaminants. Interactions with these particles will be controlled by process such as the formation of NOM surface coatings on nanoparticles (see next section), aggregation and disaggregation, aggregation structure and interactions with micro-pollutants (Christian et al. 2008). Fig. 6 shows an overview of the potential combined fate and behavior of NOM and contaminants adsorbing to engineered (carbon) nanoparticles in aquatic systems, becoming available for organisms. The upcoming sections give a better insight in these behavioral matters concerning NOM and pollutants.

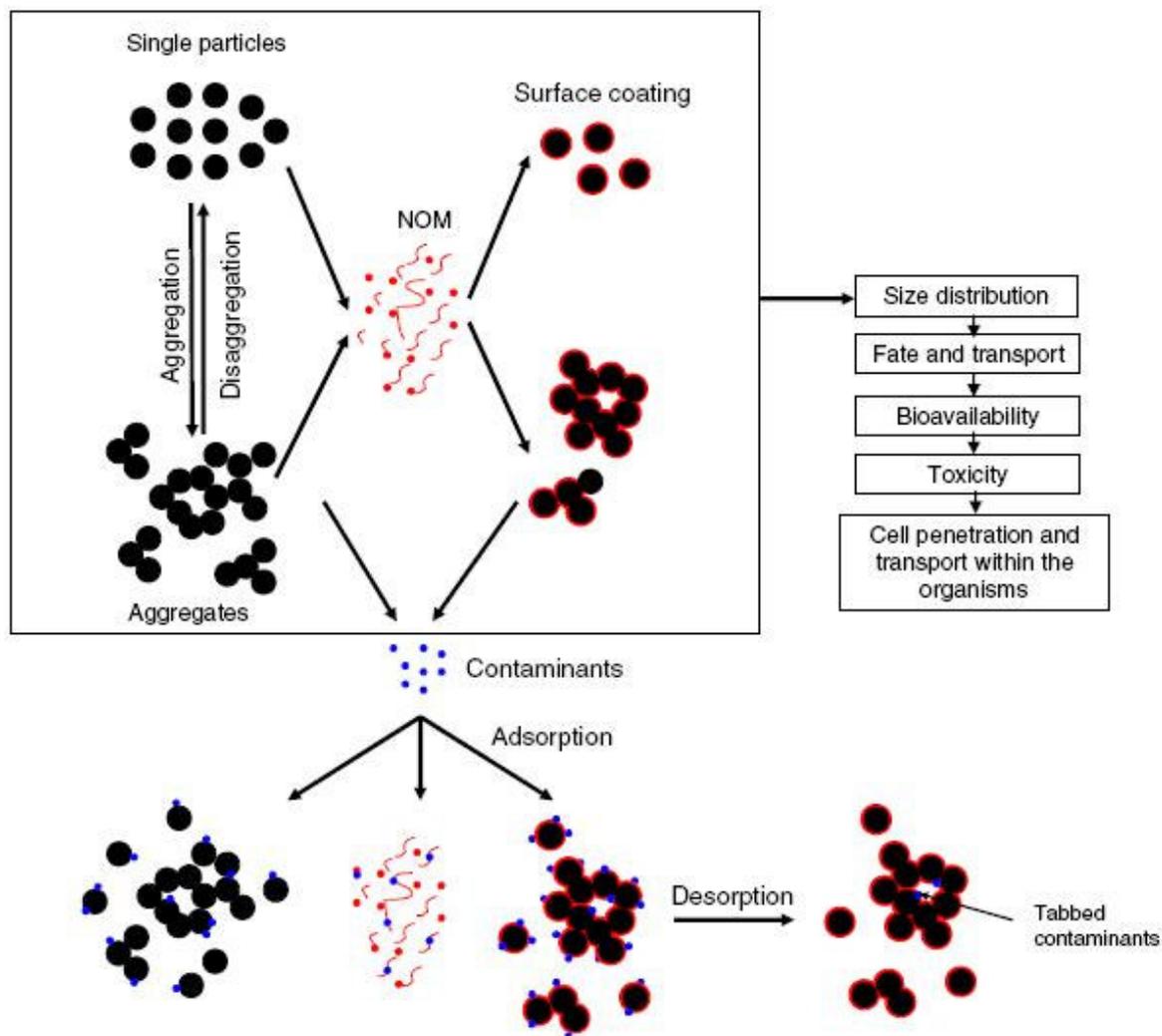


Figure 6. Schematic overview of the interaction of engineered nanoparticles with natural water components (Christian et al., 2008).

3.2.2.1. Carbon nanoparticles and contaminants

Nanoparticles entering the environment can react with many types of natural and anthropogenic substances. Since global industrial developments mankind is responsible for the presence of all kinds of unnatural substances in the environment, mostly in large concentrations. Normally, most soluble contaminants ending up in aquatic systems will bind to the surfaces of particulate or complexed matter, like humic substances (Christian et al., 2008). When engineered nanoparticles enter the aquatic environment contaminants can be sorbed onto or get stuck between aggregates of nanoparticles when adsorbed to the surface. Carbon nanotubes are found to be powerful adsorbents for a wide variety of organic compounds from water. Therefore carbon nanoparticles can have a great influence on the solid/water partitioning of pollutants in these media, when, of course, they come in large amounts (Christian et al., 2008).

In literature carbon nanotubes are reported to have been investigated in sorption processes with all kinds of organic substances, like polychlorinated hydrocarbons (PAHs) (Gotovac et al., 2007; Baun et al., 2008a), pesticides (with multi-walled nanotubes as potential solid-phase extraction (SPE) adsorbents) (Zhou et al., 2006) and metals such as copper (Liang et al., 2005) and silver (Ding et al., 2006). For this last category oxidized and hydroxylated carbon nanotubes are also very good adsorbing materials (Nowack & Bucheli, 2007).

Fullerenes, on their turn, have been used in sorption experiments with hydrophobic contaminants like dichlorobenzene and naphthalene (Cheng et al., 2005) and organic and organo-metallic compounds in aqueous solutions (Ballesteros et al., 2000). Because fullerenes can aggregate in water their interstitial spaces make good spots for contaminants to diffuse in (Cheng et al., 2005). Fullerenes were found to have a somewhat less adsorbing capacity for a wide variety of organic contaminants, like PAHs, phenols and amines, while they are very strong in removing organo-metallic compounds (Ballesteros et al., 2000).

Adsorption of metals to carbon nanotubes as well as fullerenes is pH-dependent, with increasing sorption with increasing pH found in most of the cases. Thus the properties of contaminants as well as those of the solution influence the sorption processes of these pollutants onto nanoparticles. For instance, by chemical modification carbon nanotubes become more suitable for sorption by an increase in hydrophilicity (Vermisoglou et al., 2007). The sorption of metals on MWCNTs depends on the acidity and alkalinity. With increasing pH-values metals gain better affinity and capacity, like Ding et al. (2006) showed for the behavior of metals onto natural colloids and Lu & Chiu (2008) showed for zinc ions onto MWCNTs, being excellent absorbing materials. The sorption of pollutants onto fullerenes depends also on the aggregate structure, with increasing sorption at higher dispersal.

3.2.2.2. Carbon nanoparticles and NOM

Natural organic material is ubiquitous in natural waters. Research on natural colloids showed that humic substances can adsorb to the surface of colloid particles (Baalousha & Lead, 2007), carbon nanotubes (Hyung & Kim, 2008) and fullerenes (Chen & Elimelech, 2008).

The surface coating thickness is of the order of 1.0 nm on carbon nanotubes in the presence of humic acid (10 mg l^{-1}) (Baalousha et al., 2008b). Recently it has been shown by Baalousha et al. (2008a, submitted) that NOM surface coating can change the surface charge of a nanoparticle, making it more negative, eventually affecting aggregation behavior. Believed is that the fate nanoparticles, just like natural colloids, eventually is dominated by aggregation, but significant environmental result are very scarce.

To support this hypothesis Brant et al. (2005a) found that suspensions of fullerene (salting out in solvents) become destabilized and subsequently aggregate in relatively weak (0.001 M NaCl) electrolyte, ionic solutions. This same phenomenon governs the transport and deposition of C_{60} -clusters in porous media where the increase in electrolyte concentration results in an increased affinity between the fullerene colloids and the collector surfaces, thus resulting in reduced colloid mobility (Brant et al., 2005a).

On the other hand, Chen et al. (2007) found that high concentrations of $CaCl_2$ (above 10 mM) stimulate the formation of fullerene aggregation with humic acid. These results suggest that, leaving other environmental factors out, in (weak) electrolyte natural waters typically with ionic strengths greater than 0.001 M carbon nanoparticles could easily aggregate with natural organic matter, sometimes even leading to precipitation of the materials from suspension (Blickley & McClellan-Green, 2008). Since freshwater usually has an ionic strength lower than 0.006 M (French et al., 2002) aggregation with NOM could potentially occur. So it may be expected that in sea water, usually having an ionic strength of 0.7 M (Desai et al., 1994) and probably also most brackish water, this form of aggregation certainly will take place. In line with this hypothesis, tests with artificial seawater already show aggregation of carbon nanoparticles, as will be discussed later on.

Recently, several studies have shown that nanotubes can also be stabilized in the aqueous phase by natural organic matter. In the presence of natural humic acid, which might provide sterically and electrostatically stable surfaces to SWCNTs (Baalousha et al., 2008a) and MWCNTs (Hyung et al., 2007), carbon nanotubes could be stabilized by surface coating. However, when mixed with waters sampled from a lake Hyung et al. (2007) observed that carbon nanotubes clustered (formation of floating aggregates and partial sedimentation of nanotubes), presumably due to the ionic strength and the presence of divalent cations like calcium. Apparently, sorption of humic substances enhances stability and inhibits the aggregation of carbon nanotubes to certain extend (Hyung et al., 2007). However, Baalousha et al. (2008a) added that cations, especially divalent ones like Ca^{2+} and Mg^{2+} , reduce the stability of carbon nanotubes in the absence and presence of NOM surface coating. Looking at the particles themselves the low or even absent charge on, for instance, pristine CNTs in media with neutral pH values is the main reason for their inability to form stable suspensions in water (Hu et al., 2005). The rate of aggregation formation and the increase in aggregate size expands with growing particle concentration (Phenrat et al., 2007).

Physicochemical conditions of the aqueous system, like pH, temperature (seasoning), ionic strength, the concentration of nanoparticles and natural organic matter influence the conformation and porosity of nanoparticle aggregates very significantly (Christian et al., 2008).

Nanoparticle structure controls not only their fate and behavior in an aggregation point of view, but is also believed to determine the disaggregation process (Baalousha et al., 2008a), but this has not been proven for carbon nanoparticles yet. For instance, it has been hypothesized that humic acids adsorbed to the nanoparticle surface will eventually diffuse into the aggregate pores (Christian et al., 2008) making the aggregate fall apart. But again, more research is needed to investigate possible disaggregation of nanoparticles in the natural environment and the conditions plus substances play an important role in this.

3.3. Carbon nanoparticles: assessing fate

Understanding the interactions between carbon nanoparticles and contaminants and natural colloids, as representatives of (natural) occurring substances, is not only a crucial point in the risk assessment of potential impacts of these particles but also in the comprehension of environmentally (sustainable) concentrations. The exact nanoparticle behavior in the aquatic environment will largely depend on nanoparticle properties, the type and concentrations of (organic) matter present and solution conditions in a complex manner. The coating of the surface, aggregation and disaggregation processes will eventually, for the most part, determine the bioavailability, fate and behavior of nanoparticles. The stabilization of nanoparticles by aggregation or surface coating may keep them within the water column, increasing their transportation distance at the same time.

The formation of aggregates and possible precipitation affect the concentrations of free nanoparticles. This precipitation behavior is most likely responsible for the settling of nanoparticles to the sediment and thus having the opposite effects of stabilization, reducing transport through the water phase. These two processes will eventually to a large extent decide which type of organisms will be exposed to a certain nanoparticle: those mainly residing in the pelagic zones or benthic organisms living in or on the sediment. Disaggregation will result in the formation of small aggregates that can be resuspended and may travel significant distances. Not only can contaminants, together with NOM, adsorb to nanoparticle surfaces. Also present nutrients can get attached to the particle surface and could, in this way, function not only as nutrient reservoirs for micro-organisms living in these clusters but also as available food source for particle feeding organisms (Christian et al., 2008). So, in this point of view (stabilized or precipitated) anthropogenic nanoparticles could function as nutrient transporters, increasing the bioavailability and maybe even the sustainability of life. In nutrient scarce environments, the opposite could be the case.

In the perspective of a contaminant, either nanoparticles with binding affinity for that specific contaminant can lower the contaminant's bioavailability in the water column, and thus have the potential to act as sequestering substances. This has, for example, been found for carbonaceous materials, such as soot and activated carbon (Zimmerman et al., 2004; Jonker et al., 2005; Cornelissen et al., 2006), which could also have a size fraction in the nano-range. Or nanoparticles could, in this way, be responsible for an increase in bioavailability of a specific contaminant for benthic organisms due to its insolubility and precipitation to the benthic zone (Brant et al., 2005b).

One can also look at this the other way around. When a hydrophobic contaminant sorbs onto a carbon nanoparticle or gets stuck between carbon nanoparticle aggregates it potentially could get more dispersed and be transported far further than without these interactions. When this situation occurs the bioavailability of that contaminant for benthic organisms living at spots where there normally will be a large presence of that given contaminant will be lower. On the other hand, nanoparticles are not necessarily released in the environment as single particles (Fig. 5 + 6), because in many applications nanoparticles are embedded in a matrix (Nowack & Bucheli, 2007). Nanoparticles in this appearance could also behave quite different compared to single or aggregated nanoparticles, making risk assessment even more difficult.

These findings all together suggest that the dispersal of carbon nanomaterials in the natural aquatic environment might occur to a higher extent than predicted in advance, based only on the hydrophobicity of these materials. Especially salinity, the possibility to form aggregates and the presence of substances like contaminants and NOM will largely determine the fate (stabilization or precipitation) of nanoparticles (single particles or aggregates) in the aquatic environment.

The various interactions in complex media need further investigation, just to gain more insight in how particles behave in these situations. Also the fact that there are still no reports about measured concentrations of (carbon) nanoparticles in the aquatic environment (Nowack & Bucheli, 2007) makes the risk assessment of these materials imperative. Because it is without doubt that engineered nanomaterials eventually end up in the environment the next chapter focuses on the (potential) adverse effects of organisms exposed to manufactured nanoparticles.

4. POTENTIAL UPTAKE ROUTES AND EFFECTS ON AQUATIC ORGANISMS

Carbon nanoparticles, which are almost insoluble in water in their pristine form, tend to aggregate in freshwater and seawater due to ionic strength. Also, these particles are greatly influenced by the presence of pollutants, natural organic matter and other natural occurring particles, such as colloids (Fig. 6). The environmental conditions thus determine the fate and the ways of how a given nanoparticles will be presented to aquatic wildlife. Assessing the risks of engineered nanoparticles is necessary because there are some serious ecotoxicological concerns in case of exposure: not only may the expected widespread application of carbon nanomaterials lead to substantial production volumes and consequently to increased emissions into environmental compartments, like groundwater and sediments. Also, the physical and chemical processes in the environment may alter the properties of the carbon nanoparticles, potentially making them more harmful than before emission. Carbon nanoparticles have a low biodegradability, are lipophilic by nature and could therefore eventually accumulate along various food chains, such as the human food chain (Fig. 3).

For some nanomaterials there are already some indications that they induce adverse effects to certain organisms in certain circumstances, but by far most manufactured nanoparticles have not been characterized yet. Looking at size only, nanosized particles could already pose a threat by partitioning into cell membranes and the usage of selective cellular transport mechanisms, transferring them further into organisms' systems (Oberdörster E., 2004). To gain more insight in the exposure routes of carbon nanoparticles and exposure effects on aquatic organisms this chapter focuses on (potential) impacts of carbon nanoparticles on environmental relevant species.

4.1. Uptake routes

Potential uptake routes of carbon nanoparticles (including aggregates) by aquatic biota include direct ingestion or entry across epithelial boundaries, like gills, olfactory organs (for smelling) or the body wall. At the cellular level, prokaryotes like bacteria may well be protected against the uptake of nanoparticles, since they do not have the mechanism for the bulk transport of colloidal particles across the cell wall (Moore, 2006). For eukaryotes this situation differs considerably, since they have highly developed mechanisms for cellular internalization of nanoscale and microscale (100 nm to 100 µm) particles, endocytosis and phagocytosis respectively (Moore, 2006). In invertebrates, the immune system, gut epithelium and digestive glands are likely to be targeted (Moore, 1990). More details on experimentally found exposure effects in various organisms are presented in the next section.

4.2. Hazard identification: potential adverse effects on organisms

Because carbon is an inert material, normally one would expect that carbon materials in water do not have harmful, or at least minimal, effects on organisms' health. But in view of the fact that nanomaterials often have different properties than their larger counterparts it is necessary to assess carbon nanoparticles for (new) potential risks. And since nanotubes and 'buckminsterfullerene' (C₆₀) are the most used carbon nanomaterials in research the following section summarizes the found effects in a few relevant aquatic species after carbon nanoparticle exposure. But still, available data is still limited and, as a result, not much is known about nanoparticle impact on biological systems.

4.2.1. Carbon nanotubes

Since nanotubes are not very commonly used in toxicity testing in aqueous media the following section also summarizes some nanotube exposure experiments with the pulmonary tract of rats.

Acute toxicity

No acute mortality cases were found during the exposure of juvenile rainbow trout (*Oncorhynchus mykiss*) relating to the 10-day exposure period to SWCNTs (1.1 nm) dissolved in sodium docedyl sulphate (SDS) (Smith et al., 2007). In view of the found gill injury and respiratory distress the authors expect that this species does not have the cardiovascular fitness to survive in the wild at continuous exposure. It might successfully tolerate exposure the first few days, but eventually after 1 or 2 weeks it is expected to loose the fight for survival.

In another study water fleas (*D. magna*) were exposed to SWCNTs coated with lysophosphatidylcholine (LPC) (Roberts et al., 2007). After sonication nanotubes were completely dissolved in this lipid. According to the authors solubilizing SWCNTs with lysophospholipids is more effective than with nucleic acids and surfactants and far more effective than with proteins, predicting a promising future application. Total mortality was found at 0.20 mg l⁻¹ of LPC-SWCNTs (1.2 nm) after an exposure period of 96 hours.

Oxidative stress responses

Oxidative stress is a common pathway of toxicity and disease. An organism can undergo oxidative stress through several different mechanisms (Usenko et al., 2008): First, it may be directly induced by an oxidizing agent, such as H₂O₂. Second, it may be produced through the induction of cytochrome P450 proteins. Third, a xenobiotic substance may inhibit the production of antioxidant molecules, such as glutathione (GSH) that function to maintain oxidative balance. GSH is an endogenous tripeptide enzyme and known free radical scavenger and is therefore important for detoxification of metabolites and reactive oxygen species (ROS) associated with chemical exposure and disease.

Oxidative stress is linked with lipid-peroxidation, the oxidative degradation of lipids. Exposure to carbon nanoparticles has demonstrated lipid peroxidation in mammal studies (Shvedova et al., 2003)

Results from several *in vivo* studies using intratracheal (directly into the respiratory tract) dosing of carbon nanotubes in rats, show significant acute inflammatory effects in the lungs and sometimes even mortality (Warheit et al., 2004). The used exposure doses were relatively high (1.5 to 3 mg kg⁻¹ of SWCNT (1.4 nm)). It was observed that the nanotubes aggregated and were obstructing the airways. Despite that this way of exposure does not really correspond to a realistic approach concerning the subject of this thesis (no aquatic medium involved) it does give an indication of potential effects when SWCNTs enter the pulmonary tract of rats.

In vitro studies showed similar inflammatory effects and oxidative stress for SWCNTs (Kisin et al., 2007) and MWCNTs (Witzmann & Monteiro-Riviere, 2006) in cells. Concluding, these rat studies may indicate that carbon nanoparticles could also have adverse effects on the respiratory system, like gills, of aquatic organisms.

In another study, statistically significant increased levels of GSH were found in the gills (28%) and livers (18%) of juvenile rainbow trout (*O. mykiss*) compared to solvent controls (Smith et al., 2007). Together with a found decline in thiobarbituric acid (TBARS) in the gills, liver and brain compared to controls this indicates an oxidative stress response. The authors also hypothesize that the found mucosa damage in the gut lumen can be directed to nanotube aggregates, which were present in the medium especially at low SWCNTs concentrations.

Other effects and behavioral changes

Juvenile rainbow trout (*O. mykiss*) showed signs of gill irritation and mucus secretion during exposure to SWCNTs (0.1 to 0.5 mg l⁻¹) dissolved in SDS, while in the same experiment exposure to controls with water and solvent only did not (Smith et al., 2007). Black granular deposits, which were precipitated SWCNTs, were found in the gills of exposed fish and are therefore most likely responsible for the found increase in gill pathology. Fish in the 0.5 mg l⁻¹ solutions showed a continued rise in ventilation rate with exposure time. Fish in the 0.1 and 0.25 mg l⁻¹ treatments showed sustained increases in ventilation above that of the controls until the end of experiment. Also, liver and brain pathology was found after SWCNTs exposure. No major hematological or blood disturbances were observed. Remarkably, solvent control organisms showed some increase in gill and brain pathology compared to freshwater controls.

Oberdörster E. et al. (2006) investigated the ingestion of SWCNTs by the suspension-feeding worm *Caenorhabditis elegans*. SWCNTs aggregates (10-200 nm) moved through the digestive tract and were not absorbed by the animal. Earlier, Oberdörster G. et al. (2005a) detected solubilized (bound with a synthetic peptide) as well as unsolubilized SWCNTs in fecal matter from the digestive tract of exposed fish.

In the LPC coated SWCNT study Roberts et al. (2007) observed the adsorption of these particles to the external surface of *D. magna*, which in some cases lead to immobility. Also the authors hypothesized that daphnids are able to change the solubility of LPC-SWCNTs by ingestion and further metabolism, involving digestive enzymes, and that LPC is a potential food source for these filter feeding organisms. Several findings, including nanotubes in daphnid gut tracts (Fig. 7) and precipitated nanotubes in the test systems, indicate that these daphnids do ingest and even maybe egest these particles. It shows that simple ecological relevant species could have influence on the physical properties of nanoparticles and eventually their fate in the environment.

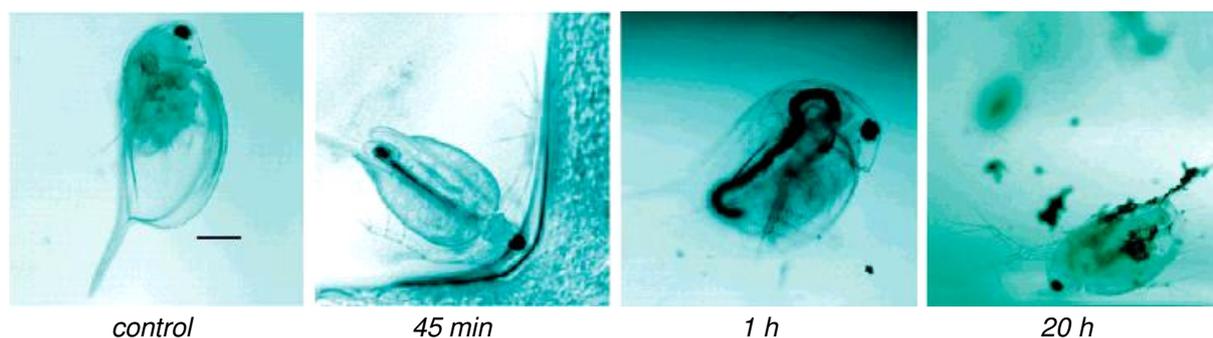


Figure 7. Time course micrographs of *D. magna* exposed to 5 mg l^{-1} of lysophosphatidylcholine coated single-walled carbon nanotubes (LPC-SWCNTs). The concentration of 5 mg l^{-1} refers to the concentration of SWCNTs in solution. Note the large numbers of tubes filling the gut tract at 45 min and 1 h. Also note the clumps of precipitated tubes around the daphnid after 20 h of exposure (bar = $200 \mu\text{m}$). (Roberts et al., 2007)

Kashiwada (2006) observed the body distribution of fluorescently-labeled polystyrene nanoparticles in transparent Japanese medaka (*Oryzias latipes*) after exposure. The gills of these medakas showed the greatest increase in particle concentration, shortly followed by the intestine.

Antimicrobial properties

It has been found by Kang et al. (2007) that highly purified SWCNTs exhibit strong antimicrobial activity. The Gram-negative *Escherichia coli* bacteria suffered cell-membrane damage resulting from SWCNT-aggregates ($0.75\text{-}1.2 \text{ nm}$; 5 mg l^{-1}), resulting in bacterial death. Recently, Kang et al. reported that the diameter of the nanotube is a key factor governing their antibacterial effects and that the main reason for cell cytotoxicity is cell-membrane contact, which lead to serious damage (Kang et al., 2008). Furthermore they demonstrated that SWCNTs are more toxic to bacteria than MWCNTs (both $0.9\text{-}30 \text{ nm}$), by the measurement of the expression of stress-related genes. The authors hypothesized that the enhanced bacterial toxicity of SWCNTs may be attributed to 1) a smaller nanotube diameter that facilitates the partitioning and partial penetration of nanotubes into the cell wall, 2) a larger surface area for contact and interaction with the cell surface, and/or 3) unique chemical and electronic properties conveying greater chemical reactivity.

Carbon nanotubes, having antimicrobial properties, may therefore be useful for inhibiting microbial attachment or formation on particular surfaces. However, the degree of aggregation (Wick et al., 2007), stabilization effects by NOM (Hyung et al., 2007) and the bioavailability (Brunet et al., 2008) of nanotubes will have to be considered with attention for these antimicrobial properties to be fully effective (Li et al., 2008).

4.2.2. Fullerenes (C₆₀)

Acute toxicity

Fullerene has been shown to induce toxicity in various cell culture and whole animal studies. Experiments with C₆₀-fullerenes (10-200 nm aggregates) demonstrate that the lethal concentration at 50% mortality (LC₅₀) for the freshwater crustacean *D. magna* is about 0.8 mg l⁻¹ for fullerene dissolved in tetrahydrofuran (THF) compared to > 0.35 mg l⁻¹ for water stirred fullerenes, after 48 hours (Zhu et al., 2006).

In the same experiment freshwater adult male fathead minnow (*Pimephales promelas*) were exposed at concentrations of 0.5 mg l⁻¹. 100% Mortality was found in the THF-fullerene exposed fish between 16 and 18 hours. These findings show that solvents used for dissolving nanoparticles are toxic themselves and biases acute toxicity tests. To exclude the negative effects of solvents on organisms, toxicity tests are therefore often performed with water-stirred fullerenes. But this will deliver some new problems as well.

Later, Oberdörster E. et al. (2006) found water-stirred fullerene (10-200 nm aggregates) uptake by *D. magna* (maximum: > 2 mg l⁻¹ / mg tissue) after an exposure period of 48 hours. Unfortunately, the researchers did not define the uptake-route and the fate of the particles in the daphnid's body (or their secretion in the feces). Mortality found was too low to determine 48h and 96h LC₅₀s from. A chronic 21-day life cycle assay exposure period was performed at a concentration up to 5 mg l⁻¹ and resulted in 40% mortality at day 5.

The same authors also reported the precipitation of fullerenes (35 mg l⁻¹) in artificial seawater due to high salt content, which supports the precipitation hypothesis in section 3.2.2.2. for waters with weak or high ionic strength. The highest fullerene-soluble concentration possible in the test set-ups was 22.5 mg l⁻¹.

Further, exposed for 48 and 96 hours were marine benthic harpacticoid copepods (species not mentioned), which barely showed mortality and did not show different behavior compared to control groups (Oberdörster E. et al., 2006).

Also for the freshwater benthic crustacean *Hyallela azteca* (scud) no acute toxicity levels could be detected since the amount of C₆₀ available for this study was limited. Fullerene concentrations could not be higher than 7 mg l⁻¹ and that was well below LC₅₀ values. Again, fathead minnows were exposed in this exposure study. The results show that fullerenes cause no significant acute toxicity in this species at 0.5 mg l⁻¹ and an exposure period of 96 hours.

At last, the authors selected freshwater Japanese medaka to research sub-lethal toxicity of fullerene by determining levels of protein-expression of certain isozymes. The results concerning this species showed that fullerene caused no significant acute toxicity at 0.5 mg l⁻¹ after 48 and 96 hours of exposure. Concluded was that medakas are probably a less fullerene sensitive species. Comparing these outcomes with those of the fathead minnow, also a fish species, the differences in interspecies sensibility could be emphasized.

The same solvent adjustments were implemented by Lovern & Klaper (2006), who researched the acute toxicity of C₆₀-fullerenes (0.72 nm) dispersed in THF (dissolved before use; 0.04 to 0.88 mg l⁻¹) on *D. magna* for 48 hours. For solubilized fullerenes (formed aggregates of 10-20 μm) NOEC (0.18 mg l⁻¹), LOEC (0.26 mg l⁻¹) and LC₅₀ (0.46 mg l⁻¹) values were determined. Total mortality was found at concentrations of 0.88 mg l⁻¹.

Baun et al. (2008a) investigated the effects of water-stirred fullerene as a contaminant carrier in aquatic systems. Several commonly found contaminants, mixed with fullerene aggregates, were exposed to green algae (*Pseudokirchneriella subcapitata*) and 4-day old *D. magna*. In algal tests the presence of C₆₀-aggregates increased the toxicity of the PAH phenanthrene with 60% and decreased toxicity of PCP about 2 times. Addition of C₆₀-aggregates (5-8 mg l⁻¹) reduced the toxicity of PCP with 25% in tests with *D. magna*, whereas a more than 10 times increase in toxicity was observed for phenanthrene. From these results it may be concluded that phenanthrene sorbed to C₆₀-aggregates is available for these organisms.

Oxidative stress responses

It has yet to be determined through which, if any, of the oxidative pathways fullerenes may act, but the physicochemical properties of fullerene (C₆₀) support the hypothesis that this carbon nanomaterial may induce oxidative stress responses (Usenko et al., 2008). An indication of this hypothesis can be found in recent research. In fish, the liver is the probable target organ following endocytotic transport across the hepatic blood system, followed by endocytosis into the liver. Research with cultured mammalian cells has shown that fullerenes can cause oxidative damage, which could lead to cell death, and that cytotoxicity is related to the lipophilicity of the particles (Colvin, 2003; Sayes et al., 2004; Sayes et al., 2007). Also, Oberdörster E. (2004) found that aggregated fullerenes, as lipophilic particles, are taken up into the brains of juvenile largemouth bass (*Micropterus salmoides*) and cause oxidative damage, altered stress response gene expression and depletion of GSH.

The same oxidative stress responses were found in adult male fathead minnows (Zhu et al., 2006), embryonic zebrafish (*Danio rerio*) (Zhu et al., 2007; Usenko et al., 2008), embryonic and larvae mummichog (*Fundulus heteroclitus*) (Blickley & McClellan-Green, 2008) and goldfish larvae (*Carassius auratus*) with increased liver activity measured for this species (Zhu et al., 2008). The oxidative effects of fullerene exposure are generally clear, but the exact mechanisms and cause are not precisely known up until this moment.

Intratracheal *in vivo* studies with high dosing of two water-soluble fullerenes (1.5 and 3 mg kg⁻¹) in rats did not indicate adverse pulmonary tissue effects or even mortality (Sayes et al., 2007). A sustained cytotoxic response was measured following pulmonary exposures to crystalline silica, as a positive control particle-type. However, no differences in cytotoxicity, inflammation parameters, cell proliferation or lung histopathological endpoints were measured when comparing the two fullerene species; C₆₀ and fully hydroxylated, functionalized C₆₀ (C₆₀(OH)₂₄). These results are not consistent with the previous *in vitro* study by this group, which showed that normal fullerene C₆₀ was 3 to 4 times more toxic than highly water-soluble fullerene (C₆₀(OH)₂₄).

This lack of correlation between *in vivo* and *in vitro* studies is, unfortunately, not a case on its own. This will make it hard to interpret results from experimental work and place them in the right perspective of risk assessment.

Other effects and behavioral changes

At a single concentration tested, C₆₀-fullerene aggregates (1.5 mg l⁻¹) exerted distinct developmental toxicity, among which delayed embryonic development and decreased hatching successes at *D. rerio* (zebrafish), whereas C₆₀(OH)₁₆₋₁₈ (50 mg l⁻¹), a derivative of C₆₀, did not (Zhu et al., 2007).

Oberdörster E. et al. (2006) showed that, despite that *D. magna* is still able to mold and reproduce (at reduced capacities) during fullerene exposure, the cumulative number of offspring was reduced at exposure levels of 2.5 mg l⁻¹ and 5 mg l⁻¹. But at the end of 21 days surviving daphnids were adapted to fullerene exposure, with ultimately the number of offspring per individual not significantly affected. This shows that, despite the found negative health effects and mortality of *D. magna*, some organisms have the ability to adapt to fullerene exposure. As long as the particle concentrations stay far under the earlier found LC₅₀ of 35 mg l⁻¹.

In the same report no toxicological effects could be observed for the freshwater crustacean *H. azteca*. Probably due to the relatively low fullerene concentration (7 mg l⁻¹) no adverse effects to the movement, the ability to molt, and feeding were found. The same authors stress that, at this moment, there are almost no proper ways to determine certain levels of relevant proteins in fish after exposure to fullerenes, making it hard to study accompanying adverse effects at the cellular and organ level.

Another study showed that immobility and disorientation of juvenile and adult *D. magna* could be observed during exposure to fullerenes, making them unable to swim to the surface (Lovern & Klaper, 2006). These authors also found behavioral changes in *D. magna* when exposed to fullerenes: heart rates of daphnids increased during exposure to C₆₀-fullerenes (dispersed in THF, followed by evaporation), indicating a higher metabolism compared to controls (Lovern et al., 2007). Also hopping rates increased in systems with plain C₆₀-fullerenes and C₆₀ with additional side-chains (C₆₀H_xC₇₀-H_x). During recovery only organisms exposed to this last group showed normal hopping rates.

Baun et al. (2008a) found increased uptake rates of phenanthrene and 1.7 times higher steady-state levels of this PAH in juvenile *D. magna* in the presence of C₆₀-fullerene aggregates. However, transferring these contaminated animals to clean freshwater resulted in no accumulation, indicating a full clearing process. The mobility of *D. magna* was not affected by these fullerene aggregates ($\leq 50 \text{ mg l}^{-1}$). Microscopic images showed that these aggregates were present in its digestive tract and on its surface (Fig. 8). For the algae (*P. subcapitata*) the growth-rate was inhibited up to 30% at a concentration of 90 mg l^{-1} , but reproduction was not found to be disordered.

Nanoparticle exposure is not necessary linked to adverse effects. It was found that fullerenes may act as antioxidants, preventing lipid peroxidation induced by superoxide and hydroxyl radicals in some liposomes (Wang et al., 1999).



Figure 8. *Daphnia magna* ingest nanoparticles from aqueous C₆₀-suspension (3 mg l^{-1}). Image made after 48 hours (Baun et al., 2008b).

Antibacterial properties

Very recently, it has been found that also fullerene has antibacterial properties. Lyon et al. (2005) showed that fullerene ($< 0.22 \text{ }\mu\text{m}$) in a low-salt medium associated with Gram negative bacteria (*Escherichia coli*) and Gram-positive bacteria (*Bacillus subtilis*). At C₆₀-concentrations of 4.8 mg l^{-1} both types of bacteria had reduced growth-rates (decreasing metabolisms by measured drop in CO₂ production) and a higher death-rate compared to the control groups. The authors emphasize that the real concentration available was lower because of the formation of aggregates and their inability to penetrate cell walls. Unfortunately, in this research the mechanism of toxicity was not studied. In this experiment, the precipitation of fullerene particles out of the suspension could also be observed at high salt levels.

By looking at C₆₀-fullerenes with (charged) functional groups Tang et al. (2007) found that positively charged C₆₀-NH₂ at concentrations of 10 mg l⁻¹ inhibited growth and reduced substrate uptake for *E. coli* and *Shewanella oneidensis* (both Gram-negative) bacteria. Damaged cellular structures were observed. Neutrally charged C₆₀ and C₆₀-OH had almost no negative effects on *S. oneidensis*, whereas the negatively charged C₆₀-COOH did not affect either microorganism's growth. The effect of fullerene compounds on global metabolism was further investigated by these researchers and they concluded that microbial central metabolism is robust to environmental stress inflicted by fullerene nanoparticles and that both *S. oneidensis* and *E. coli* bacteria can efficiently stop mechanical stress caused by C₆₀-NH by cell aggregation and precipitation of the toxic nanoparticles (Tang et al., 2007). This indicates that fullerenes can cause membrane stress and that some bacteria have the ability to adapt to some of above exposure situations.

Finally, just like invertebrates bacteria exposed to solvent dispersed C₆₀ also show increased mortality during toluene and THF exposure compared to water-stirred situations (Fortner et al., 2005).

5. CONCLUSIONS, REMARKS & RECOMMENDATIONS

5.1. *Experimental results: placed in environmental perspective*

Despite that particle toxicity has been studied for many years in mammals, mostly focusing on respiratory health and inflammation, the study of the ecotoxicity of nanoparticles is just recently emerging. This explains the relatively small number of papers published on this topic and apparently the few types of (carbon) nanomaterials and environmental relevant species used in risk assessment. Findings in mammal studies could, however, be helpful for ecotoxicological testing. Toxicity to mammal lung epithelial tissue could, for instance, be a reasonable representative or estimator for nanoparticle exposure to epithelial tissue containing organs of aquatic organisms, like gills, as Smith et al. (2007) found gill epithelial damage in rainbow trout. The ecotoxicological studies defined in the previous chapter have identified some potential target organs for carbon nanoparticles, oxidative stress responses and behavioral changes. Carbon nanoparticles at least attach to fish gills surfaces (rainbow trout) and may induce gill pathology. For carbon nanotubes the liver of fish appears to be an important target organ. Some fish studies did find inflammatory and oxidative stress responses in gill, brain and liver tissue, resulting in injuries. Also, nanoparticles could enter the digestive tract of fish (Japanese medaka) via water and food uptake and may therefore have the potential to enter even more different epithelial cells. Some studies report finding no adverse effects on organisms, but this does not mean that there are none. Regarding this issue, Handy et al. (2008a) state that other target organs for nanomaterials in fish are inferred from observations of toxic effects, simply because the techniques and measurement of nanoparticles in tissues are not yet routinely available. Nevertheless, available (sub-lethal) ecotoxicological studies do at least identify some commonly found adverse effects mechanisms (cellular injury, oxidative stress) similar to those found in mammalian studies, in vertebrates.

On the invertebrate level especially *D. magna*, as a OECD standard and more sensitive ecotoxicology test-species and an important organism in many aquatic food-webs, has been used in nanoparticle risk assessment frequently. Total mortality has been found for this species at a fullerene concentration of 0.88 mg l⁻¹, LC₅₀ values ranged from 0.46 to 0.8 mg l⁻¹. For carbon nanotubes only the acute toxicity of daphnids has been determined (0.20 mg l⁻¹). Additionally, these species-specific results are also another indication that nanotubes are more acutely toxic than fullerenes. Overall, not only may carbon nanoparticles have a potential negative impact on reproduction and molting rates of daphnids, exposure could also result in immobility and disorientation, making this species an easy target for predators when exposed. Filter-feeding daphnids are able to ingest single-walled carbon nanotubes, which could heavily increase nanoparticle exposure for this species. Remarkably, these simple aquatic organisms could modify (nutrient) coated nanomaterials, changing the behavior and effects of those particles in the environment drastically. As a result of biomodification processes solubilized nanoparticles could eventually precipitate and become available to different aquatic compartments, like the benthic zone.

On the other hand, for the freshwater invertebrate *H. azteca* no acute toxicity and negative effects on mobility, molting and feeding behavior could be found after exposure to 7 mg l⁻¹ C₆₀-fullerenes. These findings subside the impact of nanoparticles and underline, again, the varying sensibility among species.

It has been found that carbon nanotubes and fullerenes may have antibacterial properties. Reduced growth-rates and significantly more deaths were especially found at Gram-negative bacteria compared to controls. Some bacteria may also have the ability to adapt to carbon nanoparticle exposure, minimizing adverse effects. Further, Kang et al. (2008) demonstrated that SWCNTs were more toxic to bacteria than MWCNTs, which may indicate that the diameter of nanoparticles and the bacterial cell membrane structure are determining factors in toxicity. According to Neal (2008) the limited studies currently available fail to identify any significant effects at the microbial level of nanoparticles in more complex systems.

Remarkably, there have been no exposure-studies performed on aquatic plants, which also represent a large group of relevant species. In any case, the few studies on the ecotoxicology of carbon nanoparticles in the environment already indicate, in line with mammalian studies, that there may be some serious negative effects on aquatic organisms exposed to carbon nanomaterials, ranging from bacteria to (small) vertebrates. Also, these tests show that certain species could be more sensible (or do not even seem to experience hindrance) to carbon nanoparticle exposure than other resembling species, indicating interspecies sensibility. For small fish the sensibility of fathead minnows and Japanese medakas for fullerenes differs a lot, since medakas did not show an oxidative stress response and acute toxicity at particle concentrations at which fathead minnows did respond.

Besides these observed adverse effects also positive results of carbon nanoparticle exposure are reported, like the *in vitro* study of Wang et al. (1999), showing decreased lipid peroxidation in various fibroblasts due to fullerenes acting as antioxidants. This outcome is an indication that some organisms have the ability to adapt to carbon nanoparticle exposure, just as has been shown for daphnids exposed to C₆₀-fullerene. Since most of the ecotoxicological studies on carbon nanoparticle exposure are often alike, maybe new tests and accompanying endpoints will gain other, more positive insights in nanoparticle exposure of organisms.

One has to keep in mind that the tested concentrations are often mechanistically and not based on values measured in the environment. Excessive amounts of carbon nanoparticles could simply be harmful, like every other substance will be eventually. Therefore environmental carbon nanoparticle measurements, which are still not reported in literature up until this moment, have to prove how serious the real situation is in relation to the effects found at nanoparticle concentrations tested in laboratory studies. In this way ecotoxicological results found can be placed in the right perspective. In any case, Oberdörster E. et al. (2006) state that fullerene concentrations of 35 mg l⁻¹ could be reached in the field. And with the world-wide increasing nanotechnology industry and the availability of accompanying (consumer) product use it may be expected that, due to emissions, current environmental nanomaterial amounts will increase to these high levels in course of time.

Sorption to aqueous organic substances like humic acids, phytoplankton and sediments could greatly increase nanoparticle concentrations locally. Because some studies reported the inability to create nanoparticle concentrations high enough to cause 50% mortality at test organisms in some exposure media (e.g., saline), it is possible that acute toxicity will not occur in most environmental nanoparticle releases in water columns. Especially since tested concentrations are relatively high and carbon nanoparticles are very insoluble. But again, environmental conditions could lead to higher (local) concentrations, such as precipitation and accumulation in benthic zones and sub-lethal effects could potentially be induced at low concentrations. Besides this it seems that nanoparticles are less toxic to organisms living in the sand than in the aqueous phase (Crane & Handy, 2007).

Looking the other way around, nanoparticles could sorb other xenobiotics substances present in the aquatic environment, increasing nanoparticle solubility in water. On the other hand, some studies demonstrate that functionalized, water-soluble fullerene nanoparticles were less toxic than those which were not. *In vitro* studies performed by Sayes et al. (2006) show the same results for functionalized SWCNTs in human kidney cells. Baun et al. (2008a) show that C₆₀-fullerene-sorbed PAH phenanthrene was available for *D. magna* daphnids and *P. subcapitata* algae, increasing toxicity, while another sorbing contaminant (PCP) was less available, decreasing toxicity in the same species. This does not only show the inherent toxicity of manufactured (carbon) nanoparticles, but also that interactions with other compounds and characterization of nanoparticles in aqueous suspension are of importance for risk assessment of nanomaterials (Baun et al., 2008a). Carbon nanomaterials could not only function as contaminant carriers but also as contaminant sequestrates and could, in this way, have a great impact on availability of xenobiotics for organisms.

Summarizing, there are so many factors controlling and influencing (nano)particle fate and behavior in the environment. It seems almost impossible to foresee and control the effects caused by different carbon nanoparticles in the environment. Therefore, more research should illuminate these issues concerning particle behavior and fate and their impact on wildlife health.

5.2. Laboratory experiments: risk assessment and future challenges

5.2.1. Particle properties and medium conditions

From a researcher's point of view it thus seems almost impossible to mimic the precise (natural) aquatic environment for exposure assessment in the laboratory. Many factors determine the behavior and eventually the fate of a carbon nanoparticle, as has already been shown for SWCNTs, MWCNTs and C₆₀-fullerenes. Therefore generalizations on the chemistry of nanoparticles should be made with caution. Not only the properties of the particle itself, like shape, size, surface-charge, but also the environmental conditions and parameters, such as pH, salinity, the presence of NOM and pollutants are crucial fate-determining factors. Consequently, many issues arise for nanoparticle risk assessment, including aggregation formation and disaggregation of nanoparticles, dispersion by interactions with NOM in water or in solutions, the ability of (other) particles to coat on nanoparticle surfaces, and precipitation of nanoparticles.

All these factors should be taken into account in the risk assessment process of a certain carbon nanoparticle species. It is, for instance, very likely that after a given period mainly aggregates will be left in the media instead of single nanoparticles. So (test) organisms could therefore be generally exposed to particle aggregates instead of single particles.

Also, the possibility of pollutants or other substances coating the nanoparticle surface causing toxicity, instead of the carbon nanoparticles themselves, must be taken into account. As a result, researchers must be very well aware of the specific nanoparticle properties, the conditions of the media and the following nanoparticle behavior. The medium of choice will eventually be the most important factor that determines the environmental relevancy in ecotoxicity testing, since it influences particle behavior and fate very strongly.

Based on its chemistry one could make a plausible prediction of in which compartments nanomaterials will (eventually) reside in a (natural) aquatic system and consequently which organisms will be mainly at risk due to exposure. Like Blickley & McClellan-Green (2008) state: it is reasonable to assume that (fullerene) aggregates could be more readily available to pelagic organisms in the water column of freshwater systems and it is also reasonable to assume that organisms in estuarine and marine environments will encounter these materials in the sediments.

Other review papers also emphasize that the toxicity of nanoparticles for a large part depends on specific and often unknown physiochemical and environmental factors (Oberdörster G. et al., 2005a; Nel et al., 2006). Like Helland et al. (2007) propose, characterized exposure scenarios could therefore be useful when conducting toxicologic studies. Therefore, to generate reliable and relevant data on the risks of nanoparticles in the aquatic environment it seems best to observe the behavior and fate of carbon nanoparticles in sampled media or even in (potential) *in situ* situations. Not surprisingly, this last suggestion will meet the most obstacles in accomplishing this goal.

5.2.2. Dispersing carbon nanoparticles in test medium

In available ecotoxicological studies researchers struggle with the stabilizing procedure of carbon nanoparticles in test media. Water-stirring or sonication could only create low concentrations of dispersed carbon nanoparticles, without total aggregation or precipitation of the particles, limiting (acute) toxicity research. The maximum concentrations of C₆₀-fullerene dispersed in media were 35 mg l⁻¹ and 22.5 mg l⁻¹ for freshwater and full-strength seawater respectively. Concerning carbon nanoparticles toxicity testing not only the solubilization problem comes along, since dispersed carbon nanoparticles easily precipitate under marine conditions, also at nanoparticle-concentrations above 10 mg l⁻¹ there could be significant aggregation of many other nanoparticle types (Handy et al., 2008a). Besides limited solubilization, another problem with sonicating nanotubes is that, according to Oberdörster E. et al. (2006), it could be possible that the sonication process induces the release of nanoparticle-adsorbed metal catalysts, especially on SWCNTs, for which is known that they regularly contain these impurities.

A lot of carbon nanoparticle research has been performed with nanoparticles dispersed in various solvents, like SDS and THF. By using solvents low solubility, aggregation or precipitation of nanoparticles in test medium could be avoided to a large extent, creating concentrations high enough to determine far more LC₅₀s. In the concerning papers authors often claim that the use of these particular solvents has little or no effect on the results, let alone on organisms' health. But (minimal) negative effects have been found in test-organism solvent control groups (Fortner et al., 2005; Oberdörster E. et al., 2006; Zhu et al., 2006), which clearly indicate that it is not desirable to use solvents in organism toxicity testing. It is reasonable to believe that the combination of solvent and nanoparticles will amplify the damage that, for example, nanoparticles and solvent only (or effects taken together) could do, eventually biasing risk assessment. In any case, it has been shown in research that solvents bias toxicity testing outcomes. This despite of the fact that the LC₅₀ of *D. magna* exposed to, for instance, THF is far higher (5930 mg l⁻¹) (EG REACH Safety Sheet, 2007) than the concentrations of solvent it will be exposed to in these types of experiments. Here, researchers simply ignore the fact that even a small dose of solvent could induce possible adverse health effects.

Besides the chance of inducing negative impacts on organisms' wellbeing it is also known that solvents have the ability to change the ionic charge of nanoparticles, changing their 'original' state (Lovern et al., 2007). Since this issue has been a topic of discussion, solvents used for the dispersion of nanoparticles are more often evaporated before use in toxicologic exposure studies. Still, it is not exactly known if solvent-dispersed particles eventually have deviating toxicity compared to non-dispersed ones. So in toxicity testing the solubilization of carbon nanoparticles by stirring or sonication has preference above using solvent, but has its limitations. Determining LC₅₀-values could be almost impossible in this way, but assessing various sub-lethal toxicological effects must still be possible, especially for smaller organisms. Until better alternatives are at hand, the question remains if these methods are a proper translation of how these particles occur in the natural environment. A good alternative to lab-made (solvent) media could be to sample relevant natural water of interest, analyze its components plus its properties and eventually expose test-organisms to the (carbon) nanoparticles of choice in this sampled water. In future testing maybe even artificial water, corresponding to real parameters, can be used. Ultimately, in this way, more clear and straightforward results of exposure to nanoparticles under specific environmental relevant conditions could be obtained. The important question rising is which particles with which accompanying properties are to be used in this type of assessments.

5.2.3. Assessing nanoparticles of choice

When focusing on the choice of carbon nanoparticles for ecotoxicological testing some obscurity already occurs. Oberdörster E. et al. (2006) state that compared to C₆₀-fullerenes SWCNTs, both solubilized (addition of synthetic peptides) and unsolubilized, elicit fewer biochemical or gene expression level changes. On the other hand Jia et al. (2005) state that toxicity of carbon nanoparticles decreases from SWCNTs > DWCNTs > C₆₀-fullerenes. An *in vitro* mouse keratinocyte cell study by Grabinski et al. (2007) confirms that MWCNTs are more ROS inducing, and thus more toxic, than their single-walled counterparts. In the Kang et al. (2008) study MWCNTs are more toxic to bacteria than SWCNTs.

So, if Jia and coworkers are right this makes it quite remarkable, from a toxicological point of view, that C₆₀-fullerenes have been used far more in recent research than the relatively more toxic, and thus more harmful, nanotubes. Especially since literature about the toxicity of carbon nanotubes in *in vitro* testing is scarce. These two examples indicate that there could be inconsistent results in ecotoxicological studies focusing on carbon nanoparticles, probably due to inexperience in the field of nanoparticle risk assessment, and that the choice of nanoparticles in risk assessment is desirably prioritized.

The priority in the ecotoxicological risk assessment of nanoparticles should first be with the type of particles that are found in the environment most; second, (potentially) found most harmful for aquatic organisms or even humans; and third, have a (potential) high emission rate into the environment. Since the risk assessment and characterization of these relative new materials is still in its infancy, these criteria are almost impossible to determine or predict at this time. Therefore, at this point engineered nanoparticles present in the largest quantities have to be considered as potentially most harmful. In carbon nanoparticle assessment the fact that fullerenes, compared to nanotubes, are relatively cheap and easy to obtain must have played a very important role in deciding to use these carbon nanoparticles. Of course there are other carbon nanomaterials besides fullerenes and nanotubes, so it is necessary to involve these particles in upcoming risk assessment too.

Another, less acquainted factor which could make the assessment of carbon nanoparticles more difficult is the presence of impurities, like on the SWCNTs. Only a few studies give specifics about assessed nanomaterials, sometimes stating that they contain impurities, usually a very small percentage ($\pm 0.5\%$). Most of the literature does not mention the purity of the nanomaterials used. What these impurities are and if they have any effect on the outcome of ecotoxicity testing has not been reported yet, but it is something to reckon with.

Other important issues that have to be taken into account are the purity and state of a nanoparticle over time. It is known that most carbon nanotubes in various applications are probably not pristine (e.g., due to reactions with other substances starting from synthesis to the end of its life-cycle, contaminant binding) when entering the environment. And since that carbon nanoparticles are fabricated for many kinds of applications, it is very likely that there are multiple different carbon nanoparticles of one material all having different properties at the point of entering the environment. Also nanoparticle properties will change the longer the particles stay in the aquatic system. The properties of particles of a product change by external physical and chemical influences, like the weathering of oil.

Looking at the behavior of carbon nanoparticles in aquatic systems another problem raises. Several studies described above showed that carbon nanoparticles are able to aggregate, especially C₆₀-fullerenes. In these cases originated aggregates are larger in size compared to the primary carbon nanoparticles from the beginning of the experiments. Measurements show that fullerene aggregates could exceed the nanosized definition of 1-100 nm. For instance Zhu et al. (2006) found aggregates in the range of 10-200 nm and Baun et al. (2008a) even measured aggregates of 2 nm to several microns. The problem is that these authors do not report about which particles or aggregates, and in which size-range, are responsible for the effects found.

Obviously, hypothetically speaking, it are most likely the smallest particles present that are responsible for inducing responses due to exposure, since smaller particles could pass cell-membranes and enter the digestive tracts via ingestion more easily and therefore are more likely to be found in higher amounts in organisms' bodies. But, of course, larger aggregates could also play an important role in this. Because of this size-specific matter, Handy et al. (2008a) propose a broader and more flexible definition of nanoparticles for ecotoxicology, rejecting the specific nanosize range: nanoparticle aggregates with an overall dimension in the μm range but consistent of primary particles smaller than 100 nm should be regarded as nanomaterials.

So, the overall question is: which specific carbonaceous nanoparticles with which properties have to be used in future risk assessment? A good suggestion could be the tracing of the fate of nanoparticles from each stage of their lifecycle. Helland et al. (2007) add that this characterization could also be an effective orientation for prioritizing research.

Thus, more research is needed to explore the influence of nanoparticle characteristics and environmental conditions, including NOM or even present pollutants, on nanomaterial impact on the aquatic environment. By performing a lot of research on this topic consistent and complementary results can be produced, giving more insight in nanoparticle behavior and fate. Since nanoparticle risk assessment is still in its infancy these results could also be of help in prioritizing and developing research. Together with the tracing of nanoparticle fate and environmental measuring (types and concentrations) early research could be very helpful in making the choice of which precise particles to use in further, more relevant and specific risk assessment. Eventually this will help in refining optimal nanoparticle assessment strategies.

5.4. The need for new endpoints and more research

Studies focusing on the effects of carbon nanotubes on organisms are scarce and it is therefore difficult to predict the likely consequences of nanoparticle introduction into the environment at this point. Even now, most of the related literature available reports about *in vitro* experiments, mostly aiming on mammalian systems. Since the focus on potential environmental impacts of nanomaterials most ecotoxicological research is performed with mainly same test organisms, indicating that there is no research strategy for current nanomaterial risk assessment. Better coordination between researchers and authorities should provide complementary results regarding this subject. Also the investigation of new (sub-lethal) endpoints is very desirable since most ecotoxicological nanoparticle risk assessment studies focus mostly on the same endpoints, like acute toxicity and oxidative stress responses. Sub-lethal concentrations of toxicants may change individual physiological behaviors that have long-term effects at the population level (Lovern et al., 2007). So not only acute toxicological damage should be an important parameter for nanoparticle toxicity assessment, but also the damage inflicted to specific organs. Other routes of exposure, food-intake and potential exposure of the digestive tract, should be included.

As long as carbon nanoparticles are available for organisms, exposure could lead to bioaccumulation and eventually biomagnification. Therefore, it would be desirable to study organisms and particle long-term endpoints like bioaccumulation, effects on reproduction, genotoxicity and persistency of the particles and their mobility. Handy et al. (2008b) add that more ADME (absorption, distribution, metabolism and excretion) data, and a good understanding of the distribution and localization of nanomaterials in body systems are needed before a definitive list of all the target organs for different nanomaterials can be produced. Even altered organism behavior induced by nanoparticle exposure must be included, because not only reproduction, predation avoidance and food intake may be negatively influenced, but also essential food web interactions. Besides this, avoidance behavior of an organism is usually an early and sensitive indicator of toxicity at ecologically relevant concentrations and should therefore also be taken along in future risk assessment.

Standard toxicity testing appears to be appropriate for use in nanoparticle toxicity assessments, as it has been demonstrated by several published papers focusing on the ecotoxicology of (carbon) nanoparticles. But effects found at earlier assessed carbon materials, with larger non-nano particles, are expected to be quite irrelevant since these larger particles mostly have other properties compared to their nanosized counterparts.

In view of available toxicity tests usually a given protocol can not be applied to all organisms. For instance, the detection of some traditional biomarkers (like hepatic proteins and isozymes) is not always suitable for use in assessments, like Oberdörster E. et al. (2006) report. There is quite some difference in the way the metabolism of organisms anticipates on nanoparticle exposure and therefore sometimes these particular biomarker measurements are inappropriate for assessment goals.

In view of the species used in ecotoxicological testing there is quite a gap. No amphibians, reptiles, birds or even plants are tested, while these are very important relevant species being part of various food-chains. So available future tests should be adapted to (natural) available organisms and new tests should be developed, preferably focusing on real environmental conditions. It is expected that in this early stage of assessment tests will be relatively simple and mechanistic, which makes it quite difficult to compare the results with those found in other studies. Variations or even contradictions in results may be due to the degree of aggregation and adsorption of other particles or differences in nanoparticle properties. Therefore standardization of used nanoparticles and (new) test methods should be included to complement results. Some experts recommend the use of SWCNTs as they show relatively good purity (Helland et al., 2007), but before focusing on this type of 'reference' particles its relevancy in emissions should first be determined. It is far more important to study (carbon) nanoparticles that can already be found in the environment in substantial amounts than pristine particles that, in this form, basically never enter the aquatic environment. Also to avoid underestimation or even overestimation of the ecotoxicity of carbon nanoparticles experimental conditions, like the concentrations of nanoparticles or NOM in suspension, should concede the real environmental situation. For this the sampling of environmental medium is proposed, as is stated above.

Helland et al. (2007) also suggest a set of Toxic Equivalent Factors (TEFs) to be useful for comparing toxicologic effects of different types of carbon nanomaterials. These TEFs have already been developed to compare toxicity of substance classes like PAHs, which include a wide range of classes. The establishment of TEFs would need to consider nanospecific properties of the material (e.g., particle size, agglomeration state) and would require investigation of mechanisms and dose-response dependency on the cellular level (Helland et al., 2007). Complementary, characterizing (pH, DOC levels) commonly found (sampled) water types and use them as standard derivatives could also help in making future ecotoxicity testing more accessible (Werkgroep VEM, 2004). This method is applied in Dutch aquatic risk assessment. Eventually, during time available tests will become more commonly used, more extensive and more complementary, giving new and consistent results.

Realistically, at this moment, one could say that carbon nanoparticle emission to the aquatic environment is probably not a serious problem. Due to lower eventual concentrations by dispersal, probable aggregation and assumed low emission rates carbon nanoparticles in aqueous media are most likely not a real threat for aquatic ecosystems and related food-chains. In this respect the emission of natural carbonaceous nanoparticles is much larger (Koelmans et al. 2008). The area in which engineered carbon nanoparticles enter the aquatic environment is very likely a vast (accidental) spilling or dump point, in stead of a stretched exposure area. Carbon nanoparticle exposure in serious effect-inducing concentrations will, therefore, mainly be local. But then again, just like asbestos one single carbon nanoparticle could induce negative health effects. The same counts for small concentrations, especially for sensitive and smaller organisms.

Maybe the hype around nanotechnology is responsible for an 'exaggerated' concern about these very small particles. Why are researchers and authorities not more worried about other particles that are already present in the environment in substantial concentrations and thus already causing negative effects? Are risk assessors therefore not thinking clearly? On the other hand, it is still not exactly known in what concentrations carbon nanoparticles are present in the aquatic environment at this moment. Also, the behavior and fate of carbon nanoparticles in real aquatic media is still an area mainly covered in shadows. Fact is that these new carbon nanomaterials are not investigated well enough and therefore still many questions about potential risks remain. To solve them, nanoparticle risk assessment stays legitimate and desirable.

5.5. Carbon nanoparticle risks & assessments: short summary

Current carbon nanoparticle risk assessment status and future testing concerns the following points of interest:

1. Indication nanoparticle impact based on ecotoxicological testing so far:

- Nanomaterials should not be defined by their size only
- Negative exposure effects found in many trophic levels (bacteria to small vertebrates)
- Ecotoxicological studies sometimes show negative health effects in species
 - Results mainly at high concentrations, environmental relevance questionable
 - Some organism species show no adverse health effects found in others
- Interspecies sensibility shown, mechanisms unknown
- Organism adaptation to carbon nanoparticle exposure demonstrated
- Positive exposure effects on organisms' health found
- All findings together are not convincing enough to indicate that there is a major environmental problem at hand
- Lack of sufficient knowledge to properly assess the risks of nanomaterials at this point
- Risk relevancy: larger particles or other xenobiotics could be more harmful for organisms than carbon nanoparticles. Nanoparticle risk seems a hyped topic and could lead to less focus on other environment affecting substances
- To put (future) nanoparticle exposure in the right perspective more focus, research and legislation on this topic is essential

2. Mapping risks, relevancy and particle behavior:

- Exploring (potential) emission sources and pathways
 - Increasing environmental nanoparticle concentrations is assumed, not yet proven
 - Local or environmental wide problem?
- Measurements of current environmental exposure levels
- Prognosis of future nanoparticle species concentrations in the aquatic environment
- Mapping environmental emissions and concentrations must have priority

- In-depth analysis of nanoparticle-specific behavior and interactions with environmental-present substances in water under various relevant conditions (e.g., functionalization, aggregation, sequestration, solubilization)
 - Determining eventual fate per nanoparticle species, determining dispersal, accumulation, bioavailability and in which aquatic areas this occurs
- Actual risk is still predominantly an assumption
 - No serious ecotoxicological nanoparticle exposure incidents known
 - Increasing nanoparticle levels could be hazardous for ecosystems
 - Extent environmental concentrations unknown

3. Improving and expanding future risk assessment:

- Research on the influence of environmental conditions (e.g., pH, salinity) and specific physicochemical properties (e.g., shape, surface-chemistry, size, dimension) of carbon nanomaterials on ecotoxicity together is recommended

Taking into account:

- Nanoparticle characteristics and their role in particle appearance, behavior and fate
- Nanoparticles have other physiochemical properties than their larger counterparts and therefore mostly behave differently
- The toxic potential of each type of nanoparticle has to be evaluated individually
- Changes of particle properties and environmental conditions during time, starting from the point of manufacturing
 - Possible aggregation; observed ecotoxicity is assigned to what size range?
- Particle purity/contamination and coating of environmental (xenobiotic) substances.
- Characterization of relevant experimental exposure media; relevant sampled water.
 - The extend of nanoparticle solubility to expand the range of LC_{50S}, other parameters and test-organisms use in toxicity testing
- Sampled media alternatives, proper non-toxic particle dispersal solutions have to be identified
- Recommended is the use of sampled water when endpoints are achievable in this medium
- Fate tracing is helpful in mapping particular particle behavior and fate in a particular medium

- TEFs for categorizing, comparing toxicologic effects of different types of carbon nanomaterials
- More environmental relevant test organisms and carbon nanoparticle species needed for assessing ecotoxicology
 - Plants, amphibians and mainly large organisms have not yet been involved in carbon nanoparticle risk assessment
- Research strategies and coordination in test methods and endpoints is necessary
 - Goal: obtaining complementary and consistent results in nanoparticle risk assessment
 - Prioritizing particle research:
 - First: particles mainly present (in highest concentrations) in aquatic systems
 - Second: most toxicity inducing nanoparticles
 - Third: particles with (potential) high emission rates
 - More specific, long-term endpoints have to be determined and investigated per species
 - More ADME data about distribution and localization
 - Biomagnification could still affect species in higher trophic levels
 - Non-aquatic/environmental study outcomes could be helpful in prioritizing and identifying research endpoints
 - Generalization and standardization in research methods for reliable results
 - Determination of the type of tests to perform ecotoxicity tests
 - Results should be interpreted with care, avoiding contradicting results and conclusions
 - New research methods and more testing is required
 - Experimental protocols applied to one species may not work on different ones
 - Analytical method development needed (topic not discussed in this thesis)
 - Global communication and debate, criteria, methods of working and agreement about the points mentioned above by the risk assessment authorities

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