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The challenges and the limitations in Life Cycle Impact Assessment for metal oxide nanoparticles, a case study on nano- TiO_2

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Background and aim

Life Cycle Assessment (LCA) is a chain-oriented tool to evaluate the environment performance of products focussing on the entire life cycle of these products: from the extraction of resources, via manufacturing and use, to the final processing of the disposed products. Through all these stages consumption of resources and pollutant releases to air, water, soil are identified and quantified in Life Cycle Inventory (LCI) analysis. Subsequently to the LCI phase follows the Life Cycle Impact Assessment (LCIA) phase; that has the purpose to convert resource consumptions and pollutant releases in environmental impacts. The LCIA aims to model and to evaluate the selected environmental issues, called impact categories (Climate change, Human toxicity, Ecotoxicity, etc.); through the use of category indicators it portrays the overall potential environmental impact of a product system in an aggregated manner.

The LCA methodology is widely applied in several industrial sectors to evaluate the environmental performances of processes, products and services. Several reports and studies emphasises the importance of LCA in the field of engineered nanomaterials (ENMs) The ENMs offer enormous potential for the development of new products and application with improved performance as well as reduction of energy and materials. There are however unanswered questions about the impacts of nanomaterials and nanoproducts on human health and the environment. In the last decade the increasing production, use and consumption of nanoproducts, with a consequent release into the environment, has accentuated the obligation to ensure that potential risks are adequately understood to protect both human health and environment. Due to its holistic and comprehensive assessment, LCA is an essential tool to analyse, evaluate, understand and manage the environmental and health effects of nanotechnology. The evaluation of health and environmental impacts of nanotechnologies, throughout the whole of their life-cycle by using LCA methodology, is mentioned in a number of EU policy documents including the Sixth Community Environment Action Programme and the Communication on Integrated Product Policy (IPP) as well. Currently, only few LCA's studies on nanotechnology are carried out, and only fewer studies assess the aspects relating to (eco)toxicity **(Chapter II)**. This is due to the lack of knowledge in relation to risk assessment. In fact, to date, the knowledge on human and environmental exposure to nanomaterials, such nanoparticles (ENPs) is limited. This bottleneck is reflected into LCA where characterisation models and consequently characterisation factors for ENPs are missed. Therefore, ***the PhD project aims to assess limitations and challenges of the freshwater aquatic ecotoxicity potential evaluation in LCIA phase for ENPs and in particular for metal oxide nanoparticles as n-TiO_2.***

In the Life Cycle Impact Assessment phase the characterisation models have been developed to quantitatively overview the environmental pathway of a substance once released into the environment. The Characterization Factor (CF) of a chemical product for toxic impact category is evaluated as the product of Effect Factor (EF), Fate Factor (FF) and Exposure Factor (XF), [CF=EF*FF*XF]**.(Chapter V)** Since the exposure assessment of a substance requires the evaluation of fate, behavior and transport in the environmental media, currently environmental multimedia models are used to evaluate the fate factors and exposure factors of pollutants. Despite the usefulness of these models for organic substances, the exposure assessment of ENPs is still critical due to the scarce knowledge of the environmental behaviour of ENPs, of the ENP's proprieties that affect the behaviour and transport among media compartments and therefore, of their fate processes in the environment.

Therefore the evaluation of the predicted environmental concentration (PEC) of ENPs is currently mainly based on material flow analysis (MFA) where the ENPs are treated as bulk material. In such studies the important particulate nature of the material has not been considered (**Chapter III**). A correct evaluation of the environmental exposure, in principle, needs to consider all the environmental fate processes in order to estimate the bioavailable fraction. Two processes that seem to be significant are the fate processes of aggregation (incl. sedimentation) and dissolution. Environmental scientists have recently encouraged modeling of ENPs fate in freshwater based on colloidal chemistry and it has also been recognized that abiotic factors such as ionic strength and pH could influence the colloidal behavior of ENPs in freshwater (**Chapter III**).

As mentioned above, the assessment of the potential toxic impact of a substance requires the knowledge of its toxic effect. Therefore, acute toxicity tests with n-TiO_2 on *Daphnia magna* (crustaceans) and algae have been carried out. Furthermore, an extensive bibliographic review of the toxicity of metal oxide nanoparticles (in particular n-TiO_2) on freshwater organisms has been performed. The review is focused on aquatic organisms representative of reflecting the overall topic of this thesis and the experimental work undertaken during the PhD project period. The review aims to describe the current state of knowledge as well as to highlight potential relationships between particle properties and observed effects, while also drawing attention to knowledge gaps and uncertainties. The bibliographic review aims, as well, to collect~~ed~~ the effect concentrations of n-TiO_2 used into the calculation of the effect factor **(Chapter IV).**

The extensive knowledge acquired on the environmental behaviour of ENPs, on the current approach to model their environmental fate, on their ecotoxicity has induced to propose a framework for the calculation of both fate factor and effect factor for metal oxide nanoparticles such n-TiO_2 **(Chapter VI).**

Following the aim of the PhD research, limitations and challenges for LCIA have been highlighted and discussed in each chapter~~s~~ of this thesis.

INDEX

1. What are nanoparticles?

1.1 Definition and classification

Nanoparticles (NPs) belong to the wider group of nanomaterials, where the prefix 'nano' refers to infinitesimal physical dimensions and where particles are defined as a "minute piece of matter with defined physical boundaries" where "physical boundary can also be described as an interface" (ISO, 2008)

Many definitions have been proposed for nanoparticles and nanomaterials and in literature the terms "engineered nanoparticles (ENPs)", "engineered nanomaterials (ENMs)" and "nanoproducts" are not used in a uniform manner.

The chemical composition may be the same as with bulk material, but nanoparticles display totally new characteristics due to the high surface to- volume ratio and their small size (Oberdörster et al., 2007) at which quantum mechanics come into play. Thus, it is difficult to find a sound definition.

Some efforts are seen in the scientific literature to define nanoparticles based on their novel size-dependant properties. A common definition of engineered nanoparticles, combining both size and property characteristics, refers to particles with dimensions of about 1 to 100 nm, purposefully manufactured to have unique properties (Kreyling et al., 2010; Auffan et al., 2009). Hence nanoparticles possess properties that are "*qualitatively or quantitatively distinctly different from their of other physical forms*" (SCHENIHR 2006), such as those of larger-sized particles (bulk particles) made from the same materials and their water-soluble/ionic form. Size-related differences in particle properties may be due to the larger surface area per mass, resulting into an increased ratio of surface-to-core atoms and increased number of corner and edge atoms. This results in an increased reactivity (Feldheim, 2007) or an increased ion release which enables their use in novel applications.

The Scientific Committee on Emerging and Newly Identified Health Risks (SCENIHR) was set up in 2004 by the European Commission to provide scientific input on elements to consider when developing a definition of the term "nanomaterial". The SCENHIR concluded that size is universally applicable to nanomaterials. A defined size range would facilitate a uniform interpretation. A lower limit of 1 nm[1] and an upper limit of 100 nm were proposed.

Also, the number size distribution should also be considered using the mean size and the standard deviation of the size to refine the definition. The size distribution of a material should be presented as size distribution based on the number concentration (i.e. the number of objects within a given size range divided by the number of objects in total).

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[1] A nanometer is one billionth of a meter (10^{-9} m)

Recently, the European commission, on October 18th, 2011 adopted the Recommendation on the definition of a nanomaterial. According to this Recommendation "Nanomaterial" means:

“A natural, incidental or manufactured material containing particles, in an unbound state or as an aggregate or as an agglomerate and where, for 50 % or more of the particles in the number size distribution, one or more external dimensions is in the size range 1 nm - 100 nm.”

Where:

…”particle", "agglomerate" and "aggregate" are defined as follows:

(a) "Particle" means a minute piece of matter with defined physical boundaries;

(b) "Agglomerate" means a collection of weakly bound particles or aggregates where the resulting external surface area is similar to the sum of the surface areas of the individual components;

(c) "Aggregate" means a particle comprising of strongly bound or fused particles.

and also “*In specific cases and where warranted by concerns for the environment, health, safety or competitiveness the number size distribution threshold of 50 % may be replaced by a threshold between 1 and 50 %”.*

The nanoparticles can be classified, according to their origin, in natural and anthropogenic and then, intentional or unintentional (Oberdörster et al., 2007; Bhatt et al., 2011).

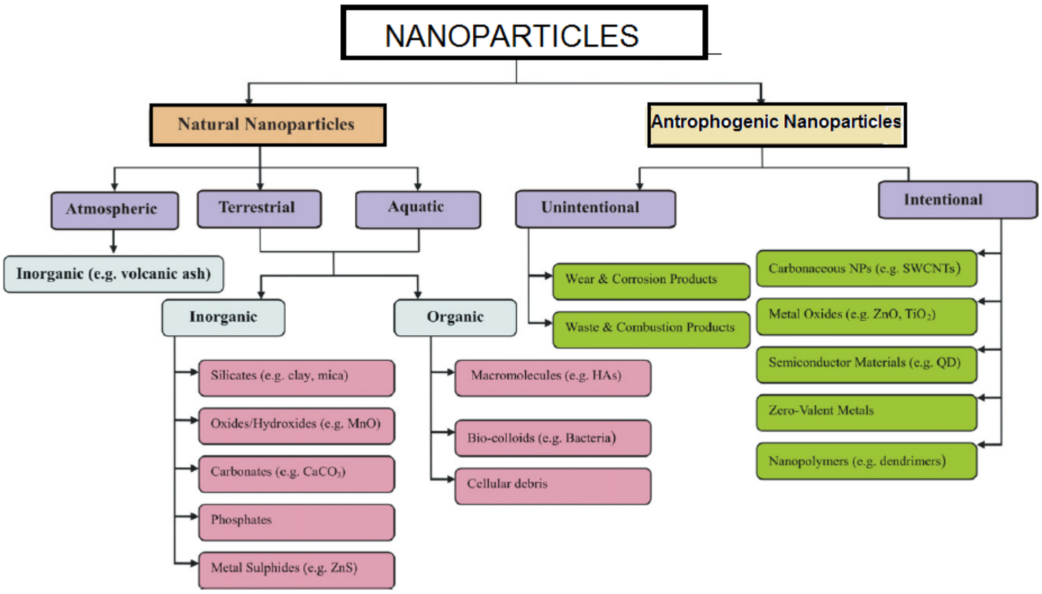

Fig.1.1 Categories of nanoparticles (Source: Bhatt et al, 2011)

The natural nanoparticles are assumed to be derived:

- From natural combustion processes;

- From geological mechanism, e.g. physic-chemical weathering, autogenesis/neoformation and volcanic eruptions;
- Many biological molecules/entities (e.g. DNA, nucleic acids, viruses) are typically nano-sized. Some of these are released into the environment directly from the organism by biological processes as nucleoprotein exudates from algae, dispersion of viruses from animals;
- From degradation of biological matters, e.g. humic and fulvic acids.

The unintentional anthropogenic nanoparticles derive from combustion processes (e.g. diesel exhausts) or waste and corrosion of products containing nanoparticles. The intentional source of nanoparticles are described by the class of engineered nanoparticles (ENPs). The ENPs are intentionally produced for dedicated applications and manufactured by two distinct methods: top-down and bottom-up. In the top-down method, particle with size lesser than 100 nm and 30 nm are produced by cutting larger pieces of source material (lithographic techniques). The bottom-up methods are based on physicochemical principles of molecular or atomic self-organization. This approach produces selected, more complex structures from atoms or molecules, better controlling sizes, shapes and size ranges. It includes aerosol processes, precipitation reactions and sol-gel processes (Bhatt et al., 2011).
Also, ENPs can be distinguished in five classes:

- **Metal oxide nanoparticles**: CeO_2, TiO_2, ZnO, Fe_2O_2, Al_2O_3, MgO, ZrO, SnO.

- **Carbon based nanoparticles**: These nanomaterials are composed mostly of carbon, most commonly taking the form of a hollow spheres, ellipsoids, or tubes. These particles have many potential applications, including improved films and coatings, stronger and lighter materials, and applications in electronics. This group comprises both fullerenes (C_{60}) and carbon nanotubes (CNTs). Two classes of carbon nanotubes are distinguished: single walled (SWNTs) and multi-walled nanotubes (MWNTs). SWCNTs are structurally single-layered graphene sheets rolled up in cylindrical shapes of approximately 1 nm diameter and several micrometers of length, whereas MWCNTs possess two or more concentric layers with varying length and diameters (Bhatt et al., 2011).

- **Zero-valent metals**: They are usually prepared by reduction of metal salts, e.g. zero-valent iron is made through the reduction of ferric (Fe^{3+}) or ferrous (Fe^{2+}) salts with a sodium borohydride. Similarly, the chemical synthesis of gold and silver ENPs involves dissolution of the metal salt in an appropriate solvent and its subsequent reduction to the zero valency.

- **Quantum dots**: They are a closely packed semiconductor crystal comprised of hundreds or thousands of atoms, and whose size is on the order of a few nanometers to a few hundred nanometers. Usually they are nanoparticles made of semiconductor materials with fluorescent properties, crucial for biological applications.

- **Dendrimers**: They are complex, multifunctional polymers with 1–10 nm diameter. The surface of a dendrimer has numerous chain ends which can be tailored to perform specific chemical functions. This property could also be useful for catalysis. Also, because three-dimensional dendrimers contain interior cavities into which other molecules could be placed, they may be useful for drug delivery.

For the aim of this thesis I will refer to ENPs, to the group of metal oxide nanoparticle and I will focus on nanoparticles of titanium dioxide (n-TiO_2).

1.2 Nanoparticles applications

In the last decade an increasing interest has been shown in nanoparticles due to their physicochemical properties that differ from those of the bulk material. The novel physicochemical properties of the ENPs are attributable to their small size, chemical composition, surface structure, solubility, shape, aggregation (Nel, 2006). The advent of nanotechnology[2] has unleashed enormous potential for the development of new products, and their applications in a number of industrial and consumer sectors (cosmetic, medicine and drugs, optical engineering). The nanomaterials and nanoparticles are widely applied in several sectors; their applications are so extended that, to date, they are largely applied in daily consumer products (clothes, sunscreens and others).

The Nanotechnology Consumer Product Inventory of the Woodrow Wilson Institute (WWI) highlights that the number of nano-enabled consumer products is increasing rapidly, where the production and distribution of nanotechnology products is increasingly global. As a result of the continuously increasing of applications of nanomaterials in consumer products, a total of 858 consumer products containing nanomaterials (nano-consumer products) were identified in the European market (2010); this result represents a six fold increase when compared to the number of 143 products in the European market

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[2] Nanotechnology is the intentional and controlled generation, or modification of materials at a nanometer (nm) scale level (Handy et al., 2008).

(2007). The product categories with the largest growth are the ‘Personal care products and cosmetics’ like sunscreens and various ‘Coating products’ such as anti-rain products for shoes and textiles.

In the following the main applications of the ENPs area described.

Metal oxide nanoparticles (n-TiO_2):

Commercial production of nano-TiO_2 between 2006 and 2010 has been estimated at 5000 tons per year, more than 10 000 tons per year between 2011 and 2014 and approximately 2.5 million tons by 2025 (Menard et al., 2011).

Titanium dioxide is a naturally occurring mineral that can exist in three crystalline forms, known as rutile, anatase and brookite, and in amorphous form. The element titanium is also found in ilmenite ($FeTiO_3$) and other minerals and ores; rutile phase is the most common form of TiO_2 found in nature (EPA, 2010). Anatase phase exhibits the highest photocatalytic activity and therefore it is used in catalysis and photocatalysis applications; rutile is known as white pigment providing opacity to paints, papers, inks, and consumer products such as toothpaste. Anatase and brookite are used as electrodes in dye-sensitized solar cells (Jiang et al., 2002). Such properties have led to the use of nano-TiO_2 for a wide variety of applications, including self-cleaning surface coatings, light-emitting diodes, solar cells, disinfectant sprays, sporting goods, sunscreens (EPA, 2009). For environmental applications, suspended TiO_2 nanoparticles have been largely used as efficient catalysts for the decomposition of organic contaminants present in water and aqueous wastes (Zhang et al., 2007).

A surface coating, for example silica and other compounds, can also be added to nanosized TiO_2 to decrease its photo-reactivity so that nano-TiO_2 can be used to protect human skin, plastic, and other objects from UV radiation (Menard et al., 2011).

Carbon based nanoparticles:

Fullerenes are applied for the sorption of organic compounds (e.g. naphthalene) and for the removal of organometallic compounds. CNTs and their derivates are used for the sorption of metals such as copper, nickel, cadmium, lead, silver, zinc, americium and rare earth metals in: electronics and computers, plastics, catalysts, battery, fuel cell electrodes, water purification systems, orthopedic implants, conductive coatings, adhesives and composites, sensors, and components in the electronics, aircrafts, aerospace, and automotive industries, as well as in sporting goods.

Quantum dots:

They are applied in medicine, e.g. medical imaging and targeted therapeutics, in solar cells, photovoltaic cells, security inks, photonics and telecommunications.

Zero-valent metals:

Zero-valent ions are used in nitrate removal from water, soil and sediments and also for detoxification of organochlorine pesticides and polychlorinated biphenyls, in bioremediation for the decomposition of molinate (a carbothionate herbicide).

Dendrimers:

They are applied in manufacture of macro-capsules, coloured glasses, chemical sensors, modified electrodes, as DNA transfecting agents, therapeutic agents for prion diseases, in drug delivery and DNA chips, in tumor treatment (used as a powerful anticancer drug).

It is worth noting that nanomaterials are associated with presumably revolutionary contributions to environment and sustainable development in terms of (Rickerby et al., 2007):

- Environmental monitoring: more sensitive detection systems for air and water quality monitoring;
- Replacement in the use of hazardous chemical substances;
- Energy and resource saving, thanks to lighter and stronger materials f vehicle production and to more efficient fuel cells;
- Environmental remediation and treatment: for example zero-valent nanoparticles (as zero valent iron) are used in water remediation nanotechnology for *in situ* application to remove a wide variety of contaminants (heavy metals, pesticides, chlorinated organic solvents ect.).

1.3 Engineered nanoparticles: environmental concerns

With the increasing production of nanomaterials and the escalating promise of new and unique nanotechnology materials, concerns of occupational, safety, and environmental hazards are raising leading to some controversies in the nanotechnology debate. In fact, with the expected benefits of nanomaterials and nanoparticles, nanotechnology is still a largely unknown area and the consequences due to the widespread production and utilization of nanomaterials are difficult to predict.

Several important aspects in regard to the environment and risk assessment of ENPs are addressed: 1) to exposure assessment, and 2) to ecotoxicity.

1.4 Environmental exposure

Among nanoproducts not all will lead to environmental exposure (e.g. a semiconductor is unlike to lead to direct exposure during its use), but materials and products with the potential to release nanoscale materials into environment, such as aereosol, powders, or suspensions of nanometer-diameter particles may lead to relevant exposure. Metal oxide nanoparticles are among the most used nanomaterials and receive attention over their potential effects. The widespread use of metal oxide nanoparticles (e.g. TiO_2) could lead to significant release of nanoparticles into the environment leading to a potential increased environmental exposure to nanoparticles (Hall et al., 2009). Particular attention has been posed on a freshwater ecosystem that seems to be an environmental compartment expecially affected by the release of these particles (Lovern and Kapler, 2006). The route of exposure of ENPs into the aquatic environment can be possible by accidental and intentional release (e.g. through environmental remediation efforts). The potential fate of nanoparticles in the aquatic environment and their interactions with aquatic organisms is illustrated in Fig. 1.2. Once there, their fate will depend on a number of factors such as presence of natural organic matter (NOM), ionic strength and pH. Currently very few data exist regarding observed environmental concentrations of TiO_2 nanoparticles.

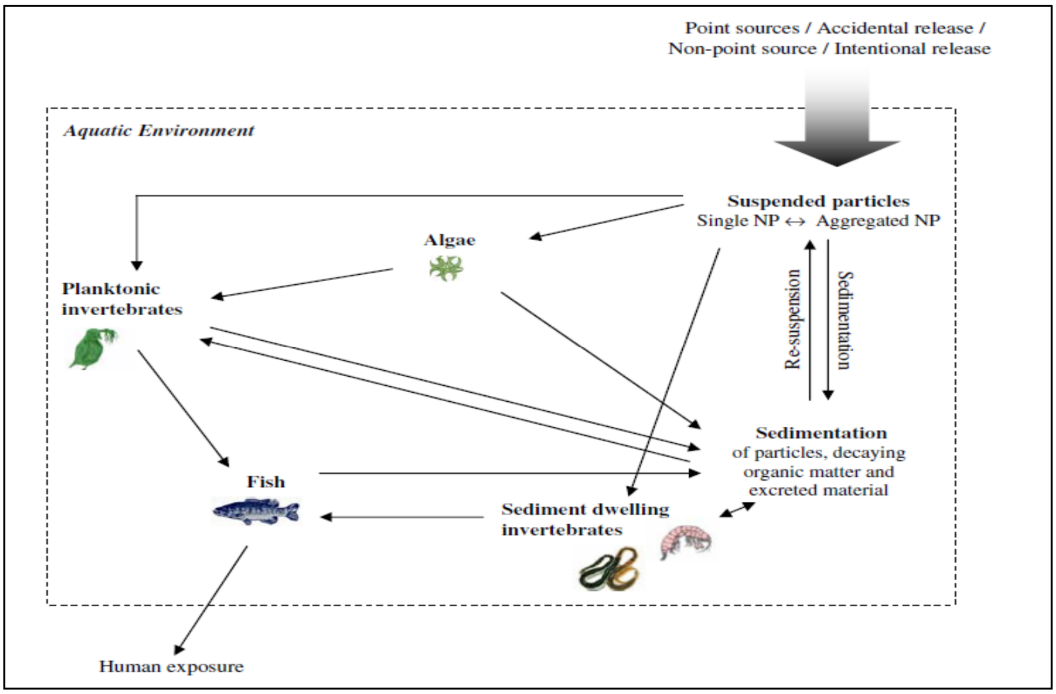

Fig.1.1: Possible route of environmental exposure of ENPs after realse into acquatic environment (Source: Baun et al., 2008)

Kiser et al.,(2009) have measured the levels of titanium nanomaterial removed and released from wastewater treatment plants. They found out that raw sewages contain 100–3000 µg/L of Ti whereas its concentrations in effluents from wastewater treatment plants ranged from <5 to 15 µg/L. As Ti is removed, it accumulates in settled solids with concentrations ranging from 1 to 6 µg/mg. Mueller and Nowack, (2008) and Gottschalk et al., (2009) modelled the quantities of TiO_2 nanoparticles released into the environment and the predicted environmental concentrations are presented in Table 1.1

Reports show that metal oxide nanoparticles (e.g. TiO$_2$) once introduced into water, will most probably aggregate and partition onto sediments and suspended particulate matter (Boxall et al., 2007; Praetorius et al., 2012). Aggregated particles are generally less mobile and can interact with filter feeders and sediment-dwelling organisms (Farré et al., 2009). It has been argued that the environmental behavior of ENPs is strongly affected by the environmental chemical condition (e.g. pH, ionic strength, humic acids), in function of which different environmental behaviours may be expected (Navarro et al., 2008; Domingos et al., 2010).

Therefore, it is not clear, at this stage, how predicted environmental concentrations for nanoparticles can be calculated. The commonly used mathematical models will need adaptation for the assessment of the environmental distribution and dispersal of nanoparticles. This implies incorporation into the models of the key physic-chemical characteristics relevant to nanoparticles such as: surface area and morphology, charge, number of particles, size, solubility and potential chemical and physical conversion into other forms, as described earlier (SCHENIHR, 2006)

Table 1.1: Predicted environmental concentrations of n-TiO$_2$ into environmental compartments in different countries; [a] Mueller and Nowack, 2008; [b] Gottschalk et al., 2009. (Source: Menard et al., 2011)

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

1.5 Ecotoxicity

The toxic potential of materials is different on a nano-scale for several reasons. Nanomaterials are theoretically expected to be more toxic than their bulk counterparts due to their greater surface reactivity and the ability to penetrate into and accumulate within cells and organisms. This can make materials more chemically reactive, and affect their functional properties such as mechanical strength or electrical properties. For example, as the size decreases, the number of atoms on the surface increases, with a conseguent increase of the biological reactivity, offering potential use in pharmaceutical industry as drugs delivers (Lovern and Kapler, 2006). Also, it is important to note that nanomaterials can be on the same scale as elements of living cells, including proteins, lipids, nucleic acids, and organelles. Therefore, one must focus particular attention on how nanoparticles can interact with or influence biological systems, which may be desirable for certain medical applications, but may cause unanticipated hazardous effects upon occupational or environmental exposure to nanomaterials. For istance, the small size of the nanoparticles increases the rate of uptake and interaction with biological tissue, raising adverse biological effects; this wouldn't be possible with the bulk material.

To date, the precise mechanisms of toxicity of metal oxide nanoparticles are largely unknown (Griffitt et al., 2008). Anyway, recent reports have shown that the toxicity of nanoparticles is generally governed by properties such as particle size, shape, chemical composition and surface properties (Crane et al., 2008; Navarro et al., 2008). For instance, n-TiO_2 is photo-inducible, redox active and thus a generator of potential reactive oxygen species (ROS) at its surfaces have been argued. However, the precise mechanisms of toxicity of nanosized TiO_2 and other metal nanoparticles are largely unknown (Griffitt et al., 2008).

1.6 Are ENPs environmental sustainable?

ENPs are expected to affect living organisms but due to the high variability of the toxic data reported, it is difficult to characterize their ecotoxicological hazard. The environmental fate of ENPs is far to be modelled and predicted, and it is still uncertain how ENPs would behave in the environment. Although environmental concentrations of manufactured nanoparticles (ENPs) have yet to be routinely measured, there are concerns that ENPs will be released from these products over their life (e.g., by erosion of the materials with use, or deliberate introduction during remediation of contaminated environmental media), or that product applications could generate wastes containing nanomaterials (e.g., domestic waste-water containing nanomaterials from household products). It is also unclear whether or not sewage treatment works could completely remove ENPs from final effluents. Therefore, despite the fact that nanoproducts are already released into environment (Som et al., 2010), environmental concerns on production, use and end of life of nanomaterials are raised. It cannot be overlooked that methods are needed to assess whether the potential benefits of nanotechnology outweigh the risks.

The benefits and potentials are currently neither completely substantiated by an assessment of ecological and human health risks or by a holistic assessment of all aspects along the life cycle of nano based products and services (Som et al., 2010).

An holistic environmental sustainability assessment of products requires the evaluation of both material and energy input and environmental releases of the life-cycle stages. Moreover, to minimise the environmental impact and achieve sustainability, material loops must be closed and it is essential to obtain an accurate estimate of the full environmental impact. Life cycle assessment (LCA) is a useful technique for calculating energy and raw material requirements for a product's manufacture, use and final disposal or re-use and for assessing the true environmental impacts (Rickerby and Morrison, 2007). In fact, due to its holistic and comprehensive perspective LCA has been recognized as a key tool for assessing the environmental performance of nanoproducts and, furthermore, for comparing a product that includes ENMs with similar products without ENMs (Klopffer, 2007).

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2 Life Cycle Assessment Methodology

Life Cycle Assessment (LCA) is a standardized methodology (ISO, 2006a,b) for determining and assessing the environmental impacts of products across their whole life cycle, for comparing different options/products with respect to their potential impacts on the environment, and for identifying the critical points within the product life cycle that contribute most to these impacts. The key environmental issues which are considered in an LCA include the following: climate change, stratospheric ozone depletion, tropospheric ozone (smog) creation, eutrophication, acidification, toxicological stress on human health and ecosystems, depletion of resources, water use, land use, noise, and ionizing radiation. This framework is applied to any kind of product and to any decision where environmental impacts are of interest and by a broad variety of actors – from governmental organisations to industry.

According to ISO Standards 14040 (ISO, 2006a,b), LCA is conducted in four main phases (Fig.1): (i) defining the goal and scope of the study, (ii) establishing a life-cycle inventory which aggregates all inputs from and outputs to the environment within the system boundaries, (iii) performing a life-cycle impact assessment which translates the inventory into potential impacts of the system on the environment and (iv) interpreting the results from the assessment to provide consistent support to decision-makers in relation to the goal and scope of the study.

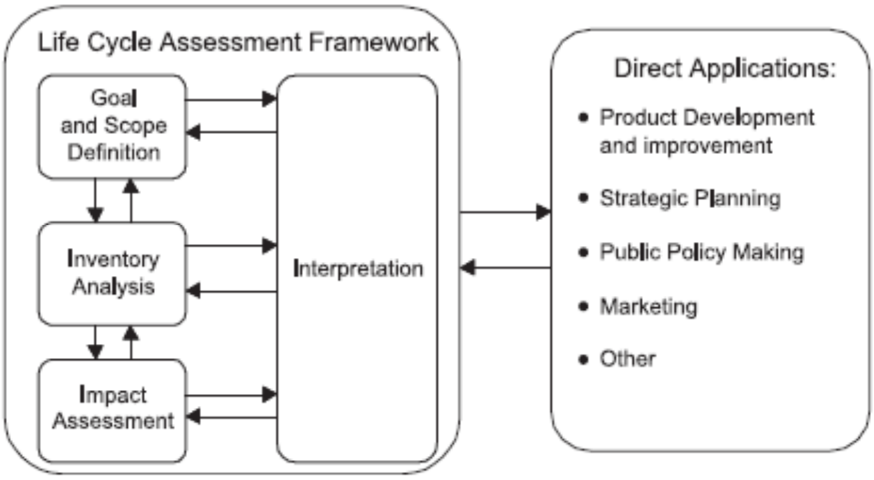


Figure 2.2: Phase and application of an LCA (Source: ILCD Handbook, 2011)

2.1 LCA: The four phases

In accordance with ISO:14040 (ISO, 2006a) the procedure of carrying out an LCA is organized in the following four steps.

2.1.1 Goal and scope definition

The goal and scope of an LCA shall be clearly defined and shall be consistent with the intended application. In defining the goal of an LCA, the following items shall be unambiguously stated: the intended application, the reasons for carrying out the study, the intended audience, whether the results are intended to be used in comparative assertions intended to be disclosed to the public. In defining the scope of an LCA. The scope of the study must clearly describe the system of the studied product or process and its boundaries, the included items and the items to be evaluated, the system functions, the functional unit, the impact categories, the methodology applied, and finally, the necessary assumptions and restrictions. Where:

Functional unit: the functional unit defines what precisely is studied and quantifies the service delivered by the product system, providing a reference to which the inputs and outputs can be related.
System boundaries: boundaries define which processes in the products life cycle are included in the LCA.
Data: the data should include all inputs and outputs from the processes. Inputs are, for example, the use of energy, water, materials, etc. Outputs are the products, co-products and emissions. Emissions can be divided into four categories: air, water, soil and solid waste depending on what the emissions affect. A lot of databases with LCA data exist and they are suitable with the LCA software. Data can also be collected through national statistics or bibliographic reviews.

2.1.2 Life cycle Inventory

In this phase all mass and energy flows into and out of the system are balanced. All these flows are listed and calculated in relation to the functional unit. For all activities throughout the product life cycle (production, transportation, use and waste treatment processes), the required materials and energy and the emissions and solid waste are assessed.

In this phase the *allocation* of sub-products may occur~~s~~; it is defined as the partitioning of the input and/or output flows of a process to the product system under study. It is required where a single production system produces more than one good, to proportion the environmental impacts of the production system to those different economic goods. Also, it becomes necessary when waste materials are recycled and reused instead of the primary materials.

2.1.3 Life cycle Impact Assessment

Life cycle impact assessment (LCIA) is the phase in LCA where the inputs and outputs of elementary flows collected and reported in the LCI are translated into impact indicator results related to human health, natural environment, and resource depletion" (ILCD Handbook, 2011). Thus, in the Life Cycle Impact Assessment phase the potential environment impacts in a number of impact categories are calculated through relating the single input and output flows to the environmental impact they may cause.

The potential impact for each of the impact category (e.g. acidification, global warming) are expressed in terms of Impact Score:

$$IS_j = CF_{i,j} \times m_{i,J}$$

Where:

CF÷ is the characterisation factor (for human toxicity case/kg) for the substance *i* for the impact category *j* (e.g. human toxicity); $m_{i,j}$ is the mass (kg emitted) of the substance emitted and classified within the impact category *j*

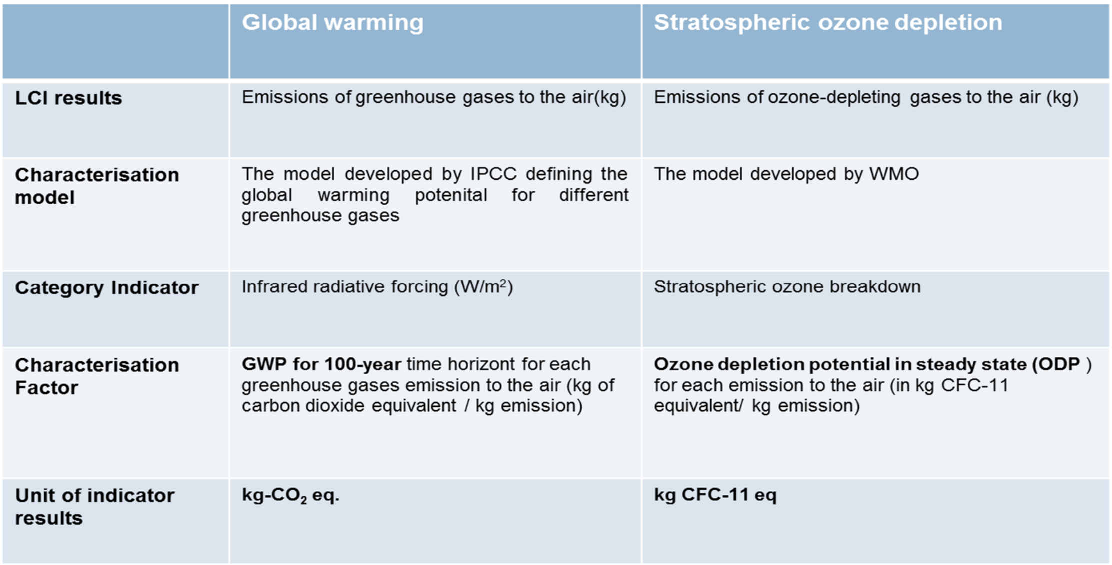
The phase of Life Cycle Impact Assessment is composed by a series of steps, some of which are compulsory whereas other are optional according to the ISO standard.

Classification (obligatory): The inventory results are classified according to the type of environmental impact that they may cause. The impact categories are identified (e.g. global warming potential, acidification, human toxicity, etc.).

Characterisation (obligatory): In this step the impacts are quantitatively characterized; all the substances contributing to the same impact category have to be translated from a mass or energy load into an impact load, ending up with one specific unit for each category.

In this step the so-called characterisation factors (CF) are applied. The characterisation factors are substance-specific and are based on models of cause-effect chains that describe the behaviour of a substance in the environment. For example, for the impact category of Global Warming Potential (GWP), all the GHG emissions (in the LCI phase express in kg) will be converted to the common unit of this impact category (e.g. CO_2 eq.). This results in a numerical indicator outcome, i.e. the LCIA profile for the product system for this impact category.

Table 2.2: Example of the characterisation steps for the impact category of Global warming and Stratospheric ozone depletion



	Global warming	Stratospheric ozone depletion
LCI results	Emissions of greenhouse gases to the air(kg)	Emissions of ozone-depleting gases to the air (kg)
Characterisation model	The model developed by IPCC defining the global warming potenital for different greenhouse gases	The model developed by WMO
Category Indicator	Infrared radiative forcing (W/m^2)	Stratospheric ozone breakdown
Characterisation Factor	**GWP for 100-year** time horizont for each greenhouse gases emission to the air (kg of carbon dioxide equivalent / kg emission)	**Ozone depletion potential in steady state (ODP**) for each emission to the air (in kg CFC-11 equivalent/ kg emission)
Unit of indicator results	**kg-CO_2 eq.**	**kg CFC-11 eq**

Normalization (optional): The impact per category can be normalized to a certain magnitude, for example to the total impacts arising in a country.

Grouping (optional): It possible to summarize the specific impact categories (e.g. human toxicity, freshwater toxicity, acidification, etc.) into the three Areas of Protection: human health, ecosystem quality and resource.

Weighting (optional): The environmental effects of chosen impact categories can be weighted; where the weights are assigned to the different impact categories and resources reflecting the relative importance, they are assigned in the study in accordance with the goal of the study.

The life cycle impact assessment covers impact categories that have been recognized as non-toxic or toxic. Within the first group for example the climate change, acidification, and resource depletion are classified. In contrast, human toxicity and ecotoxicity represent the toxic impact categories. Furthermore, the impact categories are distinguished in midpoint or endpoint. The distinction among midpoint or endpoint categories is based on the point in which the indicator is chosen along the impact pathway. Characterisation at midpoint level models the impact using an indicator located somewhere along (but before the end of) the environmental impact pathway of a substance. Characterisation at the endpoint level requires modelling all the way to the impact on the entities described by the Area of Protection i.e. on Human Health, on the Natural Environment and on Natural Resources (Fig.2.2). Also, the environmental impacts on different geographical scale are referred:

Global Impacts

Global Warming: Polar melt, soil moisture loss, longer seasons, forest loss/change, and change in wind and ocean patterns.

Ozone Depletion: Increased ultraviolet radiation.

Resource Depletion: Decreased resources for future generation.

Regional Impacts

Photochemical Smog: decreased visibility, eye irritation, respiratory tract and lung irritation, and vegetation damage.

Acidification: building corrosion, water body acidification, vegetation effects, and soil effect.

Local Impacts

Human Health: increased morbidity and mortality.

Terrestrial Toxicity: decreased production and biodiversity and decreased wildlife for hunting or viewing.

Aquatic Toxicity: decreased aquatic plant and insect production and biodiversity and decreased commercial or recreational fishing.

Eutrophication: nutrients (phosphorous and nitrogen) enter water bodies, such as lakes, estuaries and slow-moving streams, causing excessive plant growth and oxygen depletion.

Land Use: loss of terrestrial habitat for wildlife and decreased landfill space.

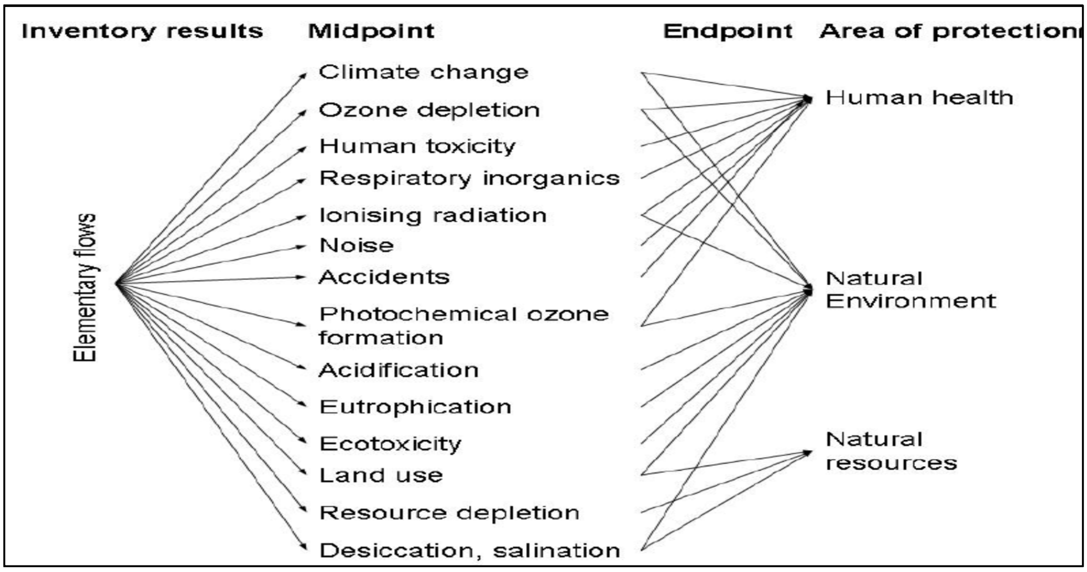


Fig. 2.2: LCIA, midpoint and endpoint impact categories (ILCD Handbook, 2011)

2.1.4 Interpretation

After establishing the inventory and assessing the environmental impacts based on the inventory the results have to be further analysed, presented and discussed. In this phase, sensitivity analysis can be performed in order to check the robustness of the result; also uncertainty analysis may be applied to interpreting the results of the life cycle assessment.

2.2 LCA and nanotechnology

As argued, LCA has been recognized as a powerful “tool” to assessing the environmental performance in the field of nanotechnology. The state of art on “LCA and nanotechnology“ in the following sub-chapter is presented and discussed.

2.2.1 Goal and scope in the nanotechnology LCA study

In an LCA study all relevant resource and energy inputs and all relevant outputs of a system are related to the functional unit which serves as the object of investigation. This functional perspective allows establishing a denominator to compare the performance of alternatives which are not comparable in absolute terms.

Among the studies conducted on nanoproducts, most of them adopt the mass as functional unit e.g. 1 kg of nanomaterial. Whereas, for comparative LCA study with the aim to compare one product based on nanomaterial with a conventional product, the weight-based functional unit is not correct since the comparison has to be carried out on the basis of a system function. For instance, the study [14] is focused on the application of carbon nanofibres in a polymer composite (before the same authors studied the production of carbon nanofibers [10]). The objective of the study is to compare traditional material such as steel with a polymer composite with a mechanical stiffness or strength equal to those of steel. The author shows that to achieve the same functionalities only 0.38 kg of polymer nanocomposite is necessary instead of 1 kg of steel. Thus, a comparison of nanofibers and steel on an equal weight basis does not reflect the actual replacement; a comparison at the level of the functionality shall be performed. However, other studies may focus on specific applications (case) of the respective materials and uses a context –related functional units (e.g. 1 m^2 of photovoltaic cell in study [15]).

Looking to the various studies on nanoproducts, the system boundaries: 1) cover all life cycle stages (e.g. cradle to grave) from the extraction of the resource until the end of life (e.g. disposal /recycling) or 2) are defined as cradle-to-gate, with the gate being on the level of factory gate of the production site of engineered nanomaterials (e.g. carbon nanotubes in study [10]) or 3) are defined on the level of the factory gate of the nano-enabled products (e.g. carbon nanofibers polymer composite as in [15]) (Fig.2.3).

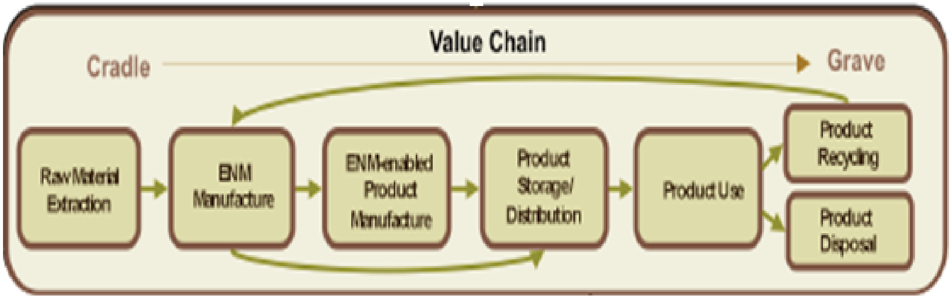


Fig.2.3: Stages of the nanoproducts life cycle.

The first life stages (extraction of raw materials, production of precursors and production of engineered nanomaterial) are covered by all the studies reported in Table 2.1. On the contrary, few studies cover the use phase and generally information on the use phase is not easily available and therefore not reported. Several environmental advantages (e.g. environmental remediation applications, energy saving) are claimed with the use of nanoproducts. However, potential release of nanoparticles during the use phase has been outlined raising concerns on the human and environmental toxicity (Gottschalk et al., 2010). Thus, further efforts on the analysis of the use phase should be carried out to evaluate the "real" benefits of the use of nanoproducts.

The analysis of the end-of-life (EOL) of nanomaterials, as disposal to landfill or incineration in waste incineration plants, is critical because it has not yet been subject of investigation. Furthermore, significant impacts for the environment may arise due to the ambient emissions and little is known about the environmental degradation (e.g. in landfill) of nanoproducts (Som et al., 2010). To date, only few LCA studies cover the phase of end of life. Meyer et al. (2010) did not consider the EOL phase because the nano-silver was assumed to have been washed off during the use phase and the EOL was assumed to be the same as for non-nano-silver product. Moreover, when a study covers the EOL, several assumptions are performed. For instance, Bauer et al. (2008) reported only a qualitative description of the EOL in the case study of CNT in electronic sector. Most of the studies (cradle-to-grave) assume as end of life the incineration in municipal solid waste incineration plants, where models for traditional chemical incineration are adopted (Hischier and Walser, 2012). Due to the lack of information about the behavior of ENM during the waste incineration, the fate of engineered nanomaterial is not accounted(e.g. Table 2.1 LCA study [6]). It is unknown what ENM fraction remains in the slag and what percentage becomes air bones or degrades under incineration condition (Som et al.,. 2010). To date, the release into atmosphere of nanoparticles due to incineration process is estimated by a fate model based on the removal efficiency of the incineration plant and by treating the nanoparticle as "particulate matter". In accordance to a fate modeling study (Gottschalk et al., 2010), the release of nanoparticles into atmosphere based on the removal efficiency (99.9%) of multistage flue gas cleaning filters for particles smaller than 100 nm has been estimated being 0.1%. Another open question is the "recyclability" of the nanostructured materials containing ENMs; little literature appears to have been evolved around nanoproducts recycling. The low number of citations may be related to the lack of development for recycling infrastructure technology or to the cost. The recycling technology for nanoproducts might benefit from further development of the technology (Asmatulu et al., 2012).

2.2.2 Life cycle inventory in the nanotechnology LCA study

An adequate LCI data on materials is necessary for an appropriate Life Cycle Impact Assessment. Therefore adequate and comprehensive LCI data for engineered nanomaterials are requested. To date, the LCA studies on nanomaterials are based on LCI data of publicly available literature or, in few cases, on pilot/commercial plants.

The input data on the extraction of raw materials, the production of precursors, the request of energy for the production of engineered nanomaterials are available in the current LCI data –bases (ETH database, Simapro7, Ecoinvent databes ecc.). In contrast, the life cycle inventory data on emissions (output data) to air, water or soil is scarcely covered (Hischier and Walser, 2012). Currently, two issues may be drawn about the life cycle inventory analysis concerning the engineered nanomaterials.

Firstly, specific physical-chemical properties may be required on the nanomaterials. The second one concerns the knowledge of the production processes of nanoproducts and the potential emissions during all life stages of the nanoproducts. In standard LCI tables, only the quantity and the chemical composition of releases are reported; generally, few chemicals require additional characteristics such as, their isotope (for radioactive releases), their stereo-isomer (for a chemical like cyclohexane) or their valence (for an ion such as chromium). On the contrary, several parameters influence fate, exposure, and effect of nanoparticles in the environment. For instance, chemical composition, particle size, shape, crystal structure, surface charge, solubility and adhesion properties likely influence the toxicity of nanomaterials. Moreover, as nanoparticles may also be coated, it is important to find out whether to report the pure material or the coated material. In this context, it is also important to know whether nanoparticles change their form (shape, coating, etc.) during their life cycle, for instance, due to aging and other influences such as weather, mechanical stress/pressure, electromechanical fields or catalysis. As a result, the elementary flows characterizing nanomaterials in the inventory may require that these additional characteristics be described. The production of data of nanomaterials and structure shall be based on precise and comprehensive LCI data with high level and representativeness. As nanoproducts are only starting to enter the market, it is at present unclear how processes related to use, maintenance and end-of-life services (e.g. disposal, recycling) will proceed. Some materials will be released during use, both intentionally and unintentionally (e.g., nano-additives in tires or nanoparticles in sunscreen). Exact release rates are not always available, especially when they are condition-dependent and the behaviour of nanomaterials discarded after use is also not yet clear. For instance, their reaction with other materials in an incinerator or at a dump site is uncertain, yet these are required data in an LCA study (Asmatulu et al., 2012).

2.2.3 Life Cycle Impact Assessment in the nanotechnology LCA study

As argued before, the LCIA phase requires the knowledge on the toxic effect following the release of nanomaterials/nanoparticles to the environment. For LCIA proposal and for the impact category of aquatic ecotoxicity, the quantification of the toxic effect is based on toxicity values collected from the main databases (e.g. IUCLID, *International Uniform Chemical Information Database).*

Currently, due to the lack of a specific database for nanoparticles and/or nanoproducts toxicity, data have to be collected by means of bibliographic reviews or literature where a strong variability of the toxic data on ENPs is reported. The high variability of the toxic data for ENPs and the lack of specific ENPs-fate models are both referred to as the reason an incomplete life cycle impact assessment phase on ENMs in the LCA studies performed until now.

The reviews performed by Hischiers and Walser (2012) and Gavankar et al. (2012) show that the phase of impact assessment is not complete in the sense of ISO 14040 series. The LCA studies on ENMs not do cover a complete life cycle of engineered nanomaterials or products. Most of the studies are cradle-to-gate and the environmental impacts are correlated to the energy and material flows for the extraction of raw materials and manufacturing phases (cradle-to-gate analysis). All this without considering the nano-specific fate, transport, and the toxicity and ecotoxicity. Although aspects relating to (eco)toxicity are usually assessed in LCA, the specific potential impacts of ENMs have not been included in the studies done so far, due to a lack of knowledge in relation to risk assessment.

This bottleneck is reflected in LCIA where characterization factors for nanoparticles are completely missed. To my knowledge only two recent studies (Eckelman, et al., 2012; Walser et al., 2011) assessed the potential toxic impact of nanoproducts. Eckelman et al. (2012) quantified and compared aquatic ecotoxicity impacts over the life cycle (production, use and release) of carbon nanotubes (CNTs) by employing the USEtox model. Walser et al. (2011) performed a cradle-to-grave LCA study to compare nanosilver T-shirts with conventional T-shirts with and without biocidal treatment (triclosan), thus assessing global warming potential, freshwater and seawater toxicity (1,4 kg-DCB-eq.).

In LCIA, the characterisation of toxic impact categories (e.g. freshwater ecotoxicity) requires the qualitative and quantitative knowledge of the exposure to a substance.

The characterisation factor of a substance is developed on the basis of 1) “fate and exposure model” (e.g. USEtox, Rosenbaum et al., 2008) which calculates the environmental concentration at which the organisms are exposed and of 2) its toxicity potential (e.g. concentration of toxic effect).

Table 2.1 (a): LCA studies of engineered nanomaterials; Source (Gavankar et al., 2012) (Hischier et al., 2012)

	Author	Type of study	Covered nanomaterials	Focus of the study	Functional unit	LCA phase	Impact assessment
[1]	Greijer 2011	LCA, cradle-to-grave	Nanocrystalline dye		1 kWh electricity output from the solar cell system	All phases	Global warming
[2]	Lloyd and 2003; Lloyd 2004	Hybrid LCA	Polymer nanocomposite (based on nanoclay)	Replacing auto-body panels made of steel with those in polymer composites and aluminium		Extraction and manufacturing	Global warming and toxic releases; Toxicity for scenario based material composition of evaluated on nano/non-nano options. Toxicity and manufacturing of the nanocomposite itself are not discussed
[3]	Steinfeldt 2004a; Steinfeldt 2004b	LCA, cradle-to-grave	(i)Nano varnish,(ii,iii) carbon nanotubes, (iv) quantum dots	Lighting, Chemical/paintings, Chemical/plastic, Electronics/displays	(i) surface treatments of 1 m^2 metal surface, (ii) 1kg Styrol, (iii) 17"flat screen, (iv) 6.579 Mio Lumen hours	All phases	Various midpoint indicators
[4]	Lloyd 2005	Hybrid LCA	Nanoscale platinum group (PGM) metal particles	Evaluating reduction in non-renewable resources like PGM via greater process control offered by nanotech	Total PGM demand for the US vehicle fleet	Extraction and manufacturing	Global warming and various (cumulative) inventory items. The focus is on how nanotechnology can help reduce the usage of precious metal within the same process with the same output as before.
[5]	Osterwalder 2006	Energy and CO_2 analysis, cradle-to-gate	Nanoparticle of titanium dioxide and zirconia	Energy comparison of wet and dry synthesis methods for oxide nanoparticle production	1 ton of nanomaterials	Manufacturing	Focus on the level of consumed energy and produced CO_2 emissions. Release and impact of emission, associated toxicity during nano-manufacturing and nano-synthesis not considered.
[6]	Roes 2007	LCA, cradle-to-grave	Polymer nanocomposite	Compare environmental impacts ad costs with nanocomposite products vis-a-vis those with conventional products	(i)amount of packaging film for 1000 bags for 200 g candies (ii) amount of fill to cover a standard greenhouse of 650 m^3 (iii) body panels to drive 150.000km	All phases	Various midpoint indicators (CML methods). Human and ecotoxicity not considered. Use phase assumed to be the same for nano and conventional
[7]	Kushnir and Sanden 2008	Energy analysis, LCA cradle to gate	Carbon nanoparticles e.g. Fullerene and nanotubes	Implications for industrial scale production	1 kg CNP	Extraction and manufacturing	Energy analysis; no discussion on nano-specific environmental impacts
[8]	Healy 2008	LCA,cradle-to-grate	Single-wall carbon nanotubes (SWCNT)	Environmental assessment of SWNT production	1 kg SWCNT	Extraction and manufacturing	Airborne inorganics, climate change, acidification. Qualitative description of EHS concerns due to nanomaterial No quantified evaluation as part of LCA study
[9]	Singh 2008; Aghooia 2005	LCA,cradle-to-grate	Single-wall carbon nanotubes (SWCNT)	EIA via LCA method (cradle-to- gate (?))	596 KG OF CARBON NANOTUBES PER HOUR	Extraction and manufacturing	Use of TRACI methods
[10]	Khanna 2008	Energy analysis, LCA cradle-to- gate	Carbon nanofiber (CNF)		1kg CNF	Extraction and manufacturing	Various midpoint indicators (GWP,AP..)from CML method. Release and impact of CNF on humans and ecosystem during manufacturing not considered

Table 2.1 (b): LCA studies of engineered nanomaterials; Source (Gavankar et al., 2012) (Hischier et al., 2012)

	Author	Type of study	Covered nanomaterials	Focus of the study	Functional unit	LCA phase	Impact assessment
[11]	Kohler 2009		Carbon nanotubes (CNT)	Potential release of CNT from the life cycle of textiles and lithium batteries		Manufacturing, use and end-of life	The study indicates that the release of CNT occurs also in the usage and disposal phase of nanotube applications
[12]	Bauer ,2008		TiN, TiAIN,Ti+,TiAIN	Examine the implications of life thinking to nanothecnology		Manufacturing	Nano-related emissions not considered
[13]		LCA,cradle-to-grave	Carbon nanotubes (CNT)		a 15" field emissions displays (FEO) television	All phases	Various midpoint indicators (CMLmethods). Potential risk at EOL noted but not quantified
[14]	Khanna and Bakshi, 2009	Energy analysis, LCA cradle-to-gate	Carbon nanofiber polymer composite	Energy implications for production and use of carbon nanofiber reinforced polymer nanocomposites	(i) equivalent of standard steel plate (ii) 150.000 vehicle miles size of 3300 lbs body panel in a midsize car	Extraction and manufacturing	Global warming potential and cumulative energy demand (in Mj) Production energy requirement comparison
[15]	Fthenakis 2009	LCA cradle-to-gate	Nano CdTe, nanocrystalline-Si and nano-Ag contact PV system		1 m^3 of PV cell		Only cumulative energy demand
[16]	Meyer 2010	Screening-LCA	Silver nano	Identify the life cycle hot spots via screening-level LCA		Extraction and manufacturing	EOL not considered; assumed to be the same as non-nano-silver socks as nano-silver assumed to have been washed off during the use phase
[17]	Roes 2010	LCA cradle to grave	Nanoscale silica		1 kg of nanomaterial and material per stiffness	All phases	Global warming and non-renewable energy use
[18]	Grubb and Bakshi 2011	Energy analysis, exergy analysis and LCA cradle to grave	Titanium dioxide	Evaluate the hydrochloride production process	1 kg of titanium dioxide	Extraction and manufacturing	Midpoint and endpoint indicators on EI 99
[19]	Walser 2011	LCA cradle-to-gate	Nano silver	Compare conventional T-shirt woth nano-silver T-shirt		All phases	Global warming potential. Freshwater ecotoxicity and seawater toxicity

2.2.4 Importance of evaluating fate and effect of nanoparticles

Focusing on nanoparticles, the assessment of the environmental exposure requires the knowledge of the environmental behaviour of the ENPs in the environment media (for example freshwater, air, soil, etc.). In the last couple of years, frameworks to assess the exposure or risk assessment of ENPs have been developed (Mueller et al., 2008; Gottschalk et al., 2010). There are evidences that the ambient chemistry parameters influence the fate processes and the toxicity of the ENPs (Levard et al., 2012). In the freshwater compartment, physical-chemical parameters such as pH, natural organic matter (NOM) and ionic strength are directly involved in the processes of dissolution and aggregation which have been referred to as important key factors in the field of nano (eco) toxicity; reports evidenced that the aggregation process leads adhesion of the ENPs (n- TiO_2) to the body of the organism (*Daphnia magna*) and influenced the mobility (Baun et al., 2008; Dabrunz et al., 2011). It was also shown that the dissolution of metallic nanoparticle (n-ZnO) could influence the toxicity due to metal ions released from metal oxide particles (Xiong et al., 2011). These researches highlight that the bioavailable forms is strictly related to the environmental chemical conditions. It is evident that in order to determine the likely bioavailability to organisms of metal oxides nanoparticles is crucial to determine the chemical fate in the environmental compartment (Johnston et al., 2010). Currently, the fate model specific for nanomaterial are still in their infancy. However, the possibility to refine the existing fate and transport models with processes that influence the fate and transport has been proposed by recent studies (Praetorius et al., 2012; Arvidsson et al., 2011; Quik et al., 2011). Whereas physical-chemical parameters of the media are required, due to the evidence that the behaviour (tendency to form aggregate, to settling, or dissolve) of ENPs is strongly affected either by the environmental conditions and physical-chemical parameters of ENPs.

The ecotoxicity of ENPs is still in debate; an high variability of data is shown in literature. For instance, the review about toxicity of nanosized TiO_2 on freshwater invertebrates conducted by Menard et al. (2011) shows that the effect values on crustaceans *Daphnia magna* range from 5.5 mg/L (Lovern and Kapler, 2006) up to 2000 mg/L (Heinlaan et al., 2008). The high variability of the toxic data is referred to be dependent by several factors, as the lack of a reference ENPs, the lack of standardized procedures for bioassay proposals. This high variability of the toxic data, the low knowledge on the mechanism of effect of ENPs to organism, the gap of knowledge on the exposure of organisms to ENPs are referred to as the main cause for which methodologies as Risk assessment are far to be applicable to ENPs. Also, this gap of knowledge is reflected in LCIA where evaluation of toxic effect is required.

2.3 Conclusion and outlook

The state of art about the studies published on "LCA and nanotechnologies" evidences **several limitations** on the LCA methodology.

- The novelty of this new technology seems not to be well represented by a weight-based functional unit when comparative LCA are performed, whereas the functionality of the engineered nanoproducts may be more appropriate for this new technology (Hischer et al., 2012).
- The inventory data used in the nanomaterial LCA studies published until now cannot be classified as comprehensive due to the lack of emission data.
- Regarding the life cycle impact assessment there is a complete lack of characterisation factors for ENMs.

Therefore, most of the studies have put a dominant focus on the assessment of energy requirements and climate change impacts, while toxicity assessment has received lesser attention. The main reason for the lack of characterisation factors, is considered as a direct consequence of the gap of knowledge on the environmental fate and behaviour of ENPs (e.g. chemical transformation of the ENP released by the nanoproducts) and the toxic effect posed by the exposure to ENPs.

A life cycle based approach could show a likely exposure scenario and identify potential receptors. Anyway several limitations to performing LCA studies have been outlined in literature. Monitoring data and simulation models could be used to refine and validate the conceptual LCA framework.

Also, new exposure scenarios over product life cycle are necessary to consider changes in exposure during manufacture, use and disposal:

- Occupational exposure during manufacturing and recycling;
- Consumer exposure: exposure pathways associated with the intended use of product, transformation and persistence in the environment, type of exposure (e.g. dermal contact with textile product);
- Transport through the environment or human body: nanomaterials/nanoparticles could be released into the environment during the use phase (e.g. abrasion, washing of textile product);
- Secondary exposure: the disposal of nanoproducts creates the possibility of transport into the environment;
- Animal species: incidental release or disposal in waste streams, bioaccumulation.

In contrast to the organic and inorganic substance, new exposure metric such as shape, surface area, chemical composition size, agglomeration state, crystal structure will be needed to characterize nanomaterials and to assess the environmental and human exposure. Furthermore, models that only account for chemical concentration in the environment may be inadequate for nanomaterials if the environmental behaviour and toxicity is related to particle size, aggregate state, surface area etc. (Abbott and Maynard, 2010).

As argued before, the LCA approach is based on a global, continental geographic scale of impact assessment. This approach may be not appropriate for nanoparticles, for which site-specific conditions strongly affected their fate, behaviour and bioavailability. A growing interest towards introducing spatial differentiation in regional impact categories emerged. Different indicators and characterization models have been proposed to calculate the site-dependent CFs for a variable number of interventions and for the following impact categories: acidification, photo-oxidant formation, terrestrial eutrophication and toxicological impacts (Zamagni et al., 2008). However, spatial differentiation requires collecting location-specific data and calculating spatially specific characterisation factors (CF). Whereas, location-specific data are rarely available for all processes within a product life cycle, but at least for processes that appear to predominate in the overall impact of a product life cycle, additional effort to collect location-specific data is advisable (Zamagni et al., 2008).

Thus, the **next challenge** for LCA in the field of nanotechnology is the development of a framework for a comprehensive and adequate modelling for nanomaterials with: 1) an implemented inventory phase, in which the emissions of nanoparticles (output data) to the environment can be assessed; 2) new exposure scenarios; 3) environmental fate and exposure models compatible with the current LCIA modelling but also considering the specific fate and behaviour of ENP in the environment; 4) criteria on how to select input data for ecotoxicological factors.

Focusing on the phase of Life Cycle Impact Assessment, in the next chapters the environmental behaviour of metal oxide nanoparticles (TiO_2) and their toxic effect on three trophic levels (algae, crustaceans and fish) will be discussed.

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3 Engineered nanoparticles (ENPs) environmental fate processes in freshwater and environmental exposure assessment

3.1 Introduction

To date, there is little known of the fate and behaviour of ENPs in the environment. However, the increasing of scientific researches on the environemntal behaviour of ENPs inidicates that careful consideration on the chemistry of nanoparticles and how it relates to their fate in surface waters and sediments is key to predicting their final fate.

Due to the rapid increase in productions rates of ENPs and inevitable release in the natural environment (Ottofuelling et al., 2011) concerns on the health and environmental potential impacts are arising. In fact, still little is known on their environmental behavior and large uncertainties on their toxic effect to aquatic organism are reported. Furthermore, the environmental concentrations of this new class of substance is difficult to predict and analyze. As consequences, methodologies as Risk Assessment and/or Environmental Impact Assessment for ENPs are still in their infancy.

This chapter aims to describe (i) the environmental fate processes of metal oxide nanoparticles in freshwater ; (ii) a brief overview on the environmental assessment of ENPs (metal oxides nanoparticles); (iii) to summarize the challenges and limitations of exposure assessment for the LCIA purpose.

3.2 Environmental fate processes

The main fate processes of ENPs in aquatic environment are: i) aggregation and precipitation to sediments, ii) transformation and degradation (dissolution, oxidation, photodegradation and/or being surface coated and passivated by coexisting matter). The fate processes are influenced both by the water chemistry (pH, ionic strength, and natural organic matter (NOM) and by the intrinsic properties of ENPs. For instance, once released in aqueous system they may aggregate but may be also stabilized and transported within water flow, depending on the properties of ENPs and the condition of water chemistry (Lin et al., 2010). The knowledge and assessment of environmental behavior in aquatic system play a key role for the exposure assessment of ENPs. Furthermore, the environmental behavior of ENPs is strictly correlated to their bioavailability and thus, toxicity.

To better understand the likely fate of ENPs in aquatic systems, it is essential to understand their interaction with the water chemistry parameters such as pH, ionic strength (I) and type and concentration of cations (Christian et al., 2008). Little information about these interactions is yet available specifically for the engineered nanoparticles. Furthermore, most studies have considered ENPs dispersed in idealized systems tending to focus on the influence of a single environmental variable (e.g., pH, ion concentration or charge, the presence or absence of NOM). It remains unclear whether trends in nanoparticle behavior established in such single variable systems will remain relevant in more complex aquatic environments where multiple factors may impact nanomaterial stability in parallel (Chowdhury et al., 2012).

3.2.1 Dissolution

The dissolution of ENPs is, in essence, the transformation of the nanoparticulate form of a chemical compound into the dissolved ionic form of a compound. For nanoparticles, especially for metal-based ENPs, the dissolution may be a critical factor for the fate in the environment. The dissolution of ENPs can depend on their properties (e.g., solute concentration, surface area, surface morphology, surface energy, adsorbing species, and aggregation) and on the properties of the solution as well (e.g., pH, ionic strength, constituent solvated molecules and concentration, and temperature).

To date, little is known about solubility and rates of dissolution of metal oxide nanoparticles in water. In absence of such knowledge, modeling dissolution remains highly speculative (Quik et al., 2011).

In principle the dissolution is described as a surface controlled processes, where the equilibrium solubility of particles increases with decreasing particle size. The driving force for dissolution depends on the metal solubility, within a given environment, as well as the concentration gradient between the particle surface and the solution phase. *Thus, the amount M (kg) of ENPs that dissolves in water per unit time t (s) is expected to be proportional to the area A (m^2) of the nanoparticles' surface and to the concentration of dissolved material near the particle's surface that should be close to the chemical's water solubility* S (kg m^{-3}) (Quik et al., 2011):

$$\frac{dM}{dt} = -kSA \quad (3.1)$$

The dissolution rate constant k (m s^{-1}) reflects the local hydrodynamic conditions near the nanoparticle–water interface, that are little known. A first order kinetic of dissolution should be expected only when area and mass are proportional. For nanoparticles this should not be expected, because the specific surface area (area per unit mass) of particles is expected to increase with the decrease in particle size, resulting from dissolution itself. However, in absence of more data on dissolution for ENPs, a dissolution rate constant of first order can be acceptable. This does indicate the large knowledge gap that remains to be filled before dissolution can be modeled adequately. (Quik et al., 2011).

Nanomaterials in aqueous suspensions, are dynamic systems undergoing simultaneous dissolution, aggregation, and sedimentation. For instance, during a 48-h exposure, the 50% of nanoparticulate copper and the 90% of nanoparticulate silver of the initial mass can be lost through aggregation and sedimentation (Griffitt et al., 2008). The formation of agglomerates can hinder dissolution by reducing the average equilibrium solubility of the nanoparticle system. Also, the adsorption of molecules and ions from the surrounding can have a significant effect on solubility and dissolution kinetics of ENPs (Lin et al., 2010). And also, the pH of the solution can definitely influence the dissolution of ENPs. It was reported that low pH (pH=2) can promote the dissolution of metal-based ENPs, such iron nanoparticles (Baalousha et al., 2009).

Dissolution affects ENPs surface properties, toxicity, and persistence. This is especially true for ENPs made of soft metal cations (e.g., Ag, Zn, and Cu) because they form partially soluble metal oxides, and because they have a strong affinity for inorganic and organic sulfide ligands. For these ENPs, the toxicity is commonly expressed through dissolution and release of toxic cations. Complete dissolution may allow prediction of their impact using existing models for metal speciation and effects. However, their high reactivity with sulfur-containing biomacromolecules and inorganic sulfur in sediments, soils, and air induce the formation of a relatively insoluble metal-sulfide shell on the particle surface that can alter the surface charge and induce aggregation

Studies on particle dissolution have been performed as part of assessing the biological effect of the dissolution of ENPs.

It was observed that the role of dissolution to toxicity of freshwater organisms (*D.magna and Zebrafish*) varies significantly with the nanoparticles composition (e.g. silver, copper, nickel, cobalt, and aluminum). Dissolution of nanosilver and nanocopper is relatively low, and therefore in these ENPs the toxicity to Zebrafish and daphnids ***is unlikely to be attributable solely to particle solubilization***. In contrast, the toxicity shown by nano nickel to daphnids could largely be attributed to the presence of dissolved nickel. In general it was found that particle dissolution explains not at all the toxicity, suggesting that the dissolution in not the mainly mechanism of toxicity. (Griffitt et al., 2008). Whereas. other mechanism can attend to the toxicity, such as the aggregation and the formation of ROS.

3.2.2 Chemical transformation: oxidation and reduction

In natural system, the chemical transformations of nanoparticles include the reduction and oxidation processes, involving the transfer of electrons to and from chemical moieties. A number of ENPs, including silver, iron and cerium may be composed of, or contain, constituents that undergo reduction and oxidation in both aquatic and terrestrial environments. In some cases, oxidation may result in the accumulation of a relatively insoluble oxide surface coating on the ENPs that passivates the surface and reduces subsequent oxidation, while also forming metal-oxide phases with a high capacity for binding ions from solution. In other cases, (e.g. Ag NPs), oxidation of Ag (0) to Ag (I) is required to dissolve and release bactericidal Ag^+.

Natural waters and aerated soils are predominantly oxidizing environments, while carbon-rich sediments and groundwater may be depleted of oxygen and result in ENPs reduction. The reduction and oxidation processes of ENPs are relevant to assess the toxicity and persistence in the environment. However, the processes depend on environmental conditions and more research should be performed ***to consider the local condition of the environmental of release.***

Photo oxidation and photo reduction, sunlight-catalyzed redox reactions, may prove to be very important transformation processes affecting ENP coatings, oxidation state, generation of reactive oxygen species (ROS), and persistence. (Lowry et al., 2012). The nanoparticles of titanium dioxide are widely applied in commercial applications (e.g. paints, sunscreens) due to their electrical and optical properties. The photocatalytic properties of nano-sized TiO_2 are used in the photo degradation of pollutants, the treatment of wastewater, and the destruction of tumor cells. Also, the photo-oxidation of nano-TiO_2 is applied as an environmental friendly application to reduce the environmental pollution in water or air. But, on the other hand, these applications rely on the ability of TiO_2 nanoparticles to form reactive oxygen species (ROS) on their surface when excited with UV light, giving rise to concerns about likely toxic effect.

3.2.3 Physical transformation: aggregation

The knowledge of aggregation processes is a key step to assess the transport, the persistence and the toxicity of ENPs in aquatic system. Aggregation may take on two forms: homoaggregation between the same ENPs, or heteroaggregation between a ENPs and another particle in the environment (e.g. Suspended Particle Matter, SPM). In most cases, the greater concentration of environmental particles compared to ENPs will result in heteroaggregation (Praetorius A. 2012). Over the time, when aggregation processes occur, the number concentration of ENPs in suspension decreases with an increase in their aggregate size which should reach a size in the order of micrometers.

The aggregation processes of ENPs in aquatic system are more deeply evaluated; to date there are evidences that the aggregation of ENPs in aquatic system is in agreement with the colloidal science and with the Derjaguin-Landau-Verwey-Overbeek, DLVO[3] theory. In fact the aggregation of ENPs in aqueous

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[3] This theory maintains that only two forces dominate interactions between particles: van der Waals (vdW) attractive and electrostatic double layer (EDL) forces.

dispersions involves the formation and growth of clusters that is controlled by both interfacial chemical reactions described by DLVO theory and particle transport mechanisms, in accordance with the colloidal science.

Theory of colloid science has been applied to nanomaterials and several experimental data show that the behavior of ENPs is in agreement with this and DLVO theory. (French et al., 2009; Domingos et a., 2010; Chowdhury et al., 2012). Therefore, the colloidal science and the DLVO are applied to predict the environmental fate of ENPs (n-TiO_2) in freshwater ecosystems (Hotze et al., 2010; Praetorius et al., 2012, Lead et al., 2006). On the basis of the colloidal science and the DLVO theory, the aggregation processes are strongly influenced by water chemistry e.g. pH, ionic strength, ionic composition and concentration (mono-divalent ions), Natural Organic Matter (NOM) and TOC (Total organic carbon). To date, there is a number of experimental studies dealing with the aggregation and deposition of ENPs (French et al., 2009; Domingos et al., 2010). These studies have been conducted under controlled test conditions by adjusting pH, ionic strength, composition of mono- and divalent ions as well as the concentration of dissolved organic matter.

Nanoparticles are surrounded by a charge layer arising from the residual ions in solution. The surface layer can be compressed or expanded depending on the total ionic concentration of the surrounding solution. The presence of calcium (Ca^{2+}) increases the average diameter of ENPs (such as n-TiO_2), in accordance to electrophoretic motilities[4] measurements, for which a decrease of motilities with the increase of calcium concentration (from 0 M up to 3.3×10^{-4} M) was observed, whereas in absence of fulvic acid the calcium appeared to be less effective in aggregating the n-TiO_2 nanoparticles. Thus, fulvic acids have the tendency to stabilize the n-TiO_2 suspension and the aggregation and the mobility of nanoparticle are affected (Domingos et al., 2010).

The ionic strength greatly influences the environmental behavior of ENPs in water suspension, in fact an increase in ionic strength of the media promote the aggregation[5]. French et al., (2009) dispersed n-TiO_2 at different ionic strength (0.0045 M and 0.0085 M). The aggregation kinetic was evaluated at a pH lower than the Point of Zero Charge (pHpzc[6]). At lowest ionic strength no significant aggregation (size distribution around 50-60 nm) occurs in the media, in contrast at higher ionic strength aggregates reach

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[4] The aggregation processes of nanoparticles in solution may be detected by electrophoretic measurements. The electrophoretic behaviour of very small particles is reversed, and larger particles are electrophoretically slower than smaller ones. And as expected by the DLVO theory the measured mobilities vary with both electrolyte type and concentration. (Agnihotri et al., 2009).

Usually, the electrophoretic mobility is detected under experimental condition at different concentration of type or ions in solution or at different pH range. This experimental set-up might not be representative of that observed in far more complex natural environments. However, the findings demonstrate that the mobility of nanoparticles greatly depend on the “experimental mixtures” studied, and thus on the natural environmental condition. (Domingos et al., 2010)

[5] In accordance with the DLVO theory an increase in ionic strength in which the ENPs are dispersed compress the diffusive layer, diminish interparticle repulsion and promote the aggregation.

[6] The pH where the net total particle charge is zero is called point of zero charge (PZC)

the size of micron. And more the aggregate size distribution became multimodal. ***This meaning that different distribution of size may be found in suspension***. Furthermore, the pH of the solution affects the surface charge of the ENPs in suspension. When the pH is close to the point of zero charge (PZC) or isoelectric point, the colloidal system exhibits minimum stability (e.g., exhibits maximum coagulation/ flocculation). When the pH is lower than the PZC value, the colloid surface is positively charged and the zeta potential will increase with a pH decreasing below the PZC.

Even if the experimental studies performed under different solution chemistry are helpful to provide a mechanistic understanding of the environmental behavior of ENPs in solution, the experimental set-up might not be representative of more complex natural environments. Where, a wide range of water conditions in regards of ionic strength , NOM and ionic composition are expected.

Fewer studies assess the aggregation and deposition on ENPs in natural aqueous matrices. Sillanpää et al., (2011) address the aggregation and deposition of n-TiO_2-particles in two different natural and brackish water samples. The experiments were performed at two particle concentrations: 100 mg/L and 1 mg/L (10 mg/L for deposition studies). The study shows higher aggregation rates in brackish waters than in natural waters, which is explained by a higher ionic strength of the water sampled. Also, the aggregation was addressed to as concentration-dependent and fast. A high aggregation was observed at the highest concentration (100 mg/L) tested and already after 3 minutes of sampling an increase from 200 nm to 1500 nm in terms of hydrodynamic diameter was detected. Also, the influence of water chemistry on the aggregation process is well demonstrated by the aggregation rate collected: a difference in the agglomeration rates between the two fresh water samples was observed (Fig.3.1). In “natural water A” (green line Fig.3.1) the aggregation, expressed in terms of hydrodynamic diameter, increases over time and gets close to 1500 nm. In contrast, in “natural water B” (green line Fig.3.1) the hydrodynamic diameter is constant over time and close to 500 nm. The difference between the two natural water samples is recognized in terms of conductivity and concentration of divalent ions. In fact the “natural water sample A” is characterized by a higher conductivity and higher concentrations of divalent ions and lower TOC, than the “natural water sample B”. Additionally, in “natural water A” the pH value is close to the isoelectric point of the nanoparticles of titanium dioxide (P25). For both brackish waters sampled, the hydrodynamic diameter increased close to 3000 nm, thus confirming that the agglomeration rate increases in high ion strength conditions (Fig.3.1).

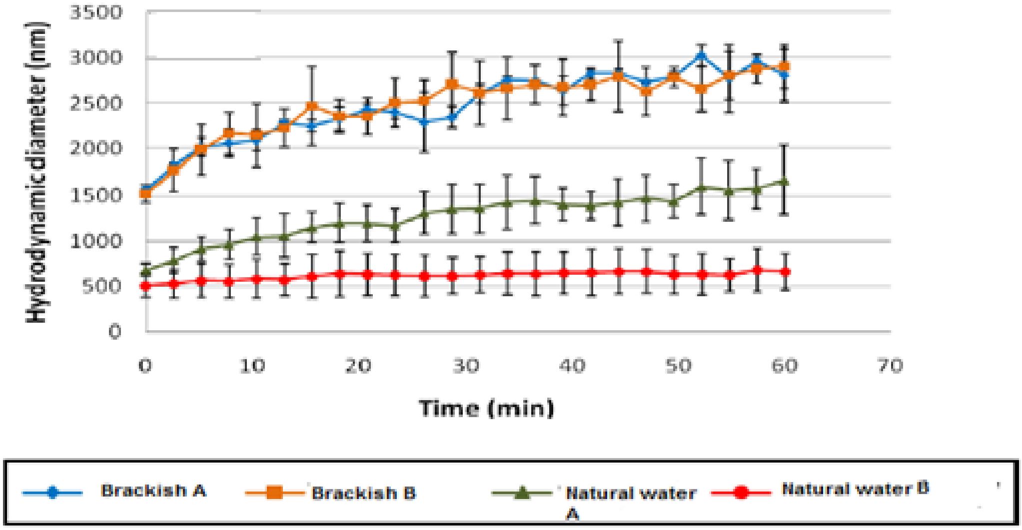

Fig. 3.1: The mean of hydrodynamic diameter of TiO_2-P25 spiked in four different natural waters (brackish and natural water) (Source: Sillanpaa M., 2011)

Furthermore, the research outlines that the aggregation is concentration–dependent. Testing a lower concentration (1 mg/L) results in lower values of hydrodynamic diameter (nm) for all the 4 water samples. This is due to the increasing distance between the suspended particles at the decreasing concentrations, which results in the decreased probability of collisions between particles. A similar result during the experimental tests on the characterization of (n-TiO_2) was observed during my experimental research on the characterization of n-TiO_2 biological test media (Salieri et al., 2012; see Chapter 4, section 4.2)

An extensive study of ENPs environmental behavior in natural water samples has been performed by Keller et al. (2010). The authors collected several water samples: one from lagoon water, one groundwater samples, one river water, one storm runoff, and one freshwater mesocosmos (common growth media for primary producers). The electrophoretic mobilities and sedimentation and aggregation rates of three metal oxide nanoparticles (TiO_2, ZnO, CeO_2) were measured. At the pH of water samples in the range of 7.07 and 8.90, the ZnO is positively charged (except at pH=8.90 which refers to lagoon water), n-TiO_2 is negatively charged and CeO_2 is close to its isoelectric point. The seawater samples show low TOC (in the range 54.0-131.1 μMC) and high value of Ionic strength (IS): 7.07×10^{-1} to 6.79×10^{-1} eq. L^{-1}.

The Santa Clara river water has an IS of 1.84 x 10-2 eq.L^{-1} and TOC of 163.8 μMC; the mesocosmos freshwater has a lower IS (7.18×10^{-3} eq. L^{-1}) value and higher concentration of TOC (5,283 μMC). At the lowest value of IS and highest concentration of TOC, such as mesocosmos freshwater, a high stability of the three nanoparticles was observed and the size of aggregates remained close to 300 nm. The stability of nanoparticles is affected by the amount of organic molecules that can be adsorbed onto the particle surface providing a barrier to aggregation. In contrast, in seawater sample with highest value of IS but lowest TOC a strong and fast aggregation was observed (Fig.3.2). The aggregation as a concentration–dependent process has been confirmed by the sedimentation rate of the three nanoparticles. At the highest concentration tested (e.g. TiO_2 200 mg/L) the sedimentation is more pronounced and faster than at lower concentration. **Furthermore the sedimentation rates differs among the water samples and also between the ENPs tested. For n-TiO_2 the sedimentation is greatly influenced by the type of water sample.** A faster and stronger aggregation in seawater (Santa Clara River), groundwater and lagoon water, resulting in a high deposition, was calculated; in contrast lower deposition was detected for mesocosmos freshwater, storm water and treated effluent water samples. The first four water samples are characterized by high IS and low TOC. The ZnO and CeO_2 nanoparticles show a similar behavior but without a remarkably difference among the water samples as detected for TiO_2.

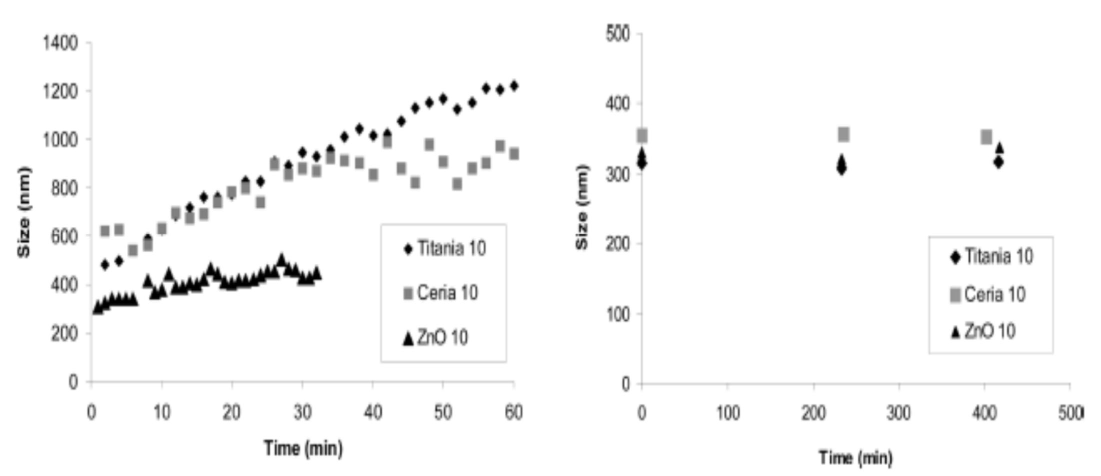

Fig.3.2: Aggregation size of TiO_2, ZnO and CeO2 at 10 mg/L in seawater (left figure) and in mesocosmos freshwater (right figure (Source: Keller et al., 2010)

Hall et al., (2009) conducted an acute toxicity test with n-TiO_2 on *Ceriodaphnia dubia* in presence (1, 5 mg/l TOC was added to the solution test) or not of TOC. In presence of TOC, the ecotoxicity tests on *C. dubia* showed much lower sensitivity to n-TiO_2 respect to conditions in which there is absence of TOC. In presence of TOC the LC50 values were always above 57 mg/L, whereas in absence of TOC the LC50 values were in the range of 3.0-13.4 mg/L. The decreasing of toxicity of n-TiO_2 with the addition of TOC, indicates that the organic carbon decrease to bioavailability of n-TiO_2; this may be due to the coating effect of organic carbon on n-TiO_2 particles. Decrease of toxicity due to a complexation mechanism has been yet recognized; for example Kolts et al., (2008) reported an average decrease in silver toxicity to *C. dubia* of 26.8-fold for tests with 2 mg/L DOC additional in test waters.

Klaine et al., (2008) and Baalousha et la., (2008) show an increase of aggregation of iron oxide nanoparticles in the presence of humic acid, further an increase of the aggregation process with the increase of the pH was reported.

3.3 Aggregation kinetics

The understanding of environmental impact of ENPs will significantly benefit from the previous knowledge about natural aquatic colloids, which are defined as materials with one dimension between 1nm and 1µm with an evident overlapping in the size domine of ENPs (1nm-100nm) (Lead et al., 2006). Due to the difference in concentration of natural colloids (present in mg/L) and ENPs (present at µg/L) in freshwater, the introduction of ENPs into the natural aquatic systems will likely to be controlled by natural colloids. A colloidal dispersion is thermodynamically unstable and will always tend to aggregate; however, it could be stabilized kinetically[7] (Handy et al., 2008; Arvidsson et al., 2011). The quantitative evaluation of the nanoparticle aggregation may be possible by following the Smoluchowsky (1917) equation which laid the foundation of the rate of aggregation (Petosa et al., 2010).Therefore, in order to describe the aggregation, it is convenient to think in terms of a dispersion of initially identical particles which, after a period of aggregation, contains aggregates of various sizes (i,j) and different number concentrations (n_i particles of size i, n_j particles of size j etc.). A fundamental assumption is that aggregation is a second-order rate process, in which the rate of collision is proportional to the product of the concentrations of two colliding species (Elimelech M.J. 1998).

Assuming that the collisions are randomly and binary, the number of collisions occurring between n_i and n_j particles in unit time and unit volume, J_{ij}, is given by:

$$J_{ij} = k_{ij} n_i n_j \quad (3.2)$$

Where k_{ij} is a second rate order constant, which depends on a number of factors, such as particle size and transport mechanism.

As far as rate of aggregation is concerned, it must be taken into account that not all collisions may be successful in producing aggregates (due to the interparticle forces, more details below). The fraction of successful collisions is given by the collision efficiency (α). If there is strong repulsion between colliding particles, there will not be any collision that give both aggregates and α= 0.001. When there is no significant net repulsion or when there is an attraction between particles, the collision efficiency can approach the unity. Following the assumption that every collision is effective in forming aggregates

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[7] Solid particles dispersed in a liquid medium represent typical sol colloids. In most cases, the dispersed colloidal particles are thermodynamically unstable, referred to as lyophobic and therefore they tend to aggregate in time.
In order to avoid aggregation, colloidal particles are typically stabilized kinetically by electrostatic repulsions. In the energy landscape the contribution of the electrostatic repulsion superimposes the Van der Waals attraction and generates an energy barrier that reduces the aggregation rate. This behaviour is quantitatively described by the DLVO theory.

(collision efficiency, α=1), the aggregation rate constant is the same as the collision rate constant. The rate of change of concentration of k-fold aggregates, where k = i + j may be expressed as (Elimelech, 1998):

$$\frac{dn_k}{dt} = \frac{1}{2}\sum_{\substack{i+j=k\\ i=1}}^{i=k-1} k_{ij}n_i n_j - n_k \sum_{k=1}^{\infty} k_{ik} n_i \quad (3.3)$$

Eq. (3.3) is based on the assumption that all particles are approximately spherical and that merging of two particles is an irreversible reaction. The first term on the right-hand side represents the rate of formation of k aggregates by collision of any pair of aggregates, *i* and *j*, such that i + j = k. Carrying out the summation by this method would mean counting each collision twice and hence the factor (-) is included. The second term accounts for the loss of k aggregates by collision, and aggregation, with any other aggregates. The terms k_{ij} and k_{ik} are the appropriate rate constants (Elimelech, 1998). **The determination of rate constants for aggregation events is dependent on two factors: (1) the mechanism by which particle collisions occur, and (2) the presence of interparticle interaction** (O' Brien, 2003).

3.3.1 Particle collision

The particle–particle collisions originate from three fundamental transport processes: 1) Brownian motion of particles leads to perikinetic aggregation; 2) particles travelling at different velocities in a shear flow experience orthokinetic (shear) aggregation; 3) particles of different size or density undergo differential settling.

3.3.1.1 Brownian motion: Perikinetic Collision Mechanism

Small particles in water can be seen to undergo random movement or *Brownian motion* (perikinetic motion). For this reason, collision between particles will occur from time to time; Smoluchowski (1917) derived an expression for collision frequency by considering the diffusive flux of the particles toward a stationary particle.

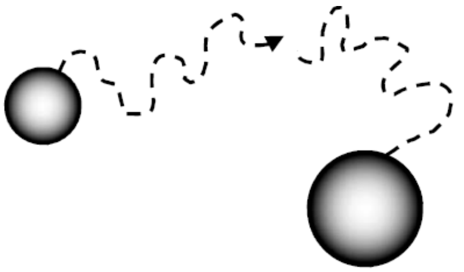


Fig. 3.3: Perikinetic motion

Using Fick's law for the number of particles J going through a unit area toward a reference particle per unit time:

$$J = -D\frac{dN}{dr} \quad (3.4)$$

where D is the diffusion coefficient of particles, N is the number concentration, and r is the radial distance from the center of a reference particle.

The diffusion coefficient of a spherical particle is given by the Stokes-Einstein equation:

$$D_i = \frac{k_B T}{6\pi\mu a_i} \quad (3.5)$$

where k_B is Boltzmann's constant, T the absolute temperature, a_i the particle radius and μ the viscosity of the suspending fluid. Smoluchowski (1917) calculated the rate of diffusion of spherical particles of type *i* to a fixed sphere *j*. If each *i* particle is captured by the central sphere on contact, then the *i* particles are effectively removed from the suspension and a concentration gradient is established in the radial direction towards the sphere, that is *j*.

After a very brief interval, steady-state conditions are established and the number of *i* particles contacting the sphere *j* per unit time is:

$$J_i = 4\pi R_{ij} D_i n_i \quad (3.6)$$

where D_i is the diffusion coefficient of particles of type *i* and n_i is their concentration in the bulk suspension. R_{ij} is the collision radius for particles *i* and *j*, which is the center-to-center distance at which they may be taken to be in contact. This is simply the sum of the particle radii, i.e.:

$$R_{ij} = a_i + a_j \quad (3.7)$$

Since that in reality, the sphere *j* is not fixed, but subject to Brownian diffusion, replacing D_i in Eq.3.6 by the mutual diffusion coefficient D_{ij} that accounts for the motion of the j particle, where:

$$D_{ij} = D_i + D_j \quad (3.8)$$

And then, if the concentration of j particles is *nj,* the number of *i-j* collisions (J_{ij}) occurring in unit volume per unit time is:

$$J_{ij} = 4\pi R_{ij} D_{ij} n_i n_j \quad (3.9)$$

Comparing the eq.(3.2) with eq.(3.9) and substituting *Rij and Dij,* with eq. (3.7), (3.8) and (3.5), the rate constant for perikinetic collisions (Elimelech MJ.,1998) is expressed as:

$$K_{ij} = \frac{2Tk_B}{3\mu}\frac{(a_i + a_j)^2}{a_i a_j} \quad (3.10)$$

For particles of approximately equal size, the collision rate constant becomes almost independent of particle size. The term $(a_i+a_j)^2/a_i a_j$ has a constant value of about 4 when $a_i=a_j$. Under these conditions, the rate constant becomes:

$$K_{ij} = \frac{8Tk_B}{3\mu} \quad (3.11)$$

Inserting values appropriate to aqueous dispersions at 25° gives:

$$K_{ij} = 1.23 * 10^{-17} \text{ m}^3\text{s}^{-1}.$$

However, the assumption of a constant value of K_{ij} is a reasonable approximation for particles which differ in size.

3.3.1.2 Orthokinetic Collision Mechanism

Brownian motion do not usually lead to the rapid formation of large aggregates. Particle transport brought about by fluid motion can give an enormous increase in the rate of interparticle collisions, and aggregation brought about in this way is known as orthokinetic collision. Smoluchowski (1917) also was the first to study the rate of orthokinetic aggregation due to uniform laminar shear forces (O' Brien, 2003).

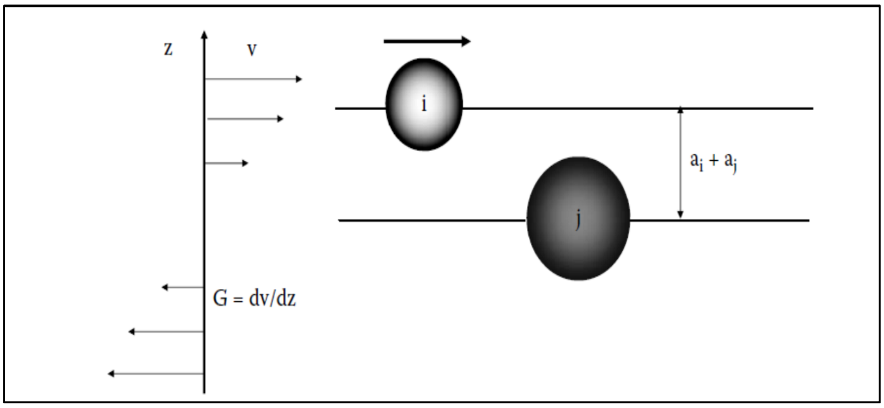


Fig. 3.4: Model for orthokinetic aggregation in uniform laminar shear (Gregory, 2006)

Fig. 3.4 shows the basic model for the treatment of orthokinetic collision rates. Two spherical particles, of different sizes, are located in a uniform shear field. A uniform laminar shear field is one in which the fluid velocity varies linearly with distance in only one direction, perpendicular to the direction of flow. The rate of change of fluid velocity in the z-direction is dv/dz. This is the shear rate and is given the symbol G. The center of one particle, of radius a_j , is imagined to be located in a plane where the fluid velocity is zero, and particles above and below this plane move along fluid streamlines with different velocities, depending on their position. A particle of radius a_i will contact the central sphere (a_j) if its center lies on a streamline at a distance a_i+a_j from the plane where u =0 (a_i+a_j is the collision radius) (Gregory ,2006).

The collision frequency depends on the sizes of the particles and on the velocity gradient or shear rate G. By considering a fixed central sphere of radius a_j and flowing particles of radius a_i, it can be assumed that those moving particles on streamlines will bring their centers within a distance (a_i + a_j). The collision frequency can then be calculated by considering the flux of particles through a cylinder of radius R $_{ij}$, the axis of which passes through the center of the fixed sphere j.

The total flux towards the central particle, J$_i$ is just twice that in one half of the cylinder and is given by (Elimelech, 1998):

$$J_i = 4Gn_i \int_0^{R_{ij}} z * \sqrt{(R_{ij}^2 - z^2)}\, dz = \frac{4}{3} Gn_i R_{ij}^3 \text{ (3.12)}$$

The total number of collisions occurring between *i* and *j* particles per unit volume and per unit time is then:

$$J_i = \frac{4}{3} Gn_i n_j (a_i + a_j)^3 \text{ (3.13)}$$

For analogies with the Eq.3.2 the rate constant for orthokinetic collisions between *i* and *j* particles is:

$$K_{ij} = \frac{4}{3} G(a_i + a_j)^3 \text{ (3.14)}$$

The rate constant of orthokinetic collisions is proportional to the cube of the collision radius, which has a major effect on aggregate growth rate. As aggregation proceeds and aggregate size increases, the chance of capture becomes greater. The most important difference with the perikinetic collision is the depedence on the size of the colliding particle. For particle of roughly equal size, the perikinetic collision rate constant is nearly independent of particle size. Whereas, for ortokinetic collision, the rate is proportional to the cube of collision radius. The great dependence of the rate constant from the radiius, means that the assumpiton of a constant value of K$_{ij}$ is not accetable.

3.3.1.3 Differential Settling Mechanism

Another important collision mechanism arises whenever particles of different size and density are settling from a suspension. Particles of different diameters settle at different velocities causing the faster moving particles to collide with slower moving particles leading to aggregation. The appropriate rate can be calculated, assuming spherical particles and using Stokes' law for their sedimentation rate. By balancing the forces of gravity (*g*), buoyancy and drag, the sedimentation velocity v$_i$ of a particle of radius a$_i$ and density ρ_s in a medium of density ρ is given by Stokes' equation (O' Brien, 2003):

$$v_i = \frac{2g}{9} \frac{(\rho_s - \rho) a_i^3}{\eta} \text{ (3.15)}$$

The relative velocity between two particles of diameters a$_i$ and a$_j$ would be u = v$_i$ –v$_j$. The rate of N$_i$ particles through a cylindrical cross section of (a$_i$ + a$_j$) is given by:

$$\frac{dN_i}{dt} = N_i \pi (a_i + a_j)^2 (v_i - v_j) \text{ (3.16)}$$

Using the last two equations, the resulting collision frequency, for particles of equal density

Is given by :

$$J_{ij} = \left(\frac{2\pi g}{9\mu}\right)(\rho_s - \rho)n_i n_j(a_i + a_j)^3(a_i - a_j) \quad (3.17)$$

where g is the acceleration due to gravity, ρ_s is the density of the particles and ρ is the density of the fluid (O'Brein, 2003). Comparing eq.3.17 with eq.3.2, the rate constant for differential settling is:

$$k_{ij} = \left(\frac{2\pi g}{9\mu}\right)(\rho_s - \rho)(a_i + a_j)^3(a_i - a_j) \quad (3.18)$$

3.3.1.4 Comparison of rate

Usually it is assumed that the three mechanisms of interparticle collisions are independent and when they operate simultaneously the aggregation rates are additive. The relative magnitudes of each contribution depend on the characteristics of the suspension and flow conditions. If the densities of the particles and the dispersing medium are nearly the same, contribution due to sedimentation can be neglected. Other factors that limit the effect of sedimentation are the high viscosity of the dispersing medium and the relatively small size of particles. (O' Brien, 2003)

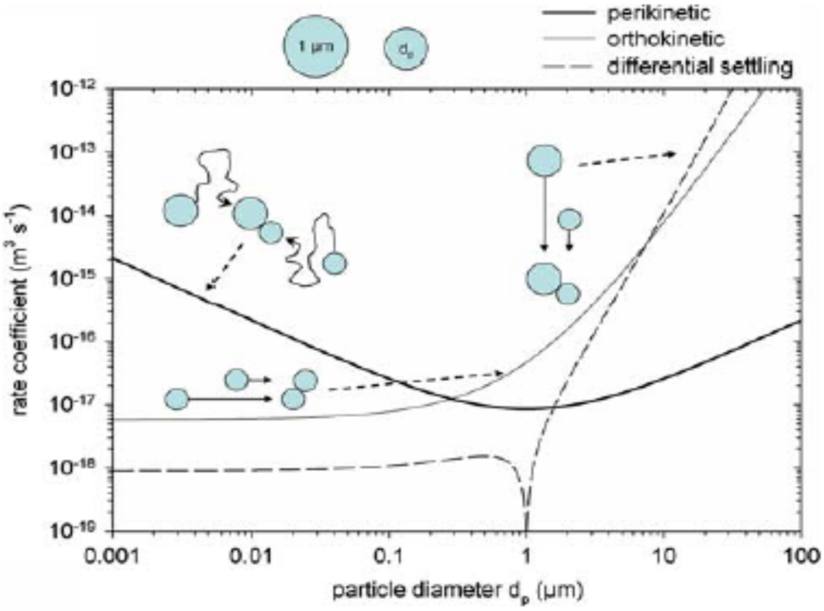


Fig.3.5: The three collision mechanisms and associated rate coefficients (Source: Handy et al., 2008)

To compare the three collision mechanisms and associated rate coefficients, one particle of fixed size (1 μm) was taken and the various rate constants as a function of the size of a second particle were evaluated. The results of such computation are shown is Fig.3.5 The computation accounts for the aggregation of 1 μm particles with particle of diameter d_p. Perikinetic mechanism gives the highest collision rates for particle less than 0.6 μm. As expected form Eq.3.10, the perikinetic mechanism has a minimum for particle of equal size. But for larger particle orthokinetic collision and differential setting become more important (Handy et al., 2008) (Elimelech, 1998).

3.3.2 Interparticle force

In aquatic system the fate and behavior of natural colloids are dominated by aggregation, which can be described with DLVO theory. The DLVO theory, developed around 1940 by Derjaguin and Landau and by Verwey and Overbeek (1948), accounts for the van der Waals and electrical double layer interactions. Other interactions affecting the colloidal stability are: hydration effects (repulsive), hydrophobic (attractive), steric interaction of adsorbed layers (usually repulsive), polymer bridging (attractive). These interactions are not taken into account in the theory; they are sometimes called non-DLVO forces.

The two major contributors to the interparticle interactions are van der Waals attractive forces and electrostatic repulsion interaction energy. The total interaction energy between the two aggregating particles will be represented by the sum of these two forces, which can be expressed mathematically as:

$$V_T(h) = V_R(h) + V_A(h) \quad (3.19)$$

Where VT is the total interaction energy for the calculation of the stability ratio, VR is the electrostatic repulsive interaction energy, and VA is the van der Waals attractive interaction energy (O'Brein). This interaction plays a key role on stability, aggregation and deposition behavior of nanoparticles in aquatic environment. The majority of studies on the environmental impact of nanoparticles have focused on qualitative interpretation of observed agglomeration behavior of nanoparticles via the classical DLVO theory. Yet, there are factors (e.g., steric, geometric, hydrodynamic, hydration, magnetic) that can impact nanoparticle agglomeration not considered by the classical DLVO theory. However, it has been generally accepted, that classical DLVO theory can provide a reasonable starting point for describing nanoparticle agglomeration in aquatic media under a wider range of environmental conditions. (Liu et al., 2011). Thus, the stability of ENPs suspended in aqueous environment, originated from forces between particle, can be evaluated as the sum van der Walls (VDW) and electrical double layer (EDL) interactions.

3.3.2.1 Electrical double layer interaction

A charged surface in contact with a solution of ions will lead to a characteristic distribution of ions in solution. If the surface is charged, then there must be a corresponding excess of oppositely charged ions (counterions) in solution to maintain electrical neutrality. The combined system of surface charge and the excess charge in solution is known as the electrical double layer. Most particles in water are charged and carry an electrical double layer. As two charged particles approach each other in water, the diffuse parts of their double layers begin to overlap and this causes an interaction. For particles with similar charge this causes a repulsion, which is the origin of colloid stability in many cases.

In aqueous media oppositely charged ions surround charged particles which balance their surface charge. The distribution of ions in the vicinity of charged particle surfaces may be described by the electric double layer theory (e.g. Stern--Gouy-Chapman).

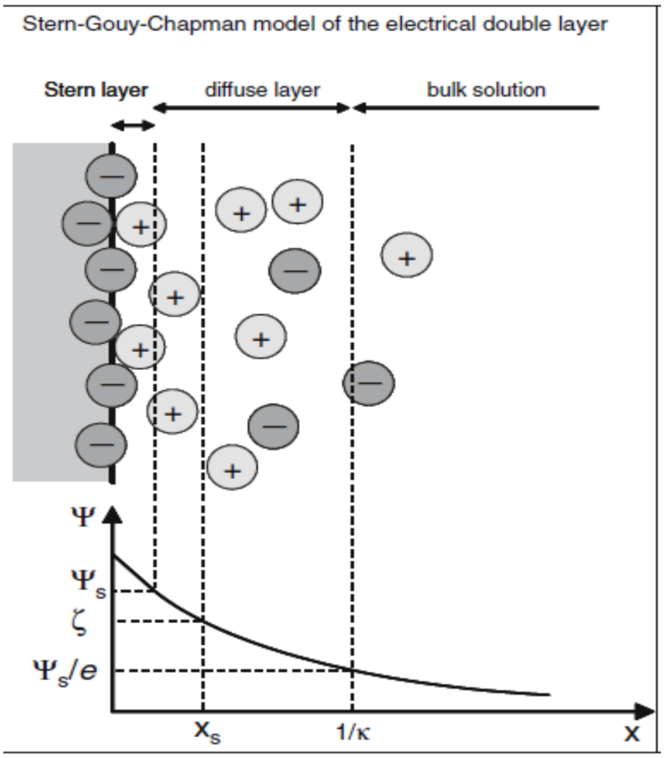


Fig 3.6: Stern-Gouy-Chapman model of the electrical double layer (Source: Handy et al.,2008)

In this model ions are distributed across two layers (Fig.3.6): on layers is a compact inner layer (called Stern layer), where the counterions are immobile and the second is a diffusive outer layer, which extends over a certain distance from the particle surface and decays exponentially with the increasing distance into the bulk liquid phase. The distribution of the ions in the diffusive layer depends on the concentration of electrolyte and the charge of the ions. The potential at the interface between the compact inner layer and the diffuse outer layer is called Stern potential (Ψs). The potential at the shear plane (transition plane from fixed ions and water molecules to those which can be sheared of by fluid motion) is called zeta potential (ζ). The value 1/ κ is the so called Debye length which is the length where the potential has fallen to a value of 1/e of Stern potential. The parameter κ plays a large part in the interaction of charged particles in water and is known as the *Debye-Hückel parameter.* (Gregory, 2006). For general cases of electrolyte solutions containing a number of dissolved salts, κ is defined as:

$$\kappa = \sqrt{\left(\frac{e^2 \sum c_i z_i^2}{\varepsilon T k_B}\right)} \quad (3.20)$$

Where n_i represents the number concentration of ion *i* in solution, z_i the valency of ions *i* in solution, k_B is the Boltzman constant, T is the absolute temperature (Kelvin), ε is the relative permittivity of the medium, *e* is the electron charge. Inserting numerical values appropriate to aqueous solutions at 25°C and converting ion concentration into molar terms (mol/L), k results in :

$$\kappa(nm^{-1}) = 3.29\sqrt{I} \quad (3.21)$$

The summation is calculated over all ions present in solution and is related to the ionic strength, I, which is defined as:

$$I = \frac{1}{2}\sum c_i z_i^2 \quad (3.22)$$

Thus, the parameter κ has dimensions of 1/length (nm^{-1}), and 1/κ (*Debye* length) *represents* the "thickness" of the double layer. The Debye length determines the extent of the diffuse layer and hence the range over which electrical interaction operates between particles. For typical salt solutions and natural waters, values of the Debye length 1/κ can range from less than 1 nm to around 100 nm or more. It can be seen from Eq.(3.20) that, as the ion concentration and/or valence increases, κ increases and hence, the Debye length, (1/κ), decreases. This effect is sometimes referred to as double layer compression and is highly relevant to the stability of colloidal particles: an increased in ionic strength will cause a charge screening of the surface and compress the Electrical Double Layer. The EDL consists of the layer of charge at the surface of a particle and the electric field generated by the charged surface. This can have a net negative or positive charge, depending on the surface ligands of the particle. These forces are generally repulsive (i.e., like charges of two identical particles will repel each other). If the repulsive forces are strong enough, the colloidal dispersion can be virtually stable. The repulsive forces are electrostatic (equal net charge), and act on fairly large length scales involving the outer layer of the particle, hence the term electrostatic double layer (EDL). If we consider the effect of adding salt ions (e.g., NaCl) to the medium, the opposite charges will be attracted and some of these salt ions will accumulate in the EDL, thus reducing the EDL thickness and the length scale that repulsive forces act on, destabilizing the ENPs in solution. Di- and trivalent ions are especially effective at charge shielding, and can act even more effective . (Handy et al., 2008).

Then, the repulsive force (Eq. 3.19) can be expressed as (Baalousha, 2009):

$$V_r(h)=32\pi\varepsilon R\,(K\,T/ze)^2\,\mathcal{Y}^2\exp(-\kappa\,h) \quad (3.23)$$

Where ε is the permittivity of the medium, R is the particle size, *Y is dimensionless function of surface potential, K is the Boltzmann constant, T* the absolute temperature, *h* is the distance between the particles (m), *e* is the electron charge and *κ* is the inverse of Debye-Huckel screening length (m^{-1}).

For small values of surface or zeta potential(ζ), for Rκ >5 and h < R, the equation is simplified to:

$$V_r(h)=2\pi eR\zeta^2\exp(-\kappa\,h) \quad (3.24)$$

3.3.2.2 Van der Walls (VDW) interaction

The attractive forces between particles favor flocculation and oppose stability. Of prime interest in colloidal systems are attractive forces between particles due to attraction between the individual molecules comprising each particle (Miller CA. 2007). The van der Waals (VDW) forces could be described following two approaches, the microscopy (classic) and macroscopic. The classical approach is due to Hamaker (1937), where the interaction between 2 macroscopic bodies is obtained by the summation of all the relevant intermolecular interactions (Elimelech,1998).

In the microscopy approach, VDW forces result from electrical and magnetic polarizations, yielding a varying electromagnetic field within the media and in the separation distance between the two surfaces. The evaluation of dispersion interactions proposed by Hamaker (1937) is based on the assumption that the potential between two surfaces could be represented as the sum of the interactions between pairs of atoms located within the two surfaces (particle or collector) and can be described in terms of geometrical parameters and a constant “A” that is called the Hamaker constant. Equations to evaluate VDW interactions are presented in Eq 3.24 and 3.26. In addition, to estimate the effect of an intervening medium (e.g. “2”) between two bodies of similar composition (e.g. “1”) or in the case of aggregation between two bodies of differing composition (“1” and “3”) Hamaker constants as, A_{123} and A_{121} are required.

Where, A_{123} is the overall Hamaker interaction parameter for the deposition of a nanoparticle of composition “1” onto a surface of composition “3” when suspended in a medium “2”. And, A_{121} is the overall Hamaker interaction parameter for the aggregation of two nanoparticles of composition “1” when suspended in a medium “2”. The Hamaker constants are readily available for a variety of materials and for example for n-TiO_2 the Hamaker constant is $A_{121} = 0.35*10^{-20}$ J (Petosa et al., 2010).

For two spheres of equal radius, R, at a surface to surface separation distance, h, the total interaction energy (VDW) is calculated as (Baalousha, 2009) :

$$Va(h) = -(A_{121}/6) * [\,(2R^2/h^2 + 4Rh) + (2R^2/(h + 2R)^2) + \ln(1 - (4R^2/(h + 2R)^2)) \quad (3.25)$$

In case of interaction between sphere and plane at distance h:

$$Va(h) = -(A_{123}/6) * [\,(R/h + R/h+2R) + \ln(h/(h + R)) \quad (3.26)$$

3.3.3 DLVO theory

Looking at the particle-particle interaction profile the van der Waals force (attractive), the electrostatic double layer (repulsive) EDL force, the total Derjaguin-Landau-Verwey-Overbeak (DLVO) forces are showed in Fig.3.7. Classical DLVO simplifies thermodynamic surface interactions and predicts the probability of two particles sticking together by simply summing van der Waals and electric double-layer potentials to determine if forces are net attractive ($-V_T$) or net repulsive ($+V_T$). For example, in Fig. 3.7, van der Waals and electric double-layer potentials are plotted as a function of separation distance between the particles. Under the experimental condition proposed by the authors, a large potential energy barrier, with a height of about 80 k_BT occurs. This means that approaching particles would have to have a combined energy exceeding this value to come into contact. Because the barrier height is so much larger than the average thermal energy of particles ($3k_BT/2$), it is extremely unlikely that colliding particles would be able to surmount the barrier. Thus, under this condition the suspension would be colloidally stable.

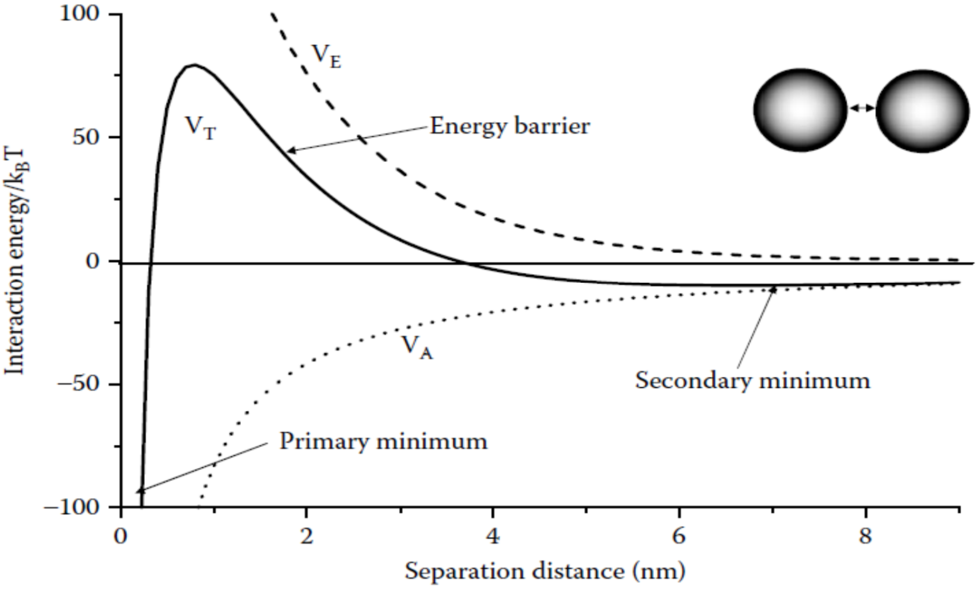


Fig. 3.7: A simplified graph summarizing the DLVO interaction energies and the resulting sum function. Potential energy diagram for the interaction of equal spheres, diameter 1μm, in 50-Mm solution of 1-1 electrolyte. The zeta potential of the particles is assumed to be 25mV, Hamaker constant is $2k_BT$. The curves show the electrical (VE), van der Waals (VA) and total (VT) interaction energy (Gregory , 2006).

These curve (V_T) demonstrates that particles can have a net attraction in a primary or secondary minimum. If the potential energy barrier could be overcome, then the particles would be held in a deep primary minimum. Particles in the primary minimum are considered to be irreversibly aggregated. At larger separations there is a shallow secondary minimum, which arises because of the different distance dependence of the two types of interaction. Electrical double layer repulsion decays exponentially with distance, whereas van der Waals attraction varies inversely with distance. It follows that, at sufficiently large distance, the attraction term will always be larger than the repulsion, hence the secondary minimum. Whereas particles in the secondary well are reversibly aggregated.

3.3.3.1 Collision efficiency

Not all collisions may successfully produce aggregates. The fraction of successful collision is called the collision efficiency (α). Under unfavorable solution chemistry conditions, where a strong repulsion between particles dominates, nanoparticle aggregation is “slow”. The aggregation rate of nanomaterials decreases in the presence of repulsive interaction. In this case, not all collisions will be successful and α =0.001. The effect of repulsive colloidal interactions on perikinetic aggregation is to give a reduction in rate. In this approach a stability ration, W, is used and expressed as $W= 1/\alpha$.

When cases only van der Waals VDW attraction and electrical repulsion EDL are considered, the stability ratio the stability ratio for spherical nanoparticles of equal size is given by (Petosa et al., 2010):

$$W = \frac{1}{\alpha_a} = 2a_p \int_0^{\infty} \frac{exp_{k_b t}^{V_T}}{(2a_p+h)^2} \quad (3.27)$$

A simple approximation of this equation is given by

$$\alpha_a = 2\kappa a_p \exp\left(- \frac{\mathrm{V_T}}{\mathrm{k_b T}}\right) \quad (3.28)$$

where a_p is the particle radius, V_T is the height of energy barriers, κ is Debye-Hückel parameter.

This equation predicts that small changes in electrolyte concentration can have a dramatic effect on the rate of aggregation.

3.4 A briefly application of the DLVO theory to predict the environmental behaviour on –TiO_2 in freshwater archetypes

Due to the evidence of a greatly influence of the environmental condition on the environmental behavior of ENPs a different behavior in 12 freshwater archetypes may be expected.

Thus, following the DLVO theory a case of study on 12 European freshwater archetypes has been conducted. The case study aims to evaluate if the DLVO theory may be applicable on environmental archetype and then to a first qualitative assessment of the environmental behaviour of metal oxide ENPs (n-TiO2) in different freshwater archetypes. The environmental behave of n-TIO2 has been evaluated by the calculation of electric repulsive energy as described in eq. 3.24. The assessment aims to draw a first approximation of the expected behaviour (here refers in term of electric repulsive energy) of ENPs in 12 freshwater archetypes (Salieri et al., 2012b).

3.4.1 Material and method

To explore the effect of variability in the environmental behaviour of n-TiO_2 with the freshwater chemistry we used the 12 sets of water-types representing of the most freshwater chemistries in EU reported by Ghandy et al., (2010). The 12 sets of water chemistries encompass different combinations of pH (5.5-8.3) and water hardness (8-224 $mgCaCO_3/L$) as shown in Table 3.1. The Ionic strength for each of the 12 freshwater sets has been calculated.

	pH	Ca	Mg	Na	K	SO_4	Cl	Hardness as $CaCO_3$	Ionic strength calculated by PHREEQCi	
		mg/L	mg/L	mg/L	mg/L	mg/L	mg/L	mg/L	mmol/L	mol/L
Eu water 1	7.4	75.8	8.5	58.4	0.1	67	102	224	9.4	9.40E-03
EU water 2	8.1	56.6	19.5	65.8	0.1	67	120	221	9.6	9.60E-03
EU water 3	7.6	42.48	6.22	26.67	3.52	48.03	32.97	132	5.4	5.40E-03
EU water 4	8.1	60.52	9.48	25.06	3.25	38.43	41.48	190	6.9	6.90E-03
EU water 5	8.2	58.51	5.59	2.6	0.78	9.61	20.92	169	5.1	5.10E-03
EU water 6	8.2	13.59	3.5	2.3	0.74	13.83	24.82	48	1.7	1.70E-03
EU water 7	7.3	52.1	8.58	11.79	0.82	109.51	20.21	165	5.9	5.90E-03
EU water 8	6.7	20.3	6.7	17	0.1	67	31	78	4.1	4.10E-03
EU water 9	6.4	6.69	2.65	7.2	2.82	85.5	5.99	28	3.1	3.10E-03
EU water 10	5.5	2.4	0.49	7.89	6.22	2.79	2.41	8	0.8	8.00E-04
Euwater 11	5.9	2.48	0.96	6.39	1.8	2.88	8.37	10	0.7	7.00E-04
EU water 12	6.3	2.2	1.12	4.09	0.51	4.8	6.98	10	0.5	5.00E-04

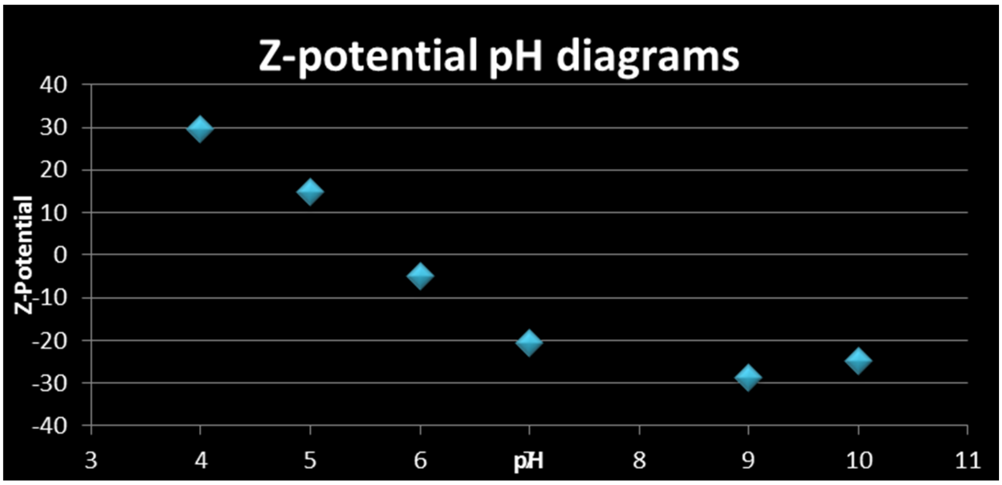
Table 3.1: Ionic strength of 12-EU freshwater archetypes.

The electric e repulsive energy of ENPs has been calculated for n TiO_2 with primary particle size of 15 nm (DLS size: Zave = 183 ± 7 nm, Polydispersion = 0.3; crystalline phase: mostly anastase; the n-TiO_2 are manufactured by laser pyrolysis at the ENEA Research Centre of Frascati, Italy). The powders of n-TiO_2 have been used in the ecotoxicity test on *D.magna* and algae describes in the next chapter. Following the DLVO theory, spherical particle and distance between particle (h= 10 nm), less than radius of the particle (r=15 nm,) have been assumed. Therefore, the electric repulsive potential energy has been calculated by the Eq. 3.24 reported in this chapter, therefore:

$$V_r(h)=2\pi e r \zeta^2 \exp(-\kappa h)$$

where: e is the electron charge, ζ is the zeta potential.

A titration of a n-TiO_2 suspension (1g/L) (background electrolyte 1mM NaCl) to get the pH dependency of the zetapotential (determined using e.g. DelsaNanoC, BeckmanCoulter or a Malvern Zetasizer) has been conducted at the Zentrum für Umweltforschung und Nachhaltige Technologien (UFT), University of Bremen, (Germany).



Figu.3.6: Zeta- potential pH diagrams

For this first application we assumed fixed distance between the particle (h) and a fixed zeta potential value that corresponds to a pH-value of 7

The Debye length (κ , nm-1), which determines the extent of the diffuse layer and hence the range over which electrical interaction operates between particles has been calculated following the E.q. 3.22:

$$\kappa(nm^{-1}) = 3.29\sqrt{I}$$

3.4.2 Result

The next figures show the relation between 1) the ionic strength and the Debye length (Fig. 3.7) and 2) the electrostatic repulsive force:

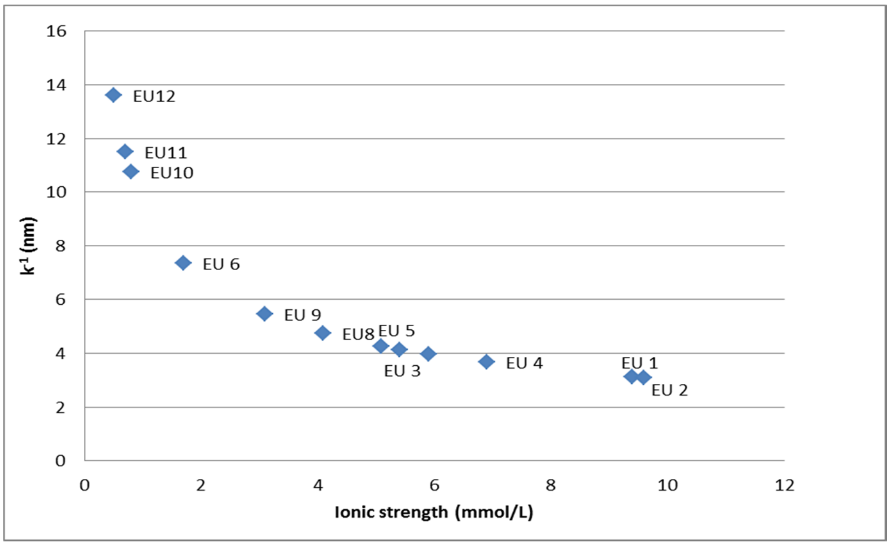


Fig.3.7: Relation between the ionic strength of 12-EU freshwater archetypes and k^{-1} is the characteristic length of the Debye length

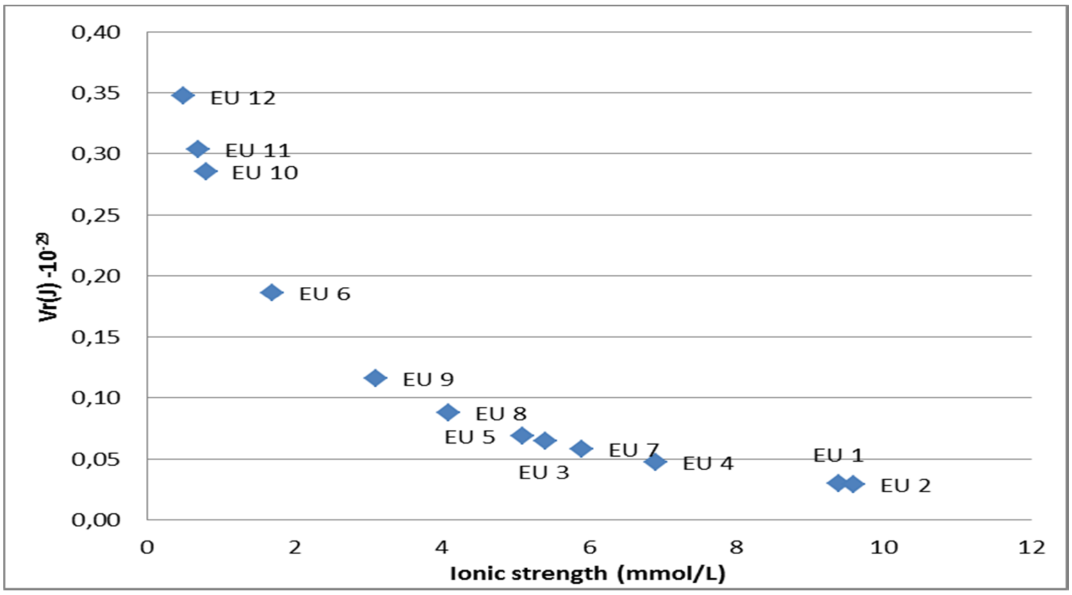


Figure 3.7: Relation between Ionic strength of 12-EU freshwater archetype and the interparticle force, such as the repulsive force Vr(J)

3.4.3 Discussion and conclusion

The result show that increasing the electrolyte concentration (thus decreasing k^{-1}) the extent of repulsive force decrease; as describes by the DLVO theory.

The qualitative assessment of the behaviour of n-TiO_2 in 12 freshwater archetypes allow to draw general conclusion. If only interparticle force act on ENPs, we can expect more aggregation in freshwater archetypes as EU1, EU2 etc. in which the repulsive force decrease due to the higher ionic strength. In contrast, less aggregation may be expected in EU 11-12 freshwater where the ionic strength in lower. As declared this case study cannot permit to evaluate the aggregation of ENPs in the 12 freshwater archetypes since that, only the repulsive force has been accounted. However, it highlight the different behaviour of ENPs in dependence of environmental condition.

Furthermore, following the purpose of the PhD research, this case of study open new question. May be the 12 freshwater archetypes as being a starting point toward a spatial differentiation for the assessment of ecotoxicity impact?

3.5 Environmental assessment

The environmental assessment of a substance requires the evaluation of ~~the~~ fate, behavior and transport in the environmental media. The environmental assessment of ENPs is still critical due to 1) the scarce knowledge on concentrations of ENPs in the environment ~~and~~ (furthermore, an estimation of production and application quantities of ENPs is difficult to obtain, 2) the lack of models to assess the concentration of ENPs in the environmental media, 3) the lack of knowledge on the environmental and ENPs proprieties that affect behavior and transport among environmental compartments. (Von der Krammer et al., 2010, Gottschalk et al.,2010).

The environmental fate models have been established and used to assess the fate, transport and exposure of organic chemicals for 30 years (MacLeod et al., 2010). Within the environmental models, the multimedia mass-balance models are widely applied to study the behavior of chemicals in the environment. They are composed of equations and algorithms that are quantitative expressions of knowledge and understanding derived from theoretical and empirical studies of chemical mass transport and degradation.

In contrast to organic chemicals, in the field of ENPs the development of environmental fate models is still in its infancy. This is due to the novel physical–chemical properties exhibited by ENPs. For instance, the assessment of fate and transport of organic chemical and inorganic substances is based on chemical properties, such vapor pressure and solubility, partitioning values that are not applicable to nanoparticles/nanomaterials due to the often low solubility and low vapor pressure. Therefore, given the particular chemical and physical properties (size, structure, high surface reactivity, catalytic, magnetic and optical properties), the classic approach and the available experience with organic chemicals may be irrelevant or not applicable to ENPs (Mackay et al., 2006).

The environmental fate modeling for ENPs requires a good knowledge of the environmental behavior of ENPs and identification, as well as assessment and inclusion of the chemical properties governing the fate processes in the environment.

To my knowledge, only few approaches in environmental modeling of ENPs have been recently proposed.

Mueller and Nowack, (2008) derived the Predicted Environmental Concentration (PEC) for: nano silver (nano-Ag), n-TiO_2 and carbon nanotubes (CNT). The environmental model is based on a substance flow analysis from products containing ENPs to air, soil, and water in Switzerland.

The PEC value is calculated considering: the worldwide production volume, the allocation of the production volume to product categories, the particle release from products, and the flow coefficients within the environmental compartments. As argued by the authors, due to the general lack of data, the flow coefficients are based on expert estimations. Furthermore, transformation, degradation and bioaccumulation processes have been neglected, even if they may play an important role in the environmental fate of ENPs.

Gottschalk et al., (2009) through a probabilistic flow analysis, modeled the environmental concentrations of nano-TiO_2, nano-ZnO, nano-Ag, CNT, and fullerenes for the U.S., Europe, and Switzerland, considering all environmental compartments including sediments. The system was described by means of 11 boxes which represented environmental compartments as homogeneous and well mixed (water, air, soil, sediment, and groundwater) and technical compartments (e.g. production, manufacturing, and consumption (PMC) sewage treatment plant (STP), waste incineration plant (WIP), landfill, and recycling processes). Depending on the ENPs different releases to environment were assumed; for instance deposition and elimination/degradation of ENPs within the compartments were modeled as constant annual flows into a sub-compartment of each box considered.

However, the models proposed do not account for specific physical or chemical transformation processes occurring when ENPs are released to environmental compartment.

Another environmental assessment for risk assessment purpose and specific for nano Ag, was performed by Blaser et al., 2008. The authors coupled a mass flow analysis (three emission scenarios were assumed) from Ag NPs containing products (plastic, textile, biocide products) with an environmental fate model in order to derive PEC values for silver in Rhine river. The environmental river model describes the Rhine river in several boxes; each box consists of a compartment of moving water (W1), a compartment of stagnant water and a compartment representing the top layer of the sediment. In this model, it was assumed that all silver that reaches the aquatic environment was considered as the environmentally most stable species, silver sulfide (Ag_2S), or sorbed to suspended sediments. The following environmental processes are included: sedimentation of particles from stagnant water into the top sediment layer, resuspension of particles into moving water, burial of the mineral fraction of particles into the permanent sediment. Diffusive exchange of dissolved fractions of silver sulfide between stagnant water and interstitial water in sediment and downstream transport of dissolved and particle–bound silver with moving water, bulk exchange between moving and stagnant water. In contrast to Gottshalck et al., (2009) and Mueller and Nowack (2009), an environment fate model was applied to model the PEC value for nano Ag in freshwater ecosystem (Rhine River). Anyway, due to the assumption that silver reaches the aquatic environment as silver sulfide, the ***specific process descriptions for Ag ENPs were by-passed***, and also this model is not directly applicable to other ENPs such as TiO_2 NPs.

In those studies, briefly described above, the ENPs are treated as bulk material, and material flow analysis was applied without taking into account the particular nature of the material. For instance, fate processes as agglomeration, or the binding to natural organic matter (NOM) and the physical-chemistry parameters of the water are not considered, even if they have been pointed out as key parameters in the exposure and bioavailability assessment of NPs. (Gao et al., 2009; Keller et al., 2010, Christian et al., 2008, Farré et al., 2009)

The need to put more emphasis on nanospecific processes was addressed by Quik et al., (2011). The authors proposed a model for estimating exposure concentration of ENPs in the aquatic environment by applying ~~of~~ kinetic equations from the colloidal chemistry to include the aggregation and sedimentation of ENPs (as n-TiO_2). The application of colloidal chemistry to environmental fate and exposure models for ENPs has been encouraged by several researches (Arvidsson et al., 2011). There are evidences, that the environmental behavior of ENPs in freshwater follows and mimics those of natural colloids (Christian et al., 2008, Petosa et al., 2010, Handy et al., 2008). Anyway, still several difficulties in modeling fate and exposure of ENPs by application of colloidal chemistry has been outlined.

Thus, the mass concentration of free nanoparticles in freshwater, C (kg/m3), due to a volume-specific release rate on nanoparticles to water (E, kg m^{-3} s^{-1}may be estimated resolving the following equation (Eq. 3.1). to

$$\frac{dC}{dt} = E - \sum kC \; with \sum k = k_{adv} + k_{vol} + k_{deg} + k_{sed} + k_{diss} \quad (3.1)$$

Formulated in this way, the challenge is to assign values to removal rate constants of first order :

Kadv (s^{-1}): advection out of system; Kvol (s^{-1}): volatilization to air; Kdeg (s^{-1}): degradation and sorption to suspended particles according to an equilibrium constant Kp (L/kg); Ksed (s^{-1}) deposition to sediment. Even if this approach provides one single approach to the modeling of conventional chemical substances and ENPs , several limitation have been argued:

- The rate constant should be assigned for each type of ENPs. For example, within the same group of ENPs such as metal oxide nanoparticles, the dissolution rate constant may be different depending on each individual (n-TiO2 or n-ZnO) metal oxide ENP s in exam
- Due to the low the scientific knowledge an high uncertainty of estimations of these rate constants is showed.

Recently, an environmental fate model for ENPs based on multimedia box model for organic chemicals and adjusted to account for ENPs specific properties and behavior in freshwater has been developed by Praetorius et al.,2012. The model permits to predict the environmental fate and transport of nano-TiO_2 accounting for the physical-chemical parameters of the system (water) and also developed on the basis of colloidal chemistry in order to consider the environmental behavior of ENPs in freshwater. A case of study on TiO_2 in Rhine River has been performed by the authors. Furthermore, the model developed demonstrates that it is possible to adjust conventional multimedia fate models for organic pollutants to account for the specific properties of ENPs.

The model considers the environmental fate processes of (i) transformation and degradation (homo-aggregation between n-TiO_2, dissolution and surface transformation) (ii) interaction with SPM, suspended particulate matter, such as the hetero-aggregation, (iii) transport (advection, sedimentation sediment resuspension, burial) .

On the basis of well-established multimedia environmental fate models for organic pollutants, the fate processes for n-TiO_2 are described as rate constants of first order, k (s^{-1}), which are derived ~~by~~ through the physical law governing the colloidal behavior in water, therefore accounting on the specific properties of ENPs.

The case study confirms the great influence of the physical-chemical properties of the freshwater, such as the SPM characteristic (density and mass concentration). For instance, with a fixed mass concentration (50mg/L) and a lower density of SPM (2.0 g/cm^3), a slower removal of free n-TiO_2 from the water column (the hetero-aggregations decrease at lower density value of SPM) and a lower settling velocity of the SPM were calculated. Therefore, n-TiO_2 can potentially be transported with the SPM in the water column over long distance.

And also, the transport of n-TiO_2 is strongly influenced by the attachment efficiency or collision efficiency (α) of the hetero-aggregation. Aggregation of ENPs in aqueous dispersions involves the formation and growth of clusters and is controlled by both interfacial chemical reactions between particles and particle transport mechanism (Brownian motion, orthokinetic motion, perikinetic motion). The attachment efficiency depends on the interfacial chemical reaction between two nanoparticles. Due to the current knowledge, assigning a value to the collision efficiency is difficult from a modeling perspective; therefore the collision efficiency (α) is approximated to a value of range from 0.0001 to 1 (Arvidsson et.al, 2011)

With the highest collision efficiency (α=1), the highest mass concentration of SPM (90 mg/L), and the highest density value of SPM (2.5 g/cm^3), the n-TiO_2 are very quickly removed from the water body, both in ~~the~~ free and SPM-bound form, and travel only about 50 km away from the source before being completely deposited in the sediment compartment.

Thus, the modeling of fate and transport of ENPs is still in its infancy and several limitations have been pointed out. Anyway general conclusion may be summarized:

- The well establish multimedia fate model can be applied to ENPs;
- The colloidal science can be applied to calculate the aggregation and sedimentation processes;
- The environmental parameters strong influence fate and transport bioavailability of ENPs.

3.6 Discussion and conclusion

Currently the environmental assessment of metal oxide ENPs is still in its infancy. The main reason is the gap of knowledge about the fate of ENPs in the environment. The fate and transport models usually applied for organic substance can be applied for the assessment on environmental fate of metal oxide nanoparticles. However, due to the different properties exhibited by ENPs, the fate models have to be adapted to the:

1) ENP specific fate processes;

2) the chemical –physical parameter governing the fate processes.

To date, the colloidal science seems a valid “tools” to develop fate and transport model specific for ENPs. In fact the nanoparticles dispersed in freshwater are comparable to colloidal systems: they are highly dynamic, with sizes changing with time due mainly to dissolution or aggregation. Most of the ENPs in aqueous environments are present as aggregates rather than as individual particles. **Thus, research on transport, transformations, and fate processes and bioavailability should focus perhaps on aggregates rather than on individual ENPs** (Lin et al., 2010). The behavior of n-TiO_2 nanoparticles in the environment is influenced by concentration of and type of ions; the presence of divalent cations enhances the aggregation in compared with monovalent cations. The fulvic acids stabilize the ENPs in suspension, leading to a lower aggregation and sedimentation rate. The strong and fast aggregation assessed in natural water samples highlights that the sediment compartments may be greatly affected by the sedimentation of ENPs such as TiO_2. Thus, more research should be focused on the sediment. Both in terms of toxicity studies on benthic organism and in the field of “fate and transport models”. The aggregation processes may have also a key role in terms of toxicity. The aggregation process can decrease the surface area of the ENPs, thereby decreasing the surface area mediated toxic response (e.g. ROS generation and dissolution). On the other hand, it increases the persistence of the ENPs, decreasing the rate of dissolution or degradation (Hotze et al., 2010).

Still, the toxic mechanism of aggregates particle is not well investigated. However, there are experimental evidences that the aggregate particle may be entrapping on the algae cells provoking a direct physical effect of disruption of the algae cell. More, as possible mechanism of toxicity the adhesion to exoskeleton of Daphnids has been suggested (Dabrunz et al., 2011). In contrast, when aggregates become too large for direct transport across the cell wall and/or membrane, uptake may be prevented. Therefore, affecting its bioavailability to organisms (Lowry et al., 2012) .

Furthermore, distinct environmental behaviors are expected in different waters (seawater, freshwater) in dependence by the different water chemistry and therefore leading to a different mobility of ENPs and exposure scenario. For instance, under environmental condition of low IS and high TOC the metal oxide nanoparticle dispersion (n-TiO_2, ZnO) is stable; thus aquatic organism may be exposed for a long time to small nanoparticle (close to 300 nm) (Keller et al., 2010). However, **under environmental condition a multimodal size distribution is expected, also indicating that the organism may be exposed to different size range of aggregates**. In general, due to the ionic strength and pH ranges of most surface waters, aggregates of several hundred nanometers to several micrometers are expected.

Furthermore the fate and bioavailability of ENPs are affected by the dissolution and transformation processes. As argued, toxic effects due to the dissolution of metal oxide nanoparticles have been reported. The dissolution processes is of more relevance for soft metal oxide nanoparticle such as, Zn, and Cu because they form partially soluble metal oxides. As well for aggregation, also the dissolution is affected by the environmental condition. Soft metal oxide nanoparticle show a high reactivity with sulphur-containing biomacromolecules and inorganic. Formation of a relatively insoluble metal-sulphide shell on the particle surface can alter the surface charge and induce aggregation. Thus, the environmental conditions (redox state and availability of free sulphide) that affect their dissolution and/or Sulfidation rates are important for assessing their potential release of toxic metal cations, and their toxicity and persistence in the environment. Even if toxic effects due to the dissolution of metal oxide nanoparticles have been reported also other fate processes such as aggregation occurring at the same time seems to be involved in the toxicity and bioavailability of NPs to aquatic organism.

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4 Ecotoxicity of metal oxide nanoparticles(n-TiO_2) on crustaceans, algae and fish

4.1 Introduction

In general, the toxicity of metal oxide nanoparticles (ZnO, n-TiO_2 etc.) is still poorly known; the studies report conflicting results and the mechanisms of toxicity are not fully understood (Baun et al., 2008). Also, there are no specific guidelines for sample preparation and testing specifically referred to nanomaterials; whereas the bioavailability and toxicity of nanomaterials may depend on the preparation methods. Several procedures for testing the nanoparticles have been described in the literature, and whereas some authors consider environmental relevance, others simulate a worst case scenario by striving for maximum dispersion (Crane et al.,2008).

The methods include stirring for varying time periods, ultrasonic dispersion or the use of organic solvents for metal oxides, sometimes followed by filtration (Hund-Rinke and Simon, 2010).

Therefore, in the REACH regulation context, the limited available data in the literature and the high variability of the data reported, do not allow to draw conclusions on the environmental impact of the use of nanomaterial (Clement et al., 2012).

The toxicity of metal oxides ENPs varies with the type of ENPs tested and, furthermore, different mechanisms of toxicity dependent on the physicochemical properties are being argued.

Metal oxide nanoparticles composed of elements that are in themselves toxic (such as Cu, Zn and Ag, Ce) show higher ecotoxicity than other types of metal nanoparticles composed of less toxic elements. The toxicity of metal oxide nanoparticles can also be explained by the release of free ions, whereas the degree to which ions are released from nanoparticles varies depending on composition (Griffit et al., 2008). It has been found that release of Ti and Ce from TiO_2 and CeO_2 nanoparticles is very low (<10 µg/L), whereas the release of Zn from ZnO nanoparticles can be set in the mg/L range (Johnston et al., 2010).

Even if the release of free ions has been argued as a possible mechanism of toxicity, furthers investigation are required to investigate the contribute in terms of toxicity. For example, Ji et al. (2010) performed toxicity test on the algae *Chlorella sp.* with bulk ZnO, and n-ZnO. The study evidences that the toxicity on the *Chlorella sp.* of the zinc materials followed an order of bulk-ZnO < nano-ZnO < Zn^{+2}. However, the authors suggest that other factors may contribute to the toxicity of Zn^{+2}, such as the aggregate present in the n-ZnO solution.

The **titanium oxide** is generally regarded as an inert material. However, for nanosized particles the high specific surface area of nanoparticles confers greater reactivity to the material with respect to its bulk

form, affecting its interaction with the organisms (Hartmann et al., 2010). The widespread use of n-TiO_2 makes exposure to the natural envirionemnt more likely. Particular attention has been paid to freshwater ecosystem that seems to be an environmental compartment particulary affected by the release of these particles as the results of their use in environment cleanup of waste (Lovern and Kapler, 2006).
The toxic effects of n-TiO_2 have been demonstrated by several studies. The n-TiO_2 have been recognized as a cause of the formation of reactive oxygen species (ROS) when exposed to UV radiation (photo-activated), causing damage to cellular and subcellular structures (Long et al., 2006). The photocatalytic activity of n-TiO_2 depends on the concentration, the crystal structure (rutile/anastase/amorphous), and the UV-A radiation (for λ <385 nm) (Hund-Rinke and Simon, 2006; Hartmann et al., 2010). It has been shown that n-TiO_2 can also cause damage to cell membranes, adhering to them (Yeung et al., 2009). Moreover, regardless of direct toxic effects, the presence of n-TiO_2 can cause indirect effects, influencing the toxicity and bio-accumulation of other pollutants present in the aquatic environment; the environmental pollutants can be adsorbed by the n-TiO_2, due to its porosity and/or surface charge. Zhang et al. (2007) reported a greater accumulation of cadmium (Cd) and arsenic (As) in carps (*Cyprinus carpio*) in the presence of n-TiO_2, thus calculating a bio-concentration factor (BCF) for Cd in the presence of n-TiO_2 10 times higher than in absence of n-TiO_2.

In this chapter **two case of study with n-TiO_2 on *Daphnia magna* and *Pseudokirchneriella subcapitata*** are reported. The ecotoxicity tests on *D.magna* have been performed during my PhD training, while the study on *P.subcapitata* is a project of research which I have supervised.

Then, the chapters aims to **review current knowledge regarding the ecotoxicity of engineered nanoparticles** with focus on particles composed of titanium dioxide (uncoated n-TiO_2). The review is limited **to aquatic freshwater ecotoxicity** with emphasis on freshwater aquatic organisms representative of algae, crustaceans and fish, reflecting the overall topic of this thesis and the work undertaken during the PhD project period. Through discussions of specific issues of interest, this review aims to describe our current state of knowledge as well as to highlight potential relationships between particle properties and observed effects, while also focusing attention on methodological difficulties and knowledge gaps.

4.2 The ecotoxicity of n-TiO_2: a case study on *D.magna*

As argued by the ecotoxicological review conducted on n-TiO_2 a high variability of toxic effect on aquatic organisms has been found. Where the high variability of the toxic results may be attributable to the type of nanoparticle tested (e.g. crystal composition), the treatments of solution to test and the exposure mode. The experimental research reported in the following has the aim to evaluate whether aquatic ecotoxicity of n-TiO_2 depends on experimental setup. Following the hypotheses that (i) the toxicity increases with the increase of the exposure duration, (ii) the toxicity is dependent on the volume of exposure due to the aggregation processes occurring in the media, several bioassays on n-TiO_2 have been performed. The research was focused on the time of exposure, the volume of exposure and the test media.

Several acute toxicity tests on *Daphnia magna* with n-TiO_2 were conducted. The acute toxicity tests were performed based on OECD guideline 202, endpoint immobilization, with prolonged exposure time of 72 h and 96 h. The particles dispersed in the test media were characterized using dynamic light scattering (DLS).

Furthermore, several treatments of the stock solution, several test media, and different test solution treatments have been applied. Three test media (ELENDT M7, ISO water and Bottle water), several treatments of n-TiO_2 in solution (stirring, sonication) and different exposure mode (exposure duration and volume of exposure) have been applied. No adverse effect was observed when 1) the organisms were exposed to 50 mL of test solution, 2) different solution treatments were applied and 3) the exposure time was prolonged up to 96 h. In contrast, with an 1) exposure volume of 20 ml, 2) prolonging the exposure up to 96 h, and 3) even if the same treatment of n-TiO_2 in solution was applied an adverse effect at 96 h was observed and EC50 values were calculated. For a better mechanistic understanding, for the experimental setup at which toxic effects were observed, we characterized the particle size of n-TiO_2 throughout the test duration. The characterization measurements show a fast and strong agglomeration in the media with a particle size in the order of micro meters. Our study remarks the exposure mode as an important parameter to assess toxic effects of n-TiO_2.

4.2.1 Material and Method

4.2.1.1 *Test compound*

Uncoated and powdered n-TiO_2 was obtained from ENEA Research Centre of Frascati (Rome, Italy). The n-TiO_2 (XRD size: 15 nm; DLS hydrodynamic diameter (z-average): 183 ± 7nm, Polydispersity: 0.3; crystalline phase: 96 %wt anatase, 4 %wt rutile) were manufactured by laser pyrolysis. The powders of n-TiO_2 purchased from ENEA Frascati were analyzed by BET before the application in the bioassay. BET measurements revealed a surface area of 67.819 (m^2/g) and a primary particle diameter of 18 nm.

4.2.1.2 *Test media*

Three types of dilution media were used for the tests. Artificial Elendt M7 and ISO water were prepared as described by OECD guideline 202 and ISO 6341, respectively. In addition bottle water (commercial water) was used for the tests. The dilution medium is the same used to culture the daphnids and to acclimate daphnids before the beginning of the test.

4.2.1.3 *Preparation of test solution*

Two experimental setup (A-B) were prepared (Table 4.1).

SETUP A, procedure 1: The experiments were conducted at Zentrum für Umweltforschung und Nachhaltige Technologien (UFT), University of Bremen, (Germany). The stock solution was prepared according to Meissner (2009) as describes below.

- A titration of a n-TiO_2 suspension (background electrolyte 1mM NaCl) to get the pH dependency of the zeta potential (determined using e.g. DelsaNanoC, BeckmanCoulter or a Malvern Zetasizer) has been conducted. From the analysis it has been seen that a good value of the zeta potential (+30 mV) to achieve a stable suspension of n-TiO_2 (Fig.4.1) could be obtained at pH = 4.
- Resulting from the titration, a stock solution (1g/L) was prepared dispersing an amount of n-TiO_2 in pure water. The dispersion was adjusted to pH = 4 using hydrochloric acid (HCl 0.1 M) and then sonicated for 12 minutes using the Branson Sonifier 450 (Branson, USA), power was 150 W at pulse rate of 0.5 s).

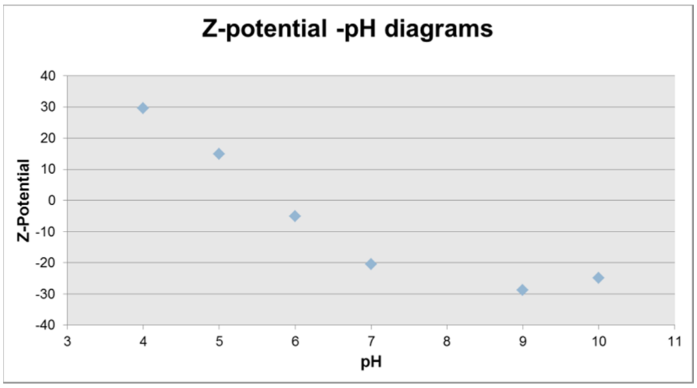

Fig.4.1: Z-potential pH diagrams

SETUP B: The experiments were performed at C.I.R.S.A, Centre of Research for the Environmental Science, University of Bologna (Italy). Methods of treatment of the stock solution and dilution procedures were varied (Table 1, procedures 2-7). A stock solution of n-TiO_2 (1.0 g/L) was prepared by dispersing the powder in MilliQ-water. Concentration series were prepared by diluting the stock solution in the media (ISO or Bottle water). In the following, the procedures 2-7 of SETUP B have been described.

2. The stock solution was stirred for 1 h with a magnetic stirrer before the preparation of the dilution series (Zhu et al., 2009). Then the dilutions at the nominal concentrations reached were stirred for 24 h with a magnetic stirrer before the test was started.
3. The same procedure as described above was followed, but the bioassay was maintained in agitation for the whole time of the biological experiment (Zhu et al., 2009).

4. The stock solution was sonicated for 1 h in ultrasonication bath. The dilution series were prepared immediately before the test and the bioassays were agitated constantly during the biological experiment.
5. 20 mg of powder were placed in 200 mL of tetrahydrofuran (THF). Gaseous N_2 was purged through the dispersion for at least 1.5 h to remove THF. Then 200 mL of deionized water was added and the suspension was placed in a Buchi rotovapor to evaporate the THF (see Lovern, 2006). Then, the solution was sonicated for 15 minutes in ultrasonication bath. The dilution series were prepared immediately before testing.

6-7 The same treatment of the stock solution as in setup A was applied. The dilution series were prepared immediately before testing. The dilution series were prepared with: Bottle water and ISO water.

SETUP	Procedure	MEDIA	Treatment stock solution	Treatment of solution to test	Modality of Exposure	Volume tested	Concentration (mg/L)	Exposure time
SETUP A	1	ELENDT M7	pH=4 sonication 12 min		24 well plate	20 mL /each conc.	10-25-50-75-100-250-500	up to 96h
SETUP B	2	Bottle water	1 h stirring	24h stirring prior to test	Beacker 100mL	50 mL	0.1-1-10-100	up to 96h
	3	Bottle water	1h stirring	24h stirring prior to test and for the whole time of bioassay	Beacker 100mL	50 mL	0.1-1-10-100	up to 96h
	4	Bottle water	1h sonication	stirring for the whole time of biossay	Beacker 100mL	50 mL	0.1-1-10-100	up to 96h
	5	Bottle water	THF+15 min sonification	stirring for the whole time of biossay	Beacker 100mL	50 mL	0.1-1-10-100	up to 96h
	6	Bottle water	pH=4 sonication 12 min		24 well plate	20 mL /each conc.	10-25-50-75-100-250	up to 96h
	7	ISO	pH=4 sonication 12 min		24 well plate	10 mL /each conc.	10-25-50-75-100-250	up to 96h

Tab.4.1: Experimental setup (SETUP A &B): treatment of stock solution and solution to test, modality of exposure (beaker, 24 well plate) volume tested, nominal concentrations tested and exposure time

4.2.1.4 *Characterization of n-TiO_2 in three media (ELENDT M7, ISO and Bottle water)*

With the aim to determine the agglomeration behavior of n-TiO_2 in test media (ELENDT M7, ISO and Bottle Water) the analyses of the size distribution of suspended nanoparticles at various concentrations have been performed using dynamic light scattering (DLS). The DLS measurements in the medium ELENDT M7 were performed at Zentrum für Umweltforschung und Nachhaltige Technologien (UFT), University of Bremen, (Germany). Here the DLS was measured using a Beckman-Coulter DelsaNanoC (Beckman Coulter, Krefeld, Germany). This device features a diode laser (30 mW, λ_0 = 658 nm) and is able to measure the scattered light at scattering angles of 15° and 165°. The experiments were carried out at the backscattering angle 165°. The scattered light is detected using a photo multiplier tube and analyzed with a digital correlator. A sample volume of 2 mL was filled in Sarstedt fluorescence cuvettes (polystyrene, d = 1 cm, Sarstedt, Nümbrecht, Germany) and was thermostated at $t = 25$ °C in the device for 4 min before the measurement time of 140 s per repetition. For the evaluation of the auto correlation functions $g(2)$ the properties of pure water for the refractive indices n(658 nm, 25 °C) = 1.3328 and for the viscosity η (25

°C) = 0.8878 cP were used as given by the Beckman Coulter Software. The cumulants method was used to calculate the z-average of the hydrodynamic diameter *d* and the polydispersity index (PDI). Zeta potential measurements were also done using the Beckman Coulter DelsaNanoC. A sample volume of 5 mL was filled in a Flow Cell and equilibrated with the same conditions mentioned in the DLS section. Here the measurement was done at the scattering angle of 15°. The measurement time was 130 s per repetition. For the evaluation additionally to refractive index and viscosity the dielectric constant of pure water was used $\varepsilon = 78.3$ as given by the Beckman Coulter software.

The DLS measurements on ISO and Bottle media waters were performed with particle analyzer unit Zetasizer Nanoseries Malvern Instrument); the light scattering unit employs a laser 4 mW He-Ne at wavelength 633 nm. The scattered light was detected at a scattering angle of 173° and each auto-correlation function was accumulated for 30 s. The DLS measurements were repeated 3 time for each sample tested (11 runs of 30 s). DLS measurements were conducted on sample of dispersed n-TiO_2 in test media. The dispersions were performed following the same procedure proposed in SETUP A for the bioassay purpose. The tested n-TiO_2 suspensions were prepared diluting the stock solution in the culture media (ELENDT M7, ISO water and Bottle water), 4 mL of each concentration tested was placed in 24 well-plates and stored in incubation chamber at the same condition of biological test (Temperature of 20 ± 2 °C and at a light regime of 16 h light: 8 h dark). The DLS measurements were performed introducing 2 mL of solution into fluorescence cuvettes; all cuvettes were used only once.

4.2.2 Acute toxicity test

Acute toxicity tests of n-TiO_2 with *D.magna* were conducted based on the OECD 202 standard procedure (OECD 2004) with extended exposure time from the traditional 48 h to 96 h. The immobilization at 24 h, 48 h, 72 h and 96 h was assessed. Animals that are unable to swim after 15s of gentle agitation of the test container are considered immobile. Neonates (less than 24 h old) were assigned randomly to the test vessels at each test start. Animals were not fed during the testing period. All tests were conducted indoors at a constant temperature (20 ± 2 °C) at a light regime of 16 h light: 8 h dark.

SETUP A: Six concentrations (10-25-50-75-100-250 mg/L) plus a control were tested. For each concentration 10 organisms (24 h old) were placed in 24 well plates and each one exposed to 2 mL of solution. The experiments were performed with an exposure length of 96 h, the well plates were stored in an incubation chamber. Three experiments in three replicates were performed.

SETUP B: Four concentrations (0.1-1-10-100 mg/L) plus a control were applied. For each concentration 5 neonates (24 h old) were placed in a 100 mL beaker containing 50 mL of test solution. The beakers were covered with a transparent plastic film and stored in incubation chamber. In case the bioassay has to be agitated for the whole time of the bioassay, the beakers were stirred at 160 rpm. All experiments were conducted with three replicates. In the procedures 6-7 the concentrations of 10-25-50-75-100-250 mg/L were tested (Table 4.1) applying ISO water and Bottle water. For each concentration tested 10 organisms (less than 24 h old) were placed in 24 well plates and each one exposed to 2 mL of solution. The experiments were conducted at least in three replicates.

4.2.2.1 *Analysis of data*

A 96 h log$_{10}$ EC50 and the associated 95% interval confidence were calculated for each replicate in SETUP A and SETUP B (Stasoft, Inc. (2001), Statistica, data analysis software system). The 96 h-EC50 (mg/L) values were calculated as arithmetic mean of three 96 h log$_{10}$ EC50. Moreover, in order to compare the results among the media applied, a statistical analysis was carried out using standard ANOVA techniques. Differences were statistically significant in case $p < 0.05$.

4.2.3 Result

4.2.3.1 Acute toxicity test on Daphnia magna

No adverse effects have been observed when the organisms were exposed to 50 mL of solution (SETUP B, procedures 2, 3, 4, 5). Whereas, adverse effect (Table 4.2) was observed only for the SETUP A and for the procedures 6 and 7of SETUP B. The calculated 96 h-EC50 values are reported in the Table 4.2 and Table 4.3.

TEST	Procedure	MEDIA	Volume tested	Concentration (mg/l)	Exposure time	EC50 (mg/l)
SETUP A	1	ELENDT M7	20 ml/each conc.	10-25-50-75-100-250-500	96 h	32 mg/l (95% CI:8-125)
SETUP B	2	Bottle water	50 ml	0.1-1-10-100	96 h	no adverse effect
	3	Bottle water	50 ml	0.1-1-10-100	96 h	no adverse effect
	4	Bottle water	50 ml	0.1-1-10-100	96 h	no adverse effect
	5	Bottle water	50 ml	0.1-1-10-100	96 h	no adverse effect
	6	Bottle water	20 ml/each conc.	10-25-50-75-100-250	96 h	82 mg/l (95% CI:2-3770)
	7	ISO	20 ml/each conc.	10-25-50-75-100-250	96 h	33 mg/l (95% CI:3-361)

Table. 4.2:EC50 values calculated in acute toxicity tests

	SETUP A Elendt M7			SETUP B ISO water			SETUP B Bottle water		
Test	EC50 (mg/l)	95% confidence interval		EC50 (mg/l)	95% confidence interval		EC50 (mg/l)	95% confidence interval	
A	17	13	22	20	12	35	176	49	634
B	45	33	60	18	13	25	226	40	1278
C	43	20	94	101	89	114	14	10	20

Table 4.3: EC50 (mg/ L) and 95% interval confidence values referred to the single tests

SETUP A: Adverse effect has been observed in the SET-UP A (Table 4.2). The average 96 h-EC50 (mg/L) value was 32 mg/L (95% CI:8-125). No or low adverse effects have been observed at 72 h, and 48 h, therefore EC50 values were incalculable.

SETUP B: No adverse effects have been observed when the organisms were exposed to 50 mL of solution (Tab.2). Adverse effects were observed when the organisms were exposed to 20 mL of test solution and the dilution series were prepared in ISO water and Bottle water. The 96 h-EC50 values (mg/L), are: 33 mg/L (95% CI:3-361) for ISO water and 82 mg/L (95% CI:2-3770) for Bottle water. The values of EC50 were incalculable at 72 h and no or low adverse effects at 48 h were observed.

ANOVA TEST: an ANOVA test was carried out to evaluate the hypothesis that the media could influence the test's result. No significant differences were found among the three media tested ($F = 1.97$; $df = 2$; $p > 0.05$): the variability among the groups is lower than the variability intra groups. The high variability intra groups evidences a low reproducibility of the test performed with ENPs.

4.2.3.2 Analysis of particles size distribution

Stock solution

The DLS measurements (D[nm]), the polydispersity index (PDI) and zeta potential measurements of the stock solution (1 g/L) were performed immediately before the preparation of the dilution series for biological bioassay and for the characterization of n-TiO_2 in suspension. The tested samples were diluted 1:50 in MilliQ water adjusted to pH 4 (using hydrochloric acid, HCl 0.1M) to reduce the multiple light scattering of the samples. Multiple scattering in highly turbid samples leads to erroneous results in DLS- and zeta potential measurements. The measurements are reported in Table 4.4.

Sample A pH=3.98			Sample A pH=4			Sample A pH=4.2		
D(nm)	PDI	Zeta potential	D(nm)	PDI	Zeta potential	D(nm)	PDI	Zeta potential
270±5	0.26±0.01	34.1±0.6	281±34	0.18±0.08	34.9±0.7	387±57	0.19±0.02	37.2±1.9

Table 4.4: DLS and zeta potential measurements of three samples of stock solution

ELENDT M7

The characterization of n-TiO_2 dispersed in ELENDT M7 water was carried out for the whole time of the bioassay. Thus, the DLS measurements (using DelsaNanoC) were performed at time 0 h, 24 h, 48 h, 72 h, 96 h. The average values of hydrodynamic diameter (µm) of n-TiO_2 are shown in Fig.4.2 and Table 4.5.

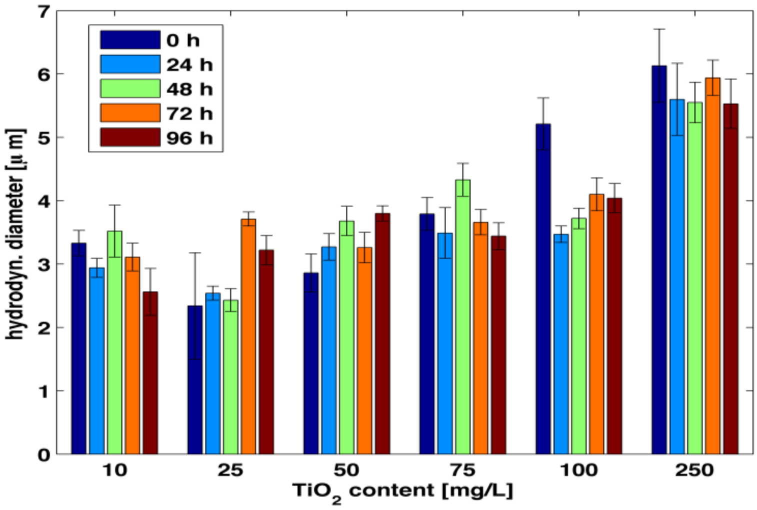

Fig.4.2: Hydrodynamic diameter (µm) of n-TiO_2 at time 0 h, 24 h, 48 h, 72 h, 96 h.

mg/L	D(µm) ±s.d time 0	D(µm) ±s.d time 24h	D(µm) ±s.d time48h	D(µm) ±s.d time72h	D(µm) ±s.d time 96h
10	3.33±0.20	2.94±0.15	3.52±0.41	3.11±0.21	2.55±0.37
25	2.34±0.84	2.54±0.10	2.42±0.18	3.71±0.11	3.22±0.23
50	2.86±0.30	3.27±0.21	3.68±0.23	3.26±0.24	3.80±0.12
75	3.79±0.32	3.48±0.40	4.33±0.26	3.66±0.20	3.43±0.21
100	5.20±0.40	3.46±1.32	3.71±0.15	4.10±0.26	4.04±0.23
250	6.12±0.58	5.59±0.56	5.53±0.31	5.94±0.28	5.53±0.40

Table 4.5: Average of hydrodynamic diameter (nm) over the concentration tested and over the time

Fig.4.2 and Table 4.5 report the hydrodynamic diameter (µm) of the n-TiO_2 in solution measured at time 0 h, 24 h, 48 h, 72 h and 96 h. The hydrodynamic diameter is in the order of micrometers, suggesting a strong aggregation of the n-TiO_2 in the media. It is not possible to detect a trend over the time for each concentration tested. Whereas a correlation among the hydrodynamic diameter with the concentration tested can be pointed out. The hydrodynamic diameter increases with increasing of the concentration tested, except at 25 mg/L and 100 mg/L. Also, the results show a fast aggregation in the media, since that values of hydrodynamic diameters in the order of micrometers are also detected at time 0.

Bottle water and ISO water

The characterization of the ENPs dispersed in Bottle water and ISO water was performed only at the concentration of 10-50-100 mg/L and at the time 0 h and 24 h. The average of the hydrodynamic diameter and of the polydispersity index (PDI) of n-TiO_2 are reported in Table 4.6 and Table 4.7. As above, a strong aggregation with hydrodynamic diameters of the ENPs in the order of micrometers has been observed. The values of PDI at time 0 h are in the range of 0.2-0.5 indicating that the solution is partially monodisperse; whereas the PDI values at 24-h are higher than 0.5 indicating that the solution is polydisperse.

	Time 0		Time 24-h	
mg/L	D(µm) ± std.dev	PDI ± std.dev	D(µm) ± std.dev	PDI ± std.dev
10	2.26±0.26	0.48± 0.10	3.63±0.66	0.93±0.05
50	5.10±0.52	0.55±0.01	7.50±0.58	0.81±0.15
100	3.50±0.45	0.49±0.10	5.60±1.33	0.71±0.07

Table 4.6: Average of hydrodynamic diameter and PDI measured at the concentrations of 10-50-100 mg/L of n-TiO_2 at time 0, 2, 4-h in Bottle water.

	Time 0		Time 24-h	
mg/L	D(µm)± std.dev	PDI± std.dev	D(µm)± std.dev	PDI± std.dev
10	1.29 ±0.06	0.31±0.03	7.12±2.34	0.76±0.22
50	3.07±0.25	0.31±0.05	8.51± 3.26	0.89±0.18
100	4.43±0.28	0.44± 0.05	4.70±0.44	0.60±0.34

Table 4.7:Average of hydrodynamic diameter and PDI measured at the concentrations of 10-50-100 mg/L of n-TiO_2 at time 0, 2, 4 h in ISO water.

4.2.4 Discussion

4.2.4.1 Time and volume of exposure

Our results show that the exposure time and the volume of exposure are important parameters in evaluating the n-TiO_2 toxicity to aquatic organisms. Adverse effects have been observed extending the exposure time up to 96 h. We found 96 h EC50 values of 33 mg/L (95% CI:3-361) in ISO water, of 82 mg/L (95% CI:2-3770**)** in Bottle water and of 32 mg/L (95% CI:8-125) in ELENDT M7. The effect concentrations (EC50) were incalculable at 72 h and no or low effect at 48 h has been observed in each procedure followed. Other reports refer that the exposure time is a key parameter for the toxicity of n-TiO_2 on crustaceans (*D.magna*).

Zhu et al. (2010) extended the acute exposure time up to 72 h and observed a higher toxicity at 72 h; the 48 h EC50 and LC50 values were both greater than 100 mg/L, in contrast 72 h EC50 and LC50 of 1.62 mg/L and 2.02 mg/L were found respectively.

Also, Dabrunz et al. (2011) prolonged the exposure time of acute toxicity test with n-TiO_2 up to 72 h and 96 h. No toxicity was observed after 48 h in any concentration tested (up to 8 mg/L) in contrast an immobilization rate of 100% at 2 mg/L was observed at 96 h. Thus, the calculated 72 h and 96 h EC50 values were 3.8 mg/L (95%; CI: 5.3–2.3) and 0.73 mg/L (95%; CI: 0.78–0.68), respectively.

Our study suggests, also, that the volume of solution at which the organism are exposed influences the test results. No adverse effect could be observed when the organisms were exposed to 50 mL of solution: no organisms immobilization was observed in all the tests performed and for the whole duration of the bioassay (24 h, 48 h, 72 h and 96 h).

4.2.4.2 Procedure followed

Since that specific guidelines to conduct eco-toxicological test on ENPs are still missing we have evaluated several treatment methods of the stock solution and several treatments of solution to test (Tables 4.1 and 4.2). Either in SETUP A and B the procedure proposed by Meissner et al.,(2009) has been applied and a stable stock solution has been obtained. We applied several treatments of the stock as well as of solution test, following the methods usually applied in the toxicity studies with nanoparticles. No adverse effect was observed at 48 h, 72 h, 96 h when the organisms were exposed to 50 mL of test solution and the bioassays were agitated constantly throughout the exposure (up to 96h) (Tables 4.1 and 4.2: setup B procedure 3, 4, 5). In contrast, Zhu et al., (2009) conducted acute toxicity test on *D.magna* with n-TiO_2, the beakers (100 mL filled with 30 mL of test solution, 10 Daphnia < 24-h old for each concentration tested) were agitated constantly at 140 rpm throughout the 48 h exposure and a 48 h EC50 for n-TiO_2 of 35.306 mg/L (95% CI: 25.627–48.928) was calculated. Lovern and Kapler (2006) prepared the titanium dioxide stock solution by placing n-TiO_2 in THF and then filtering (0.22 µm) the dispersion. The organisms (10 *D.magna* neonates, < 24h old) were placed in beakers filled with 50 mL of the test solution. An LC50 of 5.5 ppm was evaluated. The authors suggest that the treatment of filtration is an influencing parameter to the test results. In fact when they exposed the organisms to sonicated and non-filtered THF solution an LC50 value was incalculable (not in the tested concentration range). In the procedure 5 of SETUP B we followed a similar procedure to those of Lovern and Kapler (2006): we didn't filter n-TiO_2 dispersion but the bioassays were maintained in agitation for the whole time of exposure. In our study no adverse effect was observed. Thus, we suggest that even if the same test procedures are followed, the difference results may be attributable to the use of different n-TiO_2. This, underline the need of standard ENPs as reference.

4.2.4.3 Biological bioassays: analysis of data

The Table 4.3 reports the 96 h-EC50 (mg/L) values and the relative confidence of interval calculated for each dilution medium (ISO water, ELENDT M7, Bottle water) tested. For all of the single tests performed the interval of confidence is low indicating a good precision of the data and the correct execution of the test. An exception can be observed for two of the three tests performed with Bottle water, where the values of the confidence of interval are higher (Table 4.3, Bottle water: Test A-B). Probably, due to a not optimal concentration range tested. However, the confidence of interval of the mean of the 96 h EC50 (mg/L) calculated for ISO water, ELENDT M7, bottle water and (Table 4.3) on the basis on the three test performed (for each of medium applied) is higher. The result is an example of the biological variability; moreover it describes the low reproducibility of the biological tests with ENPs. The ANOVA test refused the hypothesis that the water applied could be a source of variability in fact no significant difference was found. We suggest that the high variability inside the acute tests performed, is attributable to the challenge of the organism to keep in contact with the n-TiO_2. Also other authors (Hund-Rinke and Simon, 2006) in acute toxicity test with *D.magna* on n-TiO_2 reported an high variability among the test, suggesting as reason the heterogeneous distribution of the nanoparticles and the random contact between organisms and ENPs.

4.2.4.4 Aggregation of n-TiO_2 in solution

The exposure to the ENPs seems to be influenced also by the aggregation processes that occur in solution. For instance, Hund- Rinke et al. (2006) show that the aggregates of n-TiO_2 smaller than 50 µm are easily ingested by daphnids. Furthermore, Zhu et al. (2010) exposed D.magna to particle aggregates less of 50 µm and performed a bioaccumulation test with n-TiO_2 on *D.magna* referring an high accumulation and low depuration with BCF values of 5.66 $*10^{\wedge 4}$ and 1.18 $*10^{\wedge 5}$ calculated in 0.1 mg/L and 1.0 mg/L treatment groups. Researchers underline the importance to characterize the ENPs in solution when biological assays are proposed. We characterized the n-TiO_2 solutions prior to test (stock solution) and during the bioassay time. A strong and fast agglomeration of the n-TiO_2 in solution occurred and no significant hydrodynamic diameter differences were observed among the media applied. The particle size of the n-TiO_2 in the stock solution is in the range order of several 100 nanometers, while in media (ELENDT M7, ISO water, bottle water) aggregates of the range of order of micrometer were detected. The heterogeneity of the collected data, in terms of hydrodynamic diameter, for each concentrations tested over the time, does not make it possible to detect a correlation of the particle size over time. Whereas a correlation among the particle size and the concentrations tested has been observed: in fact the particle size increase with increasing of the concentrations tested. Furthermore, the PDI detected for of the n-TiO_2 solution in the three media (ELENDT M7, ISO water and bottle water) is in the range of 0.5-1, indicating an unstable and polydispersed solution. Also, the strong and fast aggregation of nanoparticles in solution has been clearly observed during the manipulation of the 24 well plates by naked eyes. On the bottom of the 24 well plates, a white layer of n-TiO_2 has been observed since the 24 h confirming the sedimentation of the particle due to the aggregation phenomena.

4.2.5 Conclusion

Acute toxicity has been observed only when the organisms were exposed in 2 mL of solution (24 well). Also, the effects (immobilization) could be observed at 96h,. whereas at 72 h an EC50 value was incalculable and at 48 h no or low adverse effect was observed. To our knowledge, this study is the first one that use the 24 well plates and exposed the organism to 2 mL of test solution. Hund-Rinke and Simons (2006) applied the Petri dishes (Ø 55mm) filled with 20 mL of test solution in order to increase the contact between the n-TiO_2 and the test organism *(Daphnia magna)*. The EC50 values calculated with ISO and ELENDT M7 waters are more similar comparing with bottle water. The ANOVA test rejected the hypothesis that the media influences the test results. Also, the results of the ANOVA test indicate a low reproducibility within the tests (same SETUP and parameters). DLS measurements were applied due to its ability to analyze the particle sizes in solution. The physicochemical characterization of the n-TiO_2 in solution evidences a strong aggregation of the n-TiO_2 already occurring in the first hours of the experiment. Also the results suggest that the aggregation process is influenced by the concentration of the n-TiO_2 in solution. An increase in the average hydrodynamic diameter is expected with an increasing of the concentration tested, since the frequency of particle collision is a strong function of particle number concentration. Our results confirm aggregation of n-TiO_2 which result in changes in organism exposure levels (Römer et al., 2011). In fact, due to the aggregation processes the n-TiO_2 tend to settle on the bottom of the test's container (beaker or well plates), decreasing the contact between the organisms and the n-TiO_2. Our study suggests that the toxicity of the n-TiO_2 is strictly correlated with the challenge of contact of the *D.magna* with the n-TiO_2 and thus again to the modality of exposure chosen in the experimental design. A strong aggregation of the NPs occurs both in the beakers (filled with 50 mL of solution,) than in 24 well plates (each well filled with 2 mL). Anyway, in beakers the organisms have more space to swim and less challenge to come in contact with the n-TiO_2. Whereas, the decrease of volume of solution increase the contact between *D.magna* with the n-TiO_2. Applying the 24 well plates as vessel we observed a more pronounced adhesion of the n-TiO_2 to the external surface of the daphnids. The mechanism of toxicity of n-TiO_2 the is still unclear, thus, even if the experimental design that we proposed is far from being environmental realistic, it confirms the adhesion of the ENPs to the exoskeleton as a possible mechanism of toxicity. Also, the study points out that the aggregation and sedimentation processes have to be taken into account in order to reproduce the most realistic environmental condition as possible.

4.3 The ecotoxicity of n-TiO_2 on algae: a case of study with *P.subcapitata*

During my PhD training, I supervised a master thesis on "*The evaluation of the effect factor for nanoparticles of titanium dioxide for Life cycle assessment methodology: a case study on P.subcapitata*" of Tomassini Sophia.

The main aim of the thesis is to evaluate the toxicity of n-TiO_2 (ENEA-Frascati, Rome) on aquatic organism representative of the trophic level of algae, with the final goal to applied the concentration of effect (EC50 value) into the Life cycle impact assessment step.

The experiments were performed at C.I.R.S.A, Centre of Research for the Environmental Science, University of Bologna (Italy) and at C.R.S.A, Centre of Research for Centre of Research of MED INGEGNERIA (Marina di Ravenna, Italy). Even if, I worked at this project as supervisor the data will be collected and applied directly in my PhD thesis for the calculation of the effect factor for n-TiO_2; therefore, the experiment test is here briefly reported.

4.3.1 Materials and methods

4.3.1.1 Test compound

Uncoated and powdered n-TiO_2 was obtained from ENEA Research Centre of Frascati (Rome, Italy). The nanopowders of TiO_2 (XRD size: 15 nm; DLS hydrodynamic diameter (z-average): 183 ± 7nm, Polydispersity: 0.3; crystalline phase: 96 %wt anatase, 4 %wt rutile) are manufactured by laser pyrolysis. The powders of n-TiO_2 purchased from ENEA Frascati were analyzed by BET before the application in the bioassay. BET measurements revealed a surface area of 67.819 (m^2/g) and a primary particle diameter of 18 nm was determined.

4.3.1.2 Preparation of stock solution and media

A stock solution (1g/L) was prepared following the method proposed by Meissner et al., 2009. Briefly, an amount of n-TiO_2 was dispersed in Milli-Q water. The dispersion was adjusted to pH = 4 using hydrochloric acid (HCl 0.1 M) and then sonicated in ultrasonication bath for 30 minutes. The stock solution was stored at dark and a temperature of 4 °C and furthermore ultrasonicated for 30 minutes before each bioassay. Algal medium culture, that contains nutrient salts necessary for the development of microalgae, is prepared according to Algaltoxkit^{FTM} conformed to ISO 8692:2004.

4.3.2 Ecotoxicological test with algae

The ecotoxicological test with the green alga *Pseudokirchneriella subcapitata* was conducted following the guidelines ISO 8692:2004. In this test, algal growth is determined during an incubation period of 72-h. In this study, the algal growth inhibition was measured both by cell counting with optical microscope (Nikon Eclipse E600, 400x) and algal biomass was measured by chlorophyll fluorescence of the samples by fluorimetric measurements (Jasco FP-6200).

The algal stock culture for inoculation was taken from the commercial test system Algaltoxkit^{FTM} (MicroBioTests Inc., Nazareth, Belgium). The substance under investigation was incubated with *P. subcapitata* at 22 °C±2 °C for at least 72 h in 50-ml glass incubation vials containing 25 ml of algal growth medium. The vials were constantly shaken on a shaker table (about 80 rpm) and illuminated under fluorescence tubes (4000-10000 lux).

Four ecotoxicity tests were performed and all assays were run in two replicates with initial algal cell count of 10^4 cells/ ml. For n-TiO_2 inhibition tests, the concentrations of 0-1-2-4-6-8-16 mg/L were tested

Cell counting with optical microscope

After 72-h, a volume of 100µl was taken from the glass vial and the algal cells were investigated under optical microscope.

Fluorimetric measurements

After 72-h, samples of 10 mL were taken and the algal growth rates were calculated on the basis of total algal biomass in each sample quantified by acetone extractions of chlorophyll, as described by the guidelines UNI 11006:2002. Then, in order to minimize interference, samples containing n-TiO_2 particles were stored at dark at 4°C and allowed to settle for 3-h prior to fluorescence measurements. The fluorescence of the samples (4 Ml) were subsequently measured with a 10 mm square quartz cuvette, with an excitation wavelength of 430nm and emission wavelength of 671±20 nm.

4.3.2.1 Analysis of data

For each replicate performed, a 72-h log10 EC50 and the associated 95% interval confidence were calculated (Stasoft, Inc. (2001), Statistica ,data analysis software system). The 72-h EC50 (mg/L) values were calculated as arithmetic mean of two 72 h log10 EC50.

4.3.3 Result

The algal growth inhibition has been calculated both by cell counting with optical microscope (400x) and measuring the chlorophyll content by fluorimetric measurements. For each tests conducted, the concentrations of effect 72-h EC50 (mg/L) have been reported in Table 4.8

Table 4.8: Ecotoxicity test results (EC50, mg/L) on *P.subcapitata* with n-TiO_2.

TEST	EC50 (mg/L), Count cells by microscope	EC50 (mg/L) Fluorescence measurements
A	2.24 (95% CI: 1.13-4.44)	Not measured
B	2.61 (95% CI: 1.76-3.87)	5.36 (95% CI:2.45-11.71)
C	1.55 (95% CI: 1.22-1.95)	2.28 (95% CI: 1.53-3.40)
D	2.39(95% CI: 1.74-3.28)	3.81 (95% CI: 2.40-6.03)
E	2.27 (95% CI: 1.79-2.89)	3.91(95% CI: 2.41-6.33)
Geometric mean	**2.18 (95%CI:1.50-3.15)**	**3.36(95%CI :2.15-6.17)**

4.3.4 Discussion and Conclusion

The ecotoxicity results (72-h EC50) values are not significantly different among the two quantitative methods applied: count cells by microscope and fluorimetric measurements. The Table 4.8 reports the 72 h-EC50 (mg/L) values and the relative interval of confidence calculated for each test performed. For all of the single tests the interval of confidence is low indicating a good precision of the data and the correct execution of the test .Furthermore, the results confirm the validity of the two methods for n-TiO_2 testing proposal.

The 72-h EC50 values of 2.2 mg/L and 3.8mg/L calculated in this study are in the same order of magnitude of the result of Aruoja et al.,2009; who reported a 72-h EC50 values for n-TiO_2 on *P. subcapitata* of 5.83 mg/L. Among the other study on n-TiO_2 and *P.subcapitata* our results is the lower 72-h EC50 found (Table 4.8).

Further details are discussed in this thesis in the following section 4.5.1.1. To date the research conducted within the master thesis is still in progress therefore, the results will be here referred as "Tomassini, 2013".

4.4 Standard toxicity test and preparation of nanoparticles suspensions

In order to understand the potential negative environmental effects of engineered nanoparticles, appropriate tests for their hazard potential, hereby generating relevant results, are required. A key player in regulatory health and safety testing of chemicals is the OECD Chemicals Committee. One task within the remit of this committee is the development of standard test guidelines for use primarily in regulatory safety testing (OECD, 2011) 2011). To deal with the potential risks of nanomaterials, the OECD Working Party on Manufactured Nanomaterials (WPMN) has been established as a subsidiary body to the OECD Chemicals Committee. The WPMN was established in order to "ensure that the approach to hazard, exposure and risk assessment (of nanomaterials) is of a high, science-based and internationally harmonized standard" (OECD, 2010b). As part of their work, the OECD Sponsorship Programme of Testing a Representative Set of Manufactured Nanomaterials was launched in 2007 with the expected outcome of identifying intrinsic nano-specific properties of nanomaterials (OECD, 2010a). Ongoing work to develop methods and strategies has resulted in several projects and numerous publications in the Series of Safety of Manufactured Nanomaterials. This includes a Preliminary Review of OECD Test Guidelines for their Applicability to Manufactured Nanomaterials.

The preliminary review (OECD, 2010b) concerning the application of OECD guidelines to manufactured nanomaterials stated that the basic practices recommended by these guidelines are suitable for the testing of nanomaterials. However, guidelines for the delivery of substances to test systems, the quantitation of exposure, and the dose metrics, need to be adapted for the testing of nanomaterials. The guidance for sample preparation and dosimetry for the safety testing of manufactured nanomaterials does not provide detailed instructions for the application of nanomaterials in aqueous or non-aqueous media, although principal procedures are listed.

Thus, the standard test (e.g. OECD, US-EPA) and standard organisms are applied and tested with nanoparticles. Garcia et al. (2011) reported that the standard toxicity test (e.g. OCED 202) are able to provide reliable information about NPs toxicity. The acute toxicity tests (OECD 202) performed on *Daphnia magna* with TiO_2 during my PhD training, show a low repeatability. Unfortunately this low repeatability of the toxicity test with ENPs can be only argued since, no other study discussed the repeatability of their toxicity tests. Therefore, no comparison can be carried out. Even if the standard tests are largely applied in nano-ecotoxicology, several issues have been raised. For instance, the standard exposure time (48-h) proposed for acute toxicity test on *D. magna* (OECD 202, 2004) seems not be representative of the toxicity for metal oxide nanoparticle. Reports show that the standard exposure time of acute test on crustacean *D. magna* should be prolonged up to 72-h or 96-h; an increasing in the toxic effect with the increasing of the exposure time was observed (Kim et al., 2010; Dabrunz et al.,2011, Salieri et al., 2012), thus suggesting that the exposure time is a key parameter in the investigation of toxicity. Since that there are no specific guidelines for sample preparation of nanomaterials, several methods such as, stirring, ultrasonication, addition of solvents and the removal of non-dissolved test substances by filtration or centrifugation are currently applied. Several procedures for testing nanomaterials have been described in literature, as well. The bioavailability and the test results of nanomaterials may depend on the preparation method and on the procedure for testing (Hund-Rinke et al., 2010). Therefore, the lack of a standardized procedure and of a ENPs as reference has been argued as source of the high variability of toxic effects of ENPs reported in literature. The samples preparation of nanomaterials can influence the dispersion property and the toxicity of nanoparticles. For instance, the test solutions filtered exhibit a higher toxicity than unfiltered test solutions, both on algae (*P.subcapitata*) and crustaceans (*D.magna*) (Hund-Rinke et al., 2010; Lovern and Kapler 2006). The sonication and stirring of the nanoparticle suspension may influence the behavior on the ENPs in the test solution, therefore affecting the exposure mode and as a consequence the toxicity to aquatic organisms. The sonication results in an increase of the specific surfaces area by promoting disaggregation and breaking up the aggregates (Hund-Rinke et al., 2010).

Method	Advantage	Limitations
Solvent or dispersants	Fast dispersion of NPs without any other steps; Stable dispersion	Solvent may also be toxic; Toxic impurities may be present in the solvent; Extra solvents may deform some structure therby changin toxicity Need to keep a consistent ratio of solvent and test materials
Ultrasonication	No solvent are involved No extra toxicants are added No solvent control needed	Sonication times are variable with the concetration; Ultrasonication can produce change of shapes and consequently in toxicity; Unstable dispersions
Stirring	No solvent are involved No controls are needed	Long stirring times are needed Unstable dispersions
Shaking	No solvents are involved; No control needed	Long shaking times are needed; Unstable dispersions; Shaking can produce change of shapes and consequently in toxicity; Additional steps coul be required

Fig. 4.3: Advantages and limitations of dispersion preparation methods (Farrè et al., 2009)

Furthermore, there are evidences that the toxicity of metal oxide nanoparticles such as Ag, ZnO, CeO and CuO to aquatic organisms (e.g. *D. magna*) may be due to the ions (e.g. Ag^+ and $Cu2^+$) released from the nanoparticle surface; whereas it is believed that the dissolved concentration released from nanoparticle suspension may vary with respect to the preparation methods and act as a crucial determinant of the acute toxicity. Following this hypothesis, Jo et al. (2012) investigated how different methods of preparing the test solution of Ag and CuO nanoparticles would result in different acute response on *D.magna*. The authors have applied several methods of treatment (such as filtering, un-filtering, sonication and stirring) showing that:

- Unfiltered solutions of both Ag and CuO nanoparticles were much less toxic than filtered solutions both of Ag and CuO nanoparticles, apparently because of the aggregation of nanoparticles limiting the toxicity of nanoparticles;
- The Ag nanoparticle suspensions dispersed by sonication have a higher acute toxicity than suspensions dispersed by stirring when based on total Ag concentrations;
- The initial concentration largely affects the toxicity: for Ag nanoparticles the suspensions prepared with a higher initial concentration have lower toxicity than suspensions prepared with a lower initial concentration;
- Little differences in the toxicity among the different dispersion methods were observed when toxicity was evaluated in terms of the dissolved concentrations;

- Regardless of dispersion methods, CuO nanoparticle suspensions have a similar toxicity based on both total and dissolved Cu concentrations.

Thus, for certain nanoparticles such as Ag, the application of different dispersion methods, such as stirring, sonication and solvent addition, could lead to dissimilar acute toxicity results when the toxicity is estimated in terms of their total concentration instead of dissolved concentration.

Also, for metal oxide ENPs such as n-TiO_2, it is well known that nanomaterial properties such as surface area, agglomeration size, deposition and resuspension behavior depend on environmental conditions. Therefore the ENPs toxicity may depend both on the employed methodology and on the properties of the medium supporting the sample under consideration (Hund-Rinke et al., 2010). Thus, characterisation procedures, through the recent technological advances in areas such as transmission electron microscopy (TEM), scanning electron microscopy (SEM), confocal microscopy, light scattering, atomic force microscopy should be applied. To date, the charaterisation of metal oxide ENPs such as TiO_2, is scarsly applied. This leads to a scarce knowlege on the exposure scenario and on the bioavailable form to which the organisms are exposed.

Furthermore, for toxicity tests with ENPs, a spanning of acute factors (mortality) and chronic factors (growth or reproductive inhibition, changes in morphology) among similar species are reported in literature. Therefore there is significant variation in the toxicological endpoints considered. Also, among the same nanoparticles tested (e.g. n-TiO_2) the reports are inconsistent in the type of ENPs used as toxicants; thus, a large variation in physicochemical properties (e.g. crystal size, primary size) among the toxicity studies is reported.

Concerning the endpoint type and the methodology of assessment some considerations should be made.

In the acute toxicity tests performed with n-$TiO_{2,}$ carried on the organisms representative of algae, strong phenomena of aggregation of the particles in test media have been observed . The aggregates may be entrapped to the algal cells and reducing the light availability for the algal cell. The inhibition of growth rate is usually the endpoint measured in algae tests (OECD 201) which is assessed counting the algal cells using a microscope. Due to the entrapment mechanism, the measurements of the endpoint, through the measurement of chlorophyll by fluorimetric measurements, may be leading to less bias (Carini M., 2011; Salieri et al., 2013a)

Also sub-lethal investigations are useful to evaluate the ENPs toxicity; in Kim et al. (2010) even if the acute effect concentration (LC50) value was incalculable, an increasing activity of antioxidant enzymes with an increase of n-TiO_2 concentrations was observed.

For the aquatic organisms representative of the trophic level of fish, the state of art shows that most nanoparticle types (e.g. n-TiO_2) do not exhibit or have low direct toxicity, but sub-lethal effects are displayed (Jovanovic and Palic, 2012). However, biochemical studies of the n-TiO_2 oxidative stress and/or of other sub-lethal effects are still limited (Kim et al., 2010).

4.5 A bibliographic review on n-TiO_2 toxicity

4.5.1 Toxicity of n- TiO_2 to algae

Algae play an important role in the aquatic ecosystem, not only producing biomass forming the basic nutriments for food webs, but also oxygenating the water for animal life to thrive. Algae occupy a unique position in the aquatic world because they utilize light energy in the process of reducing CO_2 to the oxidation state of cellular carbon. Algae are the ultimate source of both cellular carbon and chemical energy for other organisms, and because of this are often called primary producers.

Therefore, algae are commonly used as model organisms for the toxicity examination of toxicants and nanoparticles as well. The bibliographic review on the effects of n-TiO_2 on algae is presented in Tables 4.8 and Table 4.9. Most of the studies have been performed on *Pseudokirchneriella subcapitata* (Table 4.8), while fewer have been conducted on *Desmodesmus subacapitatus, Chlmydomonas reinhardatii, Chlorella sp* (Table 4.9).

Extremely variable 72-h EC50 and LC50 values were reported for TiO_2 nanoparticles tested with *P. subcapitata* (Table 4.8) as well with other algal species (Table 4.9). The high variability of the toxicity metal oxide ENPs (such as n-TiO_2) could be related to several parameters such as: test method, particle size, surface area, crystal form.

4.5.1.1 Test method

Most of the studies on the toxicity of n-TiO_2 on algal species have been performed in accordance with ISO or with OECD standard procedure both procedures have the growth inhibition percentage after 72-h of exposure as an endpoint. Sometimes the duration of the exposure has been prolonged up to 96-h (Griffitt et al., 2008) or up to 144-h (Ji et al., 2011); however the exposure duration does not seem to be an influent parameter on the toxicity of metal oxide NPs, since the trend of toxicity is similar among the exposure times (2-4-6 days).

The toxicity tests performed with n-TiO_2 on *P. subcapitata* (Table 4.8) show heterogeneous EC50 values: from 5.83 mg/L (Aruoja et al., 2009) to 241 mg/L (Hartmann et al., 2010). However, a lower effect concentrations 72-h EC50 of 2.2 mg/L and of 3.65 mg/L have been calculated in the toxicity tests performed at the Centre of Research of MED INGEGNERIA (Marina di Ravenna, Italy) under my supervision. The result (72-h EC50: 2.2 mg/L) obtained by counting of algal cells in this study is not accordance with a previous study (Carini et al., 2012) conducted on the same nanoparticles (n-TiO_2, ENEA.-Frascati), on the same algae P.subcapitata at C.R.S.A, Centre of Research for Centre of Research of MED INGEGNERIA (Marina di Ravenna, Italy). In those study a 72-h EC50 of 422 mg/L was found. Also, this result is the highest value reported in literature. In the ecotoxicity test of Carini et al., (2012) the bioassays were performed by inoculating 2 mL of solutions to test in 24-well plate. During the 72 h incubation, the 24 well plate have been kept at controlled temperature (22 ± 2°C) and at constant agitation on shaker table (about 80 rpm) and under continuously illumination.

During the experiments large aggregates of n-$Ti0_2$entrapped to algal cell and fragments of algae cell dispersed into the solution has been observed.

The observations of Carini et al., 2012 confirms the 1) formation of aggregates in the algae medium, 2) the mechanism of entrapping of aggregates to algal cells and 3) the disruptions of membranes cells due to the contact with ENPs. However, this observation are not in accordance with the EC50 calculated; in fact a lower EC50 should be expected since several mechanisms of toxicity have been observed. Therefore, we hypothesize that the cell count measurement by human error has been affected due to the high turbidity of the solutions tested and the presence of fragments of the algal membrane cells.

However, the study conducted by Carini M, (2011) evidences the difficulties of testing low soluble ENPs such as n-TiO2 and furthermore, as the lack of standardize protocol for testing ENPs can increase the variability of the toxicity results.

Thus on the basis of the practical experience in laboratory a combination of several techniques is recommended. Such as, the combination of visual inspection, cell counting and fluorescence measurement which should give additional insight into the nature of the observed effects.

4.5.1.2 Particle size

The relationship among algal toxicity, primary particle size and/or aggregation state, also defined as secondary particle size of the n-TiO_2 in solution, is not yet clear.

Primary particle size

No clear relationship between the primary size of particles and effects on algae *P. subcapitata* could be discerned. For example, particles described as less than 100 nm in diameter after filtration were not at all toxic to these algae (Blaise et al., 2008), while sonicated particles with diameters between 25 and 70 nm in diameter were very toxic (Aruoja et al., 2009).

Hund-Rinke and Simon (2006) tested two different n-TiO_2 products on *Desmodesmus subspicatus*: product 1 with a primary size of 25 nm (crystalline form: mainly anatase), while the particle size of product 2 was 100 nm (crystalline form: 100% anatase). Product 1, smaller in primary particle size, exhibited the highest toxicity (endpoint: inhibition of growth rate) and a 72-EC50 of 44 mg/L was observed. In contrast to product 1, the 72-h EC50 of product 2 resulted not determinable. Similarly, Hartmman et al. (2010) suggest that the primary size of the ENPs may influence the toxicity results. The authors performed acute toxicity tests on *P.subcapitata* testing 6 concentrations (0.6–250 mg/L) of three different n-TiO_2 with particle size respectively of: <10 nm, 20 nm and 300 nm. At the lower concentration tested the highest growth inhibition was observed with the smaller n-TiO_2 tested, (<10 nm). Anyway, the 72-h EC50 values do not reflect this trend: the smallest particles (n-TiO_2, <10 nm) showed a lower toxicity (72-h EC50 of 241 mg/L) than the ones with a higher primary particle size (Table 4.8). Thus, the toxicity of n-TiO_2 may be influenced also by other factors such as the different crystal form. In fact the NPs tested in the study differ from each other by the content of amorphous TiO_2.

The toxic effects data (72-h EC50) collected in this thesis and the primary size of the n-TiO_2 have been correlated and proposed in Fig. 4.4. No clear relationship between the primary size of particles and effects on algae *P. subcapitata* could be discerned.

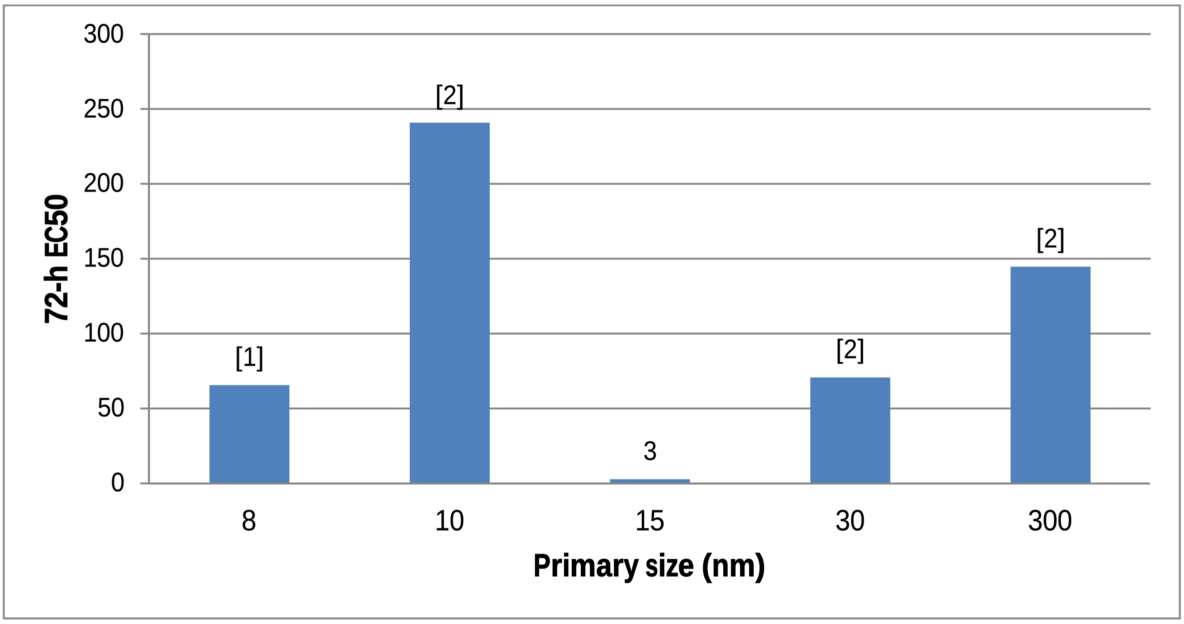

Fig.4.4 Relation between 72-h EC50 values and primary size for algae *P.subcapitata* [1] Hund-Rinke et al., (2010) [2] Hartmann et al., (2010); [3] “Tomassini 2013”

Secondary particle size

Generally, metal oxide particles have been found to form larger aggregates >100 nm in both algal test media and freshwater (Hartmann et al., 2010; Keller et al., 2010; Aruoja et al., 2009); observed n-TiO_2 cover a larger extent of the algal surfaces than the bulk particles where more particle-free algal cells were seen. With regard to the formation of aggregates in test media and their influence on toxicity, Aruoja et al. (2009) and Ji et al. (2010) speculated that the aggregates in aqueous solution form an opaque solution reducing the incident light beam (therefore leading to “shading effect”) and influencing the activity of photosynthesis cell and the production of chlorophyll with a consequent inhibition of cell growth. Thus, several studies have been designed to elucidate the possible shading effect of nanoparticles (e.g. n-TiO_2) in algal growth tests, most of these have applied a physical separation of algal cells and nanoparticle suspensions (Hund-Rinke and Simon, 2006; Aruoja et al., 2009). By using this method, shading has been rejected as a cause of the observed reductions in growth. However, there are evidences that the particle aggregates may be entrapped on algal cells and reduce the light availability to the entrapped algae cells, thus inhibiting the algal growth (Hartmann et al., 2010; Sadiq et al., 2011; Ji et al., 2010). Particle adhesion may also lead to direct physical effects such as disruption of cell membrane as a result of their surface structure or due to photochemical reactions taking place on or near the cell surface.

The toxic effects data (72-h EC50) reported in literature and the secondary particle size of the n-TiO_2 in the test media have been correlated, as proposed in Fig. 4.5. The effect concentrations (72-h EC50) for *P. subcapitata* reported in literature could not be linked to the median values of particle size in media measured by dynamic light scattering. Toxicity increases with the decreasing of the particle size in the test media.The 380 nm as well as the 140 nm diameter particles in media have similar toxicities to algae *P. subcapitata* with 72-h EC50 of 16 mg/L and 21 mg/L (Warheit et al., 2007). Furthermore, the 380 nm particles in media are much more toxic to algae *P. subcapitata* than those of 416 nm and 1261 nm.

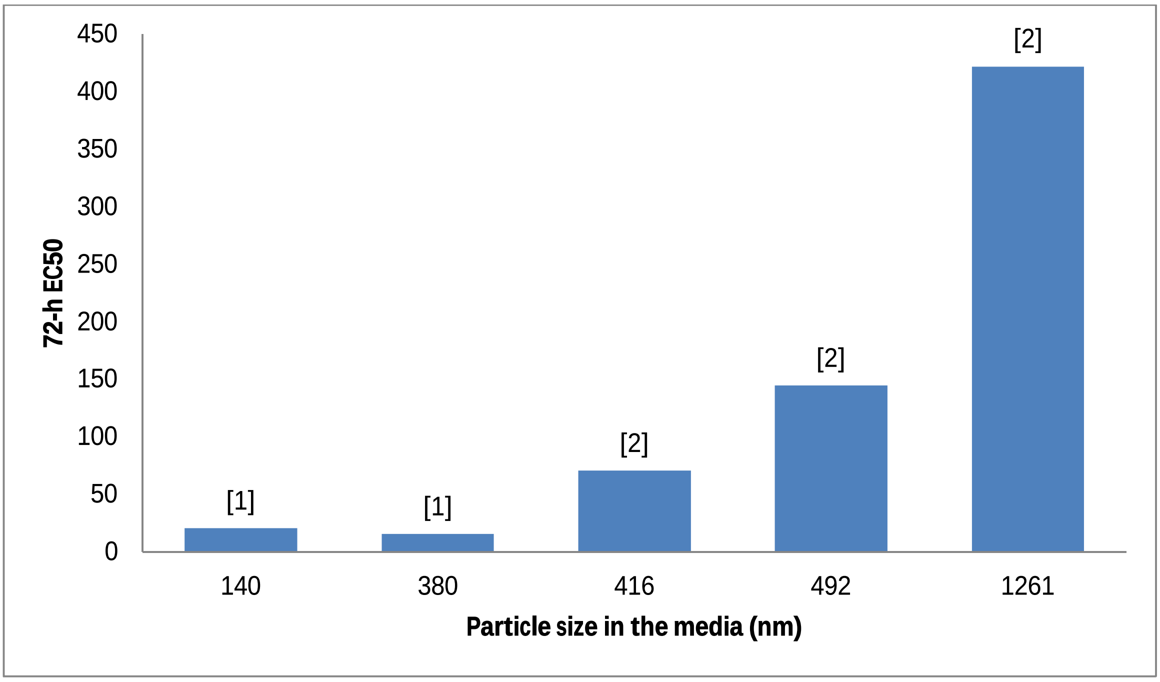

Fig.4.5: Relation between 72-h EC50 values and particle size in the media (n-TiO2) determined with dynamic light scattering (DLS) for algae *P.subcapitata*; [1] Warheit et al., 2007 [2] Hartmann et al., 2010.

4.5.1.3 Surface area

Wang et al. (2008) report that the formation of aggregates increases the size of ENPs reducing the exposed surface area and consequently reducing the chemical reactivity of ENPs on algal cells.

A correlation between the surface area and the 72-h EC50 values for *P. subcapitata* was found by (Menard et al., 2011). In this review, updating and adding more data, a correlation was not found (Fig.4.6).

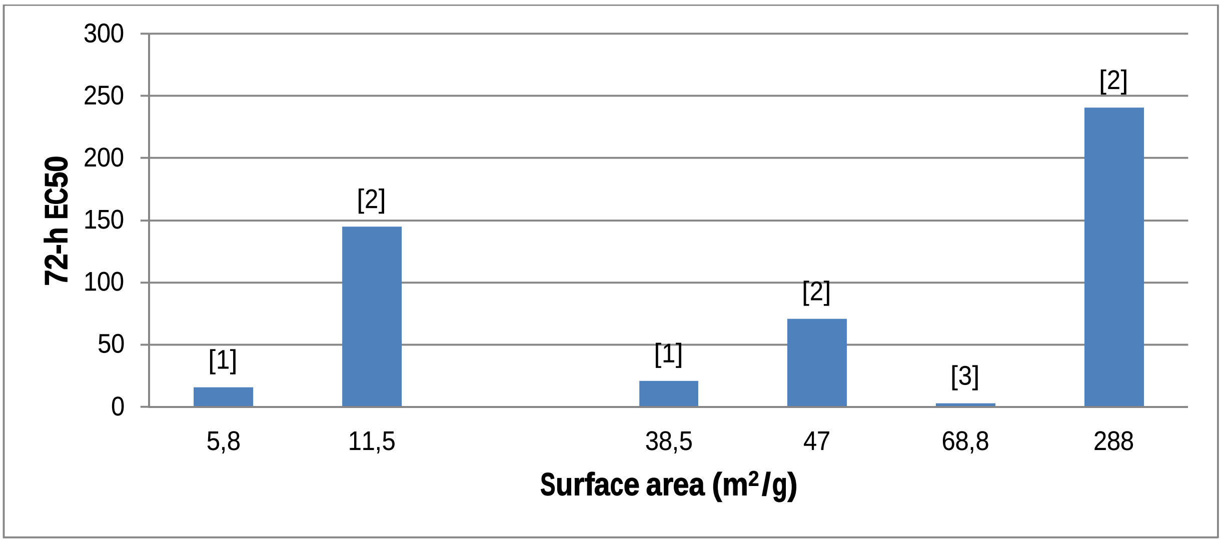

Fig.4.6: Relation between 72-h EC50 values and surface area (n-TiO2) for algae *P.subcapitata* [1] Warheit et al., 2007; [2] Hartmann et al., 2010; [3] "Tomassini, 2013".

4.5.1.4 Crystal form

The toxicity of n-TiO_2 on algae could be influenced by the crystal form: the anatase greatly inhibits the algal growth, whereas the rutile suspension shows no significant difference in term of toxicity with respect to the control, showing, moreover, a similar toxicity to the suspension of the bulk form (Ji et al., 2010).

However, the effect concentrations (72-h EC50) for *P. subcapitata* reported in literature could not be linked to crystal form used in the toxicity tests. Hartman et al. (2010) reported a 72-h EC50 of 71.1 mg/L testing a n-TiO_2 composed by 72.6% of anastase, whereas a 72-h EC50 of 21 mg/L of n-TiO_2 (79% of anastase) was found by Warheit et al. (2007).

Table 4.8: Chemical properties of n-TiO$_{2,}$ treatments of ENPs for bioassay proposal, toxicity test applied and toxicity values on *Pseudokirchneriella subcapitata*

Author	NP	Primary size (nm)	Surface Area(m^2/g)	Particle dimension DLS (nm)	ZP	Treatment of NPs	Methods	Toxicity test	Toxic value EC50 (mg/L)
Warheit 2007	Aluminium surface coating	Refers as median particle in water sampling (DLS)	5.8	380		N.R	OECD n°201	inhibition of growth rate (72-h)	16 (12-22)
Warheit 2007	(79%rutile;21 %anastase		38.5	140	/	N.R	OECD n°201	inhibition of growth rate(72 h)	21 (16-26)
Blaise, 2008	TiO$_2$	<100	/	/	/	Filtrate	/	inhibition of growth rate(72 h)	IC25> 100
Griffitt, 2008	P25	20	45.41	687.5	-25.1	Stock suspension: 10 mg powder to mL of MilliQ water, sonicated with a probe sonicator.	U.S EPA	Chronic 96-h;Algal growth	N.M
Aruoja, 2009	TiO$_2$	25-70	/	/	/	Stock suspensions were prepared in algal medium immediately before each experiment before use they were ultrasonicated for 30 min	OECD n°201	inhibition of growth rate(72 h)	5,83
Hall, 2009	TiO2 (99%)	10 nm				NPs dispersed in hard water (USEPA), stirring for 30 min. The test solutions were agitated to maintain the NPs in suspension	U.S EPA	Chronic (96-h)-Cell production	IC25=1.0-2.0 mg/L
Hartmann, 2010	TiO$_2$67,2%anastase	<10	288	1261	-23	Stock solutions were prepared by suspending TiO2 particles in algal test medium; 10 min sonication in a water bath. These suspensions were kept at 5 ◦C in the dark and sonicated again 10 min prior to preparation of test suspensions.	ISO 8692	inhibition of growth rate(72 h)	241 (95.6-609)

Hartmann, 2010	P25 TiO_2 72,6%anastase;18,4rutile; 9%amorfa	30	47	416	-21	Stock solutions were prepared by suspending TiO2 particles in algal test medium; 10 min sonication in a water bath. These suspensions were kept at 5 ◦C in the dark and sonicated again 10 min prior to preparation of test suspensions.	ISO 8692	inhibition of growth rate(72 h)	71.1 (59.4-85.1)
Hartmann, 2010	TiO_2	300	11.5	492	-25	Stock solutions were prepared by suspending TiO2 particles in algal test medium; 10 min sonication in a water bath. These suspensions were kept at 5 ◦C in the dark and sonicated again 10 min prior to preparation of test suspensions.	ISO 8692	inhibition of growth rate(72 h)	145 (112-188)
Hund-Rinke, 2010	TiO_2	8 nm	250 (m2/g)			The dispersion was prepared in according to OECD test guideline no. 201. The NPs were suspended by stirring and/or ultrasonication in a bath sonicator	OECD 201	inhibition of growth rate (72-h)	EC20 values reported
Hund-Rinke, 2010	TiO_2	150 nm	8 (m2/g)			The dispersion was prepared in according to OECD test guideline no. 201. The NPs were suspended by stirring and/or ultrasonication in a bath sonicator	OECD 201	inhibition of growth rate (72-h)	EC20 values reported
Hund-Rinke, 2010	TiO_2	8 nm	250 (m^2/g)			The dispersion was stirred vigorously(700 rpm) for 48 h. Non-dissolved solids were then removed by passing the dispersion through a 0.22 µm-filter	OECD 23 (2000)	inhibition of growth rate (72-h)	65.8
Tomassini 2013	TiO_2 (ENEA); anastase	15	68.81	/		Stock solution in MilliQ-water (pH=4) ultrasonication in a bath sonicator(30 min);	ISO 8692:2004.	growth inhibition rate (72-h)	2.2 (cell counting by optical microscope) 3.3 (fluorescence measurement)

Table 4.9: Chemical properties of n-TiO2, treatments of ENPs for bioassay proposal, toxicity test applied and toxicity values on algae

Autore	Specie	NP	Primary size	Surface Area	Particle dimension on DLS (nm)	ZP	Treatment of NPs	Methods	Toxicity test	Toxic value EC50 (mg/L)
Hund-Rinke, 2006	*Desmodesmus subspicatus*	Anastase TiO_2	25 nm and 100 nm	N.R	N.M	N.M	NPs were dispersed in test medium and dispersion was achieved by ultrasonic The mixture was continuously stirred on a magnetic stirrer. During incubation, the plates were shaken.	ISO 6341, OECD 202, DIN 38412-30.	inhibition of growth rate (72-h)	25 nm: 44 100 nm: EC50 could be not calculated
Wang, 2008	*C. reinhardtii*	79% rutile; 21% anastase TiO_2	21 nm	50	N.M	N.M	/	/	inhibition of growth rate(72 h)	10
Sadiq, 2011	Scenedesmus sp.	TiO2	<25nm	200-220	72-h: 517 nm	N.M	Stock suspensions prepared in algal medium before each experiment Sonicated for 30min before use	OECD 1984	inhibition of growth rate (72-h); chlorophyll content at 24,48,72-h	21.2
Sadiq, 2011	ChLorella sp.	TiO2	<25nm	200-220	N.M	N.M	Stock suspensions prepared in algal medium before each experiment Sonicated for 30min before use	OECD 1984	inhibition of growth rate (72-h); chlorophyll content at 24,48,72-h	16.1
Ji, 2010	*ChLorella sp.*	Anastase TiO_2	5-10nm	324	260	-21,6	sonication 30 min	/	inhibition of growth rate (144 h)	EC50 (144-h) =120
Ji, 2010	*ChLorella sp.*	Rutile TiO_2	50nm	167	280	-33,8	sonication 30 min	/	inhibition of growth rate (144 h)	not reported

4.5.2 Toxicity of n - TiO_2 to freshwater invertebrates

Ecotoxicological effects of a number of metal oxide nanoparticles have been investigated for several freshwater crustaceans species, with *Daphnia magna* as the most common test organism.

Planktonic crustaceans, like the daphnids, are generally the food and energy link between the primary producers (algae) and secondary consumers (fish and fish larvae). Furthermore, daphnids are filter feeders and have been found to feed on particles in the size range of 0.4– 40 µm (algal cells, larger bacteria). Thanks to their filtering capacity, organic and inorganic particles can be intake. There is evidence of intake of nanoparticles aggregates when *D. magna* is exposed to n-TiO_2 and nC_{60}. In fact, the single nanoparticle tends to aggregate in water solution reaching a size in the order of micrometers and can be ingested by filter feeding organism as *D. magna* (Baun et al., 2008; Salieri et al., 2012).

The ecotoxicological effect of n-TiO_2 on crustaceans (*D. magna, Daphnia pulex* and *Ceriodaphnia dubia*) are reported in Table 4.10 and Table 4.11 An extremely high variability of the effect is attributed: the effect concentrations range from 5.5 mg/L (Lovern and Kapler, 2006) to 20000 mg/L (Heinlaan et al., 2008) and most studies found an acute toxicity higher that 100 mg/L. Anyway, it cannot be overlooked that in some case a high toxicity of n-TiO_2 to freshwater invertebrates has been detected. Dabrunz et al. (2011) reported an acute 72-h EC50 of 3.8 mg/L and an acute 96-h EC50 of 0.73 mg/L for *D. magna*. Similarly, a 72-h EC50 of 1.62 mg/L was found by Zhu et al. (2010). However, the same authors referred a 48-h EC50 of 35 mg/L. This result is in the same order of magnitude of the toxic value (96-h EC50 = 32 mg/L) observed during the acute toxicity test on *D. magna* with TiO_2 performed during my PhD training. (Salieri et al., 2012;Chapter 4, section 4.2). Furthermore in some studies 48-h LC50 of 9.2 mg/L for *D. pulex* and 7.6 mg/L for *C. dubia* were found (Hall et al., 2009; Table 4.11). Chronic studies using total number of living offspring as the endpoint, reveal EC50 for total number of living offspring of 0.46 mg/L and LC50 of 2.62 mg/L after an exposure of 21 days (Zhu et al., 2010).

4.5.2.1 Test method

Most of the studies on the toxicity of n-TiO_2 on crustaceans species are in accordance with OECD standard procedure (OECD 202, 2004) or to the U.S. EPA method (US EPA, 1993). However, different treatments on test solution (sonication, filtration and others) or test designs are applied.

Experimental design

As argued above, the treatments of the ENPs may influence the test result (Baun et al., 2008).

Lovern and Klaper (2006) have studied the effect of the preparation of TiO_2 ENPs suspension on its toxicity. After filtration of the suspension (0.22 µm) in the presence of tetrahydrofuran (THF), they reported a high acute toxicity to *D. magna* (LC50 = 5.5 mg/L); whereas the EC50 is 100 times greater when the suspension was prepared by sonication. In contrast, in Wiench et al. (2009), no adverse effects on mobility were observed when the organisms (*D. magna*) were exposed to 0.22 µm filtered n-TiO_2 test solution. An higher effect concentration (LC50 = 143 mg/L) for the same level of n-TiO_2 was found by Zhu et al. (2009) when the test solutions with *D.magna* were shacked for all the time of the bioassay. The results of Lovern and Kapler (2006) may also be due to the residual THF present in the test medium.

The influence of the dispersion treatments on the toxicity is difficult to identify since a direct comparison is not possible due to different type of ENPs or test design applied in the different studies.

Also, the exposure mode may influence the toxicity results. The acute toxicity tests on *D. magna* with n–TiO_2, performed during my PhD training, point out the time and volume of exposure as influential parameters. (Salieri et al., 2012, section 4.2).

The volume of exposure seems to be a key parameter within the toxicity test. No toxicity was observed when the organisms were placed in beacker and exposed to 50 ml of solution to test; in contrast when the organism were placed in 24-well plates and exposed to 20 ml of solution to test, effect concentrations were found. Dabrunz et al. (2011) exposed *D.magna* to n-TiO_2 prolonging the standard test duration (24-h, 48-h) up to 72-h and 96-h. The authors reported a lower acute toxic value (0.73 mg/L) at 96-h than at 72-h (3.8 mg/L) (Table 4.10). Also, the results of Zhu et al., (2009) suggest a time dependent toxicity.

4.5.2.2 Particle size

Primary particle size

In order to evaluate a possible correlation between the primary size and the toxic effect, acute EC50 values reported in literature and the primary size of the n-TiO_2 tested have been compared.

Fig. 4.7 shows the acute toxicity values reported in several studies and the primary size of the n-TiO_2 tested; the toxicity values higher than the maximum concentration (for instance EC50 >100 mg/L) tested are not used. Formulated in this way, only three toxic values are available and, therefore, due to the few data available, it is difficult to evaluate a possible correlation between the primary particle size and the toxicity. No correlation between the primary particle size and the toxicity data (48-h) has been found. However, mammalian studies evidence that the small particle size, with a large reactive surface area, can lead to toxicological injury through the production of reactive oxygen species (ROS) and oxidative stress. The toxicity mediated by the production of ROS is not always well described by *in vivo* toxicity tests, as revealed by the biochemical investigation of Kim et al. (2010).

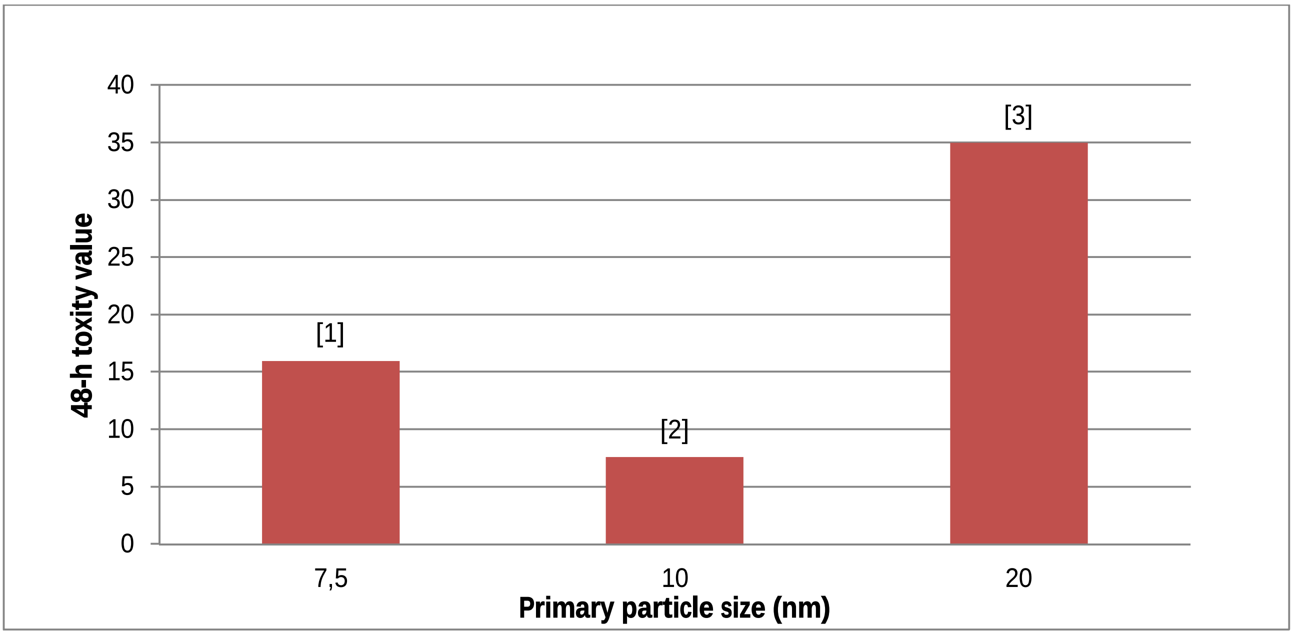

Fig.4.7: Relation between 48-h toxicity values(mg/L) reported for Crustacean and primary particle size. [1] Garcia et al., 2011; [2] Hall et al., 2009; [3] Zhu et al., 2009.

Secondary particle size

As before, a possible correlation between the particle size in suspension and the toxicity may be only hypothesized (Dabrunz et al., 2011; Lovern and Kapler, 2006) mainly due to the fragmentary information on the characterization of particle size distribution in the test media.

Lovern and Klaper (2006) conducted 48-h acute toxicity tests with *D. magna* and n- TiO_2 ; the pre-treatment of the test dispersion was either a 30 min bath sonication in deionized water or an elaborate multistep procedure involving dispersion in tetrahydrofuran (THF), sparging with nitrogen, stirring and passing through a 0.22-µm filter. Transmission electron micrographs (TEM) of the dispersions showed an average diameter of 30 nm for the filtered TiO_2 suspension and aggregations of 100–500 nm for the sonicated dispersion. The results showed a strong influence of particle size on the toxicity of the dispersions: the 50% lethal concentration for filtered TiO_2 was 5.5 mg /L while no significant effects were observed at the highest concentration of 500 mg/L in the sonicated, unfiltered dispersions. Dabrunz et al. (2011) exposed *D. magna* at 2 mg/L of two test solutions with average diameter of 100 nm and 200 nm, observing an higher acute toxicity for the 200 nm suspensions (Fig. 4.8), and suggesting a correlation between the particle size in suspension and the toxicity.

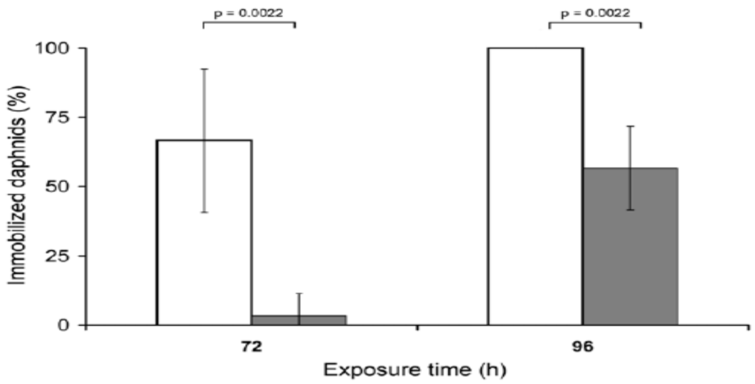

Fig.4.8: Mean of immobilization of *D.magna* exposed to 2 mg/L of n-TiO2 of different size (100 nm white bars; 200 nm grey bars) Source: Dabrunz et al., 2011.

The data on the particle size in solutions to test have been collected (Table 4.10 and 4.11). Fig. 4.9 features the relationship between EC50 (*D. magna*) values and the particle size (µm) of n-TiO_2, characterized by Dynamic light scattering (DLS) in test media.

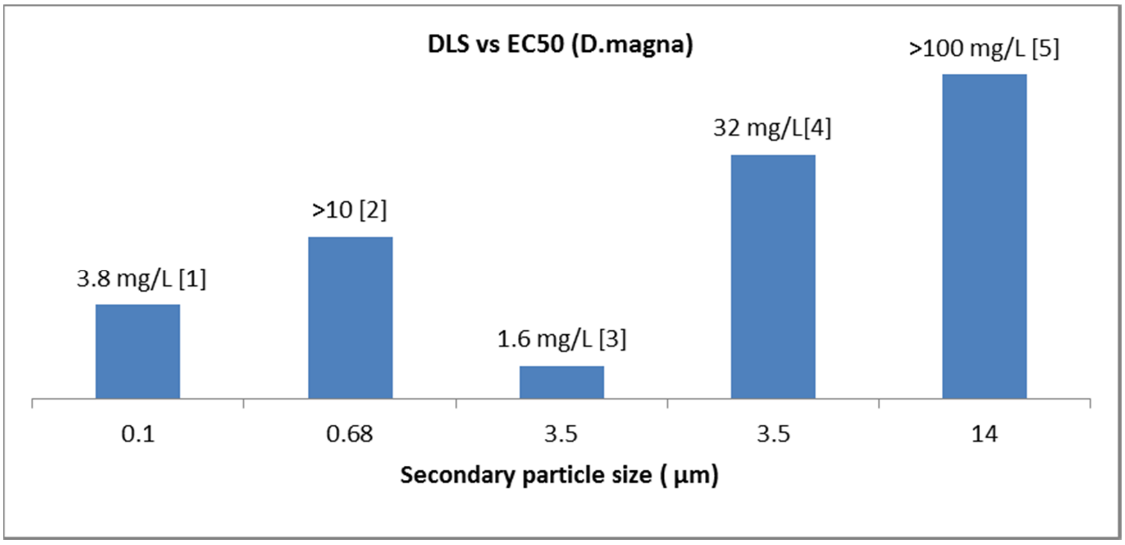

Fig. 4.9:Relation between EC50 (mg/L) values and particle size in the media tested.[1] Dabrunz et al., 2011 [2]Griffit et al.,2008; [3] Zhu et al.,2009; [4] Salieri et al.,2012; [5] Wiench et al.,2010

Apparently, with the exception of the third study, an increase of toxicity with the increasing of the size of the particle in suspension is showed. Anyway, the inhomogeneity of the acute EC50 values collected has to be observed. This inhomogeneity is ascribed to the different exposure length applied by the authors; the acute EC50 values are observed at different exposure times (48-h, 72-h and 96-h)

To my knowledge, the characterization of the ENPs in suspension in only a few eco-toxicological studies on crustaceans has been performed. (Wiench et al., 2010; Griffit et al., 2008; Kim et al., 2010).Griffit et al. (2009) characterized the size distribution of several ENPs (n-TiO_2, nanocopper, nanosilver, nanocobalt, nanoaluminium) in test media at time zero. The characterization measurements show higher values for particles of nano-aluminum (DLS: 4442 nm) and n-TiO_2 (DLS: 687nm). The toxicity to *D. magna* of n-TiO_2 is lower than the other nanoparticles with lower value of particle size, suggesting an influence of the particle size on the adverse effects. Anyway, it has not been possible to exclude toxic effects dependent on the type of NPs tested. Kim et al. (2010) performed acute toxicity tests with n-TiO_2 on *D.magna*; both the acute toxic values (48-h LC50) and chronic values were incalculable, but at the higher concentrations tested (5 and 10 mg/L) an increase of mortality was observed. Further, biochemical parameters such as antioxidant enzymatic activity of GPX (glutathione peroxidase) and GST (glutathione-S-transferase) were measured in *D. magna* exposed to 5 mg/L TiO_2 NPs after size fractionation to <200, <400, and <800 nm. The size analysis by Dynamic light scattering reports an accumulated volume percentage of 30, 37, and 71% in the size fractions of <200, <400, and <800 nm, respectively; the relative percentages of TiO_2 ENPs in size <200, 200<size<400, 400<size<800, and size >800 nm were 30, 7, 34, and 29%, respectively. The activities of GPX and GST increased with the increasing size fraction. The enzymatic responses in *D. magna* of GPX and GST were closely related to the specific size range of 400 < size < 800 nm suggesting the biomarker investigation may better evidence a possible particle-size dependent toxicity.

Currently, systematic investigations concerning particle size distribution in test media (at which the organisms are exposed) are scarcely available. More efforts should be put into the characterization of the physicochemical properties of ENPs in the test media with the aim to report the effect to a substance of known characteristics (Menard et al., 2011). Furthermore, it has to be remarked that the exposure of the test organism occurs in a dynamic way: the ENPs such as n-TiO_2 in aqueous solution tend to aggregate and settle down on the test vessels changing the exposure mode. Thus, the evaluation of the exposure is not easily to perform and the particle size characterization over the time exposure should be performed.

Table. 4.10: Chemical properties of nano-TiO_2, treatments of NPs for bioassay proposals, toxicity test applied and toxicity values for crustacean *D.magna*

Author	Primary size **and composition**	Surface Area (m^2/g)	Particle dimension DLS (nm)	Treatment of NPs	Method	Toxicity test	Toxic values EC50 (mg/L); LC50 (mg/L)
Lovern and Kapler 2006	10-20 nm	N.R	N.M	sonication and THF solvent + filtered		Acute test 48-h. 10 organism/50 ml . Endpoint: mortality	Filtred:LC_{50} 5, 5 ppm. Sonication LC50 was incalculable
Hund-Rinke 2006	particle size of 25 nm (Mainly anatase), Product 2: 100 nm (100% anastase).	N.R	N.M	NPs dispersed test medium and dispersion was achieved by ultrasonic dispersion. Mixture was continuously stirred on a magnetic stirrer. The dispersion was illuminated with simulated sunlight (300–800 nm).	ISO 6341, OECD 202, DIN 38412-30.	Acute test 48-h. Test performed in Petri dishes made of glass (Ø 55 mm):20mL/5 neonates. Endpoint: immobility	No concentration-effect curves were determined; In the tests without pre-illumination of dilution the immobilization rates were lower than after pre-illumination.
Warheit 2007	79% rutile, 21% anatase Primary size refers as median particle size (DLS) in water	38.5	140		OECD 202: 2004	Static acute test 48-h	>100
Heinlaan 2008	25–70 nm	N. R	N. M	Dispersed in MilliQ-water-sonicated	Daphtoxkit (1996)	Acute test 48-h Endpoint: immobility	> 20000
Lee 2009	7 and 20nm	300.8 and 66.6	N. M	Test solutions prepared in culture media and dispersed for 15 min using a sonicated (to prevent aggregation)	(OECD, 1984, 1998)	Mortality, growth, inhibition	EC50 not reported

Stigul2009	6 nm		N. M	N. R	Daphtoxkit OECD 202	Acute test 48-h Endpoint: mortality	LD_{50} not reach
Wiench 2009	20–30 nm; 70/30 anatase/rutile;	48.6	5 min ultrasonication(M4 water) : D10%(0.05 µm) D50% (1.5 µm)D90% (14 µm)	NP dispersed in several test media; Several treatments tested: sonication, stirring.	Acute test: OECD 202.	Acute 48 ore, 10 neonates/10 mL. Endpoint: immobility	> 100
Zhu, 2009	<20 nm; purity 99.5%	N. R	N. R	NP dispersed in reconstituted water (OECD 202) and suspension shacked	Acute test:OECD 202.	30mL/10 neonates in Beacker. Endpoint: immobility, mortality	35.306 LC50=143.38
Garcia 2010	7,5 nm	N. R	N. R		OECD 202	Acute test 48-h; 10 organism/20 mL Endpoint :immobility	LC_{50}= 16
Kim 2010	<40 nm (rutile/anastase: 30/100)	N. R	The accumulated volume percentage was 30, 37, and 71% in the size fractions of <200, <400, <800 nm, respectively	Stock solution: NP dispersed in water (pH=2).Test solution: Stock solution diluted in moderately hard freshwater	U.S. EPA method (US EPA, 1993)	Test acute 48-h. 25mL/5 neonates Endpoint: mortality-Chronic test 21 days, Endpoint reproduction-Biomarker investigation	LC_{50} impossible to calculate
Zhu 2010	21 nm (P25) 20%rutile; 80% anastase	50	Particle size distributions at 0, 12 and 24 h were determined and found to be 580.5, 2349.0, 3528.6nm respectively	Stock solution: NP dispersed in MilliQ-W, sonicated for 10 min Test solutions were prepared immediately prior to use by diluting the stock solution with standard culture medium(prepared according to ISO standard 7346-3:1996 and OECD Guideline 202)	OECD 202	Acute test: 72-h. 30mL/10 neonates in Beacker. Endpoint: immobility and mortality Chronic test (OECD 204) :21-days; Endpoint: total living offspring	Acute:EC50(48-h), LC50(48-h) >100 EC50 (72-h)= 1.62 LC50 (72-h)= 2.02 Chronic:EC50 0.46 mg/L; LC50=2.62

Dabrunz 2011	6 nm pure anastase	230	Size distribution on stable monodispersed stock solution showed an average diameters of 100 nm and 200 nm(ISO media)	NPs stable suspension obtained by stirred media milling	OECD 202 exposure time prolonged up to72-h, 96-h	Acute test: 5 organism / 50 mL; Plusa cute tests were performed exposing the organism to 100nm size particle and 200 nm size particle	Acute 72-h: EC50 =3, Acute 96-h:EC50= 0.73 100 nm suspension more toxic than 200 nm suspension
Salieri 2012	18 nm 96%wt of anastase, 4%wt rutile	68.81			OECD 202; exposure time prolonged up to72-h, 96-h	Acute test (96-h) 10 organism/20 mL. Endpoint immobility ; three media ELENDT M7,ISO water, Bottle water	32; 33 82

Table 4.11: Chemical properties of nano-TiO2, treatments of NPs for bioassay proposals, toxicity test applied and toxicity values on crustaceans *D.pulex* and *C.dubia*

Author	Specie	Primary size	Surface Area (m2/g)	Particle dimension DLS (nm)	Treatment of NPs	Method	Toxicity test	Toxic value EC50 (mg/L); LC50 (mg/L)
Hall 2009	*D pulex*	10			NPs dispersed in hard water (USEPA), stirring for 30 min. The test solutions were frequent agitated to maintain the NPs in suspension	U.S. EPA (2002 a,b)	Acute (48-h)-Mortality	LC50= 6.5 and 13.0 Geometric mean 7.6
Hall2009	*C.dubia*	10			NPs dispersed in hard water (USEPA), stirring for 30 min. The test solutions were frequent agitated to maintain the NPs in suspension	U.S. EPA (2002 a,b)	Acute(48-h)and Chronic (96-h)-reproduction;	LC50 (acute) 3.0-13.4-11.0-3.6-15.9 Geometric mean= 9.2; IC25 (chronic)=2.5-9.4-26.4 mg/L
Griffit 2008	*C.dubia*	20.	45.41	687.5	Stock suspension: 10 mg powder to mL of MilliQ-water, sonicated with a probe sonicator.	ASTM	48-h static; death/immobilization	>10
Griffit2008	*D pulex*	20	45.41	687.5	Stock suspension: 10 mg powder to mL of MilliQ-water, sonicated with a probe sonicator.	ASTM	48-h static renewal; Mortality; 5 adults in 200 mL of test solution in filtered (0.45 µm) test water;	>10

4.5.3 Toxicity of n-TiO_2 to freshwater vertebrates (fish)

In contrast to algae and crustaceans, a fewer number of studies are focused on the acute or chronic toxicity of metal oxides nanoparticle on fish (Table 4.12). On the other hand, an higher number of studies are focused on the biological investigations.

Effect concentrations are available for *Danio renio* (Griffit et al., 2008, Zhu et al., 2008, Xiong et al., 2011), fathead minnow *Pimephales promela*s (Hall et al., 2009), rainbow trout *Oncorhynchus mykiss* (Federici et al., 2007; Warheit et al., 2007).

4.5.3.1 Acute and chronic toxicity

That "state of art" leads to a fragmentary information on n-TiO_2 toxicity of to fish. Xiong et al. (2011) tested n-TiO_2 and ZnO on *Danio renio* with several endpoints: lethality, oxidative stress and oxidative damage. The authors referred a dose-dependent toxicity both for n-TiO_2 and ZnO, with a 96-h LC50 value of 124.5 mg/L and 4.92 mg/L, respectively. The bulk form of TiO_2 was lower toxic than n-TiO_2 even if the particle size in the test media reached an average particle size similar to their bulk particle in suspension. Regarding the remarkable difference of toxicity between the TiO_2 and their bulk form, the difference of structure of n-TiO_2 may be a possible explanation. In fact even if the average hydrodynamic diameters are similar for both of the two forms, the hydrated particles of n-TiO_2 are composed of numerous nano-sized particles (Xiong et al., 2011). Griffit et al. (2008) did not evidence a 48-h acute toxicity for n-TiO_2 on *Danio renio*; the 48-h LC50 value is higher than higher concentration tested (10 mg/L). Similarly, Warheit et al. (2008) reported a 48-h and 96-h values >100 mg/L, which was the highest concentration tested. Hall et al. (2009) reported a 48-h and 96-h LC50 higher than 1000 mg/L and a chronic value IC25 of 451 mg/L.

On my knowledge, no acute-chronic studies have been performed over the 96-h of exposure. Due to the evidence that the exposure mode influences the test results with ENPs, further researches may be performed on this way. For instance, prolonging the time of exposure, further phenomena may be possibly observed, as the direct ingestion of aggregates or the ingestion of aggregates particle entrapped on the algae food. A possible relation between the particle size at which the fish species are exposed with the toxic value is far to be known. Anyway, there are evidences of oral uptake of particle aggregates.

4.5.3.2 Sub-lethal effect

Most nanoparticle types present in the aquatic environment, such as n-TiO_2, do not exhibit or have very low direct acute toxicity. Instead, they display silent or concealed sub-lethal effects on the immune system with serious implications (Jovanovic et al., 2012). Xiong et al. (2011) have exposed *Danio renio* to 50 mg/L of n-TiO_2 and to 5 mg/L of ZnO nanoparticles testing the oxidative stress and oxidative damage. Biomarkers of oxidative effect e.g. superoxide dismutase (SOD) and catalase (CAT), reduced glutathione (GSH) were investigated. With n-TiO_2, no significant changes in SOD activities were observed in gill tissues, but a significant decrease of SOD activity in liver tissues and an increase of SOD activity in gut tissues were displayed. The exposure to 5 mg/L of ZnO reduced SOD activity in liver tissue and augmented it in gut tissue. The catalase (CAT) activity was higher in liver tissue, followed by gut tissue, and then gill tissue for both of the NPs tested. The same trend was observed for GSH. Thus, all biomarkers measured in liver tissue were sensitive to 50 mg/L TiO_2 ENPs and 5 mg/L ZnO ENPs suspension, as well as 50 mg/L TiO_2 ENPs in the dark.

Federici et al. (2007) exposed rainbow trout (*Oncorhynchus mykiss*) to n-TiO_2 (0.1, 0.5, or 1.0 mg l^{-1}) for 14 days. The research showed that TiO_2 may induce oxidative stress in the brains of rainbow trout as well as a significant decrease in Na^+K^--ATP activity. Furthermore, injury to gill (oedema and thickening of the Lamellae) and areas of erosions in the intestinal epithelium were observed suggesting the oral ingestion of n-TiO_2 suspended in water as probably route of intake. Anyway, the accumulation of the n-TiO_2 in the internal organs of rainbow trout has not been observed.

Accumulation of n-TiO_2 in the fish tissues has been also investigated by other several authors (Warheit et al., 2008; Zhang et al., 2007; Johnston et al., 2011). For instance, Zhang et al. (2007) reported that carps exposed to 10 mg L^{-1} n-TiO_2 for 25 days accumulated a whole body concentration of about 3.39 mg g^{-1} as TiO_2. This study provides evidence of that n-TiO_2 have a strong adsorption capacity for Cd and, due to the facilitated transport, fish accumulate much more Cd in the presence of n-TiO_2. Hence, the study of Zhang et al. (2007) provides evidences of accumulation of n-TiO_2 to carps, but also highlights that the research should be addressed to the potential of the facilitated transport of other trace toxic pollutants when they co-exist with ENPs.

4.6 Discussion and conclusion

An high variability of toxic effect of n-TiO_2 for the species representative of the three trophic levels investigated has been reported; also, the mechanism of toxicity of nano-TiO_2 is far to be known.

As far as algae is concerned, evidences of entrapping of particle aggregates to algal cells have been observed. The entrapping of aggregates could also provoke physical effects such the disruption of cell membrane (Aruoja et al., 2009; Sadiq et al., 2011). Furthermore, the aggregates of NPs in aqueous suspension may reduce the light availability to algae cells by “a shading effect”. In contrast, the larger size of the aggregates reduces the exposed surface area and thus the chemical reactivity of NPs (Wang et al., 2008). Regarding the crystal phase, the anastase was found more toxic than rutile (Ji et al., 2010). Anyway, on the basis of the toxicity data collected in this bibliographic review, a toxicity dependent on the crystal form has not be proved.

As far as crustacean is concerned, ingestion of aggregates and/or adhesion of ENPs to body surface has been pointed out. Moreover, toxic effects could be attributed either to the ingestion of contaminated algae, or to the absorption of nanomaterial dispersed in the daphnia medium after algae addition, or a combination of both. Also, the oxidative stress as mechanism of toxicity of nano-TiO_2 has been investigated. Due to its photo catalytic properties, Radical Oxygen Species (ROS) can be produced and damages to DNA can occur. Anatase TiO_2 was considered to be particularly reactive, producing much more ROS than rutile TiO_2 and resulting in toxicological effects; meanwhile, an increase in ROS generation up to a size of 33 nm and then a decrease beyond this size of nano-TiO_2 was predicted. This suggests that consideration for the size range is important in predicting TiO_2 ENPs toxicity when the ENPs are agglomerated in the water phase. Therefore, describing the toxicity of nano-TiO_2, particle size and crystal form must be taken into account (Ji et al., 2010). The biochemal biomarkers should be also applied (Klaper et al., 2009). In fact, Kim et al. (2010) have indicated antioxidant enzyme activities as biomarkers to assess the toxicity of TiO_2 to *D.magna*. Moreover, the biochemical investigation may be helpfully to investigate and understand possible explanations of toxicity.

Fewer studies are available for fish as opposed to algae and freshwater invertebrates. During this review, only five studies with acute or chronic endpoint have been found. As above, an high variability of toxic data is reported, the acute toxicity values ranging from LC50 > 10 mg/L to LC50 >1000 mg/L. Due to the low number of available data and the different species treated, a comparison among the results was not performed. Anyway, a dose-dependent toxicity (Xiong et al., 2011), a time-dependent toxicity (Chen et al., 2011) and a toxicity of the aggregates form of the ENPs have been reported (Johnston et al., 2010). Accumulation of NPs in the fish tissue and the ENP capacity to facilitate other contaminants intake have been investigated (Zhang et al., 2007). Chen et al. (2011) have reported an increase of the weight of the gills and liver in Zebra fish exposed to nano–TiO_2; and moreover gill and liver injuries were found. On the trophic level of fish, more data are referred to sub lethal endpoint.

Aggregation of n-TiO_2 is a general issue that is observed and described in the majority of published studies. Furthermore, even in studies focused on one specific particle type, differences in media composition and suspension preparation procedures introduce significant variability into nanoparticle characteristics and behavior. Time- and media-dependent aggregation is a problem that has to be overcome in order to obtain reliable and comparable test results. In fact aggregation likely influences test results. In relation to the characterization of particle size distribution in the test media suspension, it has to be remarked that: 1) characterization is not usually performed 2) when it is conducted, it is not always repeated over the exposure time (e.g. particle size measurements are not performed up to 96-h). The particle size may change over the time affecting the exposure mode to aquatic organism. In fact, over the time the ENPs in water tend to aggregate (particles of different dimension are expected over the time) and therefore to settle down on the test vessel, leading to a “dynamic” exposure which may vary over time in dependence of the rate of aggregation. Thus, a dynamic evaluation is not easy to perform and the particle size measurements are usually not referred to the whole time exposure. More efforts have to be performed to characterize the ENPs in tests media and during the whole exposure time. No correlation has been found with the secondary particle size both for algal species and for crustaceans, mainly due to low data available. There are evidences that the duration of exposure influences the test results: chronic test or acute test with prolonged exposure time should be preferred. However, it should not be overlooked that the reproducibility of toxicity tests with NPs may be affected by concentration-dependent aggregation of the nanoparticles, subsequent sedimentation, and possible attachment to vessel surface (Hartmann et al., 2010; Salieri et al., 2012).

Therefore, general conclusions cannot be drawn relative to nanoparticle ecotoxicity due to the great diversity in material types and particle properties. Some studies have found acute and sub-lethal ecotoxic effects at concentrations in the µg/L range, whereas others have found low or no toxicity at mg/L concentrations. Furthermore, in many studies it has been possible to establish dose-response relationships but large variations in effect concentrations have been found even in seemingly comparable tests using nanoparticles of the same material and the same test species.
The review on the toxicity focused on n-TiO_2 shows that nanoparticles may differ in their toxicological effects in dependence on particle variety and size, test organism species, and test methods (e.g. treatment of ENPs, exposure mode). There are different research results and understandings on the toxicity mechanism of oxide nanoparticles. Further studies are clearly needed to clarify both toxicological effects and underlying mechanism of nanoparticles. Different mechanisms of toxicity on the aquatic organism have been argued. According to the literature data, the specificities that distinguish the toxicity of n-TiO_2 particles are correlated to (i) the suspension preparation method, (ii) the particle shape and crystalline structure, and (iii) the exposure time duration with the tested organism. The limited available data in the literature does not allow to draw conclusions on the ecotoxicological impact of the use of nanomaterials. So, there is a need for additional ecotoxicological studies and physicochemical characterization of nanoparticles to ensure consistency of results

Table 4.12::Chemical properties of nano-TiO_2, treatments of NPs for bioassay proposals, toxicity test applied and toxicity values on fish

Author	Specie	NP	Primary size (nm)	BET (m^2/g)	Zeta potential (mv)	DLS (nm)	Treatment of NPs	Method	Toxicity test	Toxic value
Warheit 2007	*Oncorhynchus mykiss*	79 %rutilio, 21 % anatase	Refers as median particle size in water (DLS)	38.5		140		OECD 203	48-h and 96-h; endpoint immobilization	LC50 >100 mg/L
Griffitt 2008	*Danio renio*	TiO_2-P25	20	45.41	-25.1	687.5	Stock suspension: 10 mg powder to mL of MilliQ water, sonicated with a probe sonicator	ASTM	48-h static renewal; Endpoint: survival; organisms exposed in 12-wellplates with 4 mL of solution	LC50 48-h > 10 mg/L
Hall 2009	*Pimephales promalas*	TiO_2 (99%)	10				NPs dispersed in hard water (USEPA), stirring for 30 min. The test solutions were agitated to maintain the NPs in suspension		USEPA protocol; Acute (96-h- growth as dry weight); Chronic (7-days) two replicate of 10/exposure; feed	Acute (96-h) LC50>1000 mg/L; Chronic IC25 =342-597 mg/L
Xiong2011	*Zebrafish*	TiO_2 (purity 99%); Anatase	30		-13.1	251-630 (403)	Dispersed with a bath sonicator for 20 min instead of using stabilizing agents.	Test suspensions were prepared and dispersed using a sonicator for 20 min immediately prior to use	Acute test (96-h); unfed Endpoint :Lethality and oxidative stress and damage	96-h LC50 of 124.5 mg/L.

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5 The characterisation of freshwater toxic impact: the USEtox model

5.1 The characterisation step: a qualitative description

The characterisation of toxic impact as the human toxicity and ecotoxicity encompass characterisation models, which describe the cause-effect chain of an emission into the environment. The characterisation of toxic impacts is realised on substance specific characterisation factor (CF) that combine exposure potential and toxicity to represent the relative contribution of the substance to health and environmental impacts.

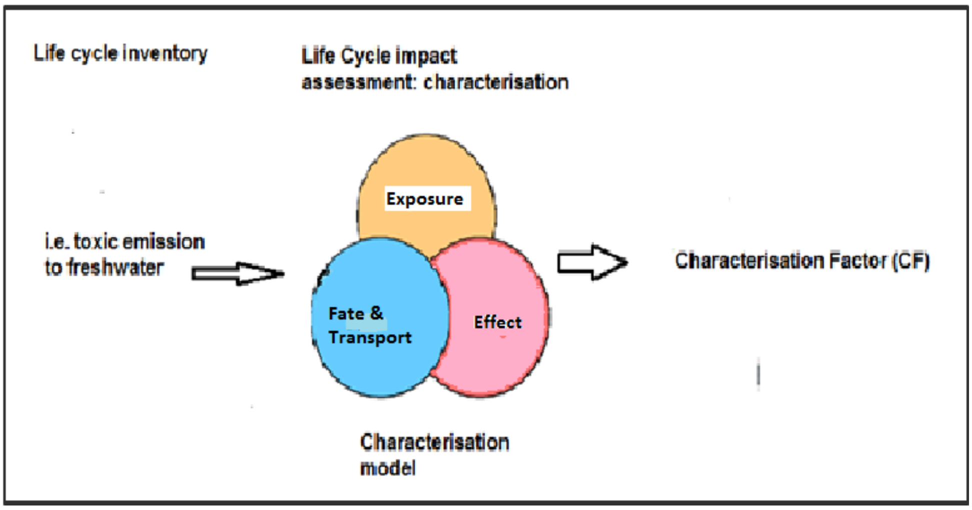

Fig.5.1: The scope of the characterization step.

The conceptual model usually applied for the characterisation of freshwater toxic impact is presented in Fig.5.2.

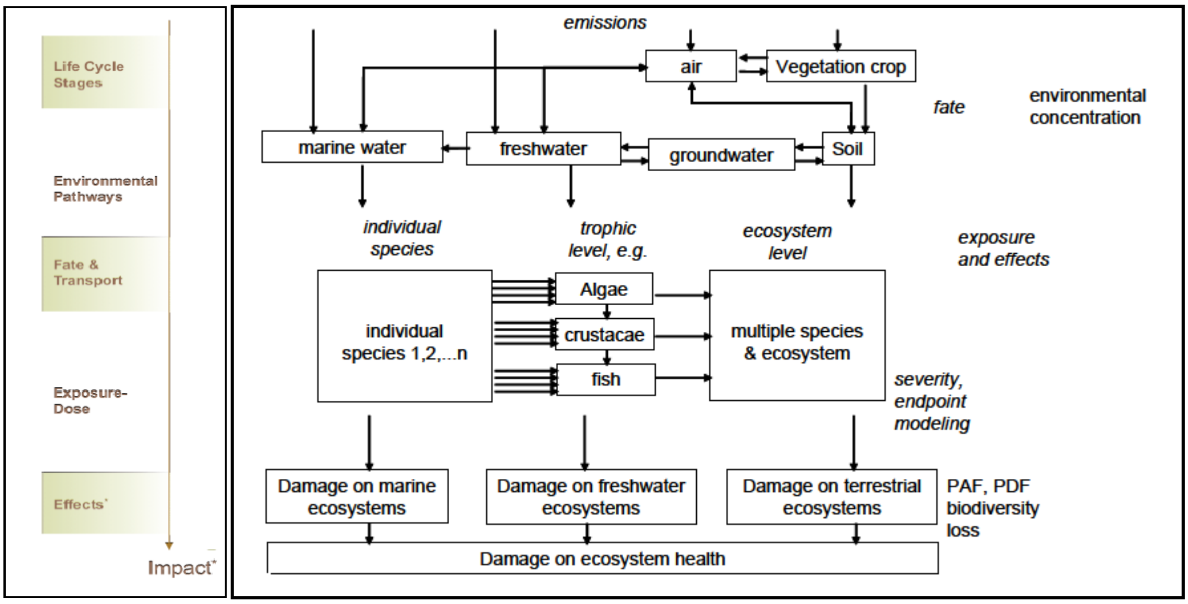

Fig.5.2: Framework for ecotoxicological impact assessment (ILCD Handbook, 2011)

Following the chart proposed in Fig. 5.2 the characterisation models describe the environmental pathway of a substance in environmental compartments therefore, they estimate environmental concentration and account the toxic effects correlated to its exposure.

In summary, the characterisation models apply

- Multimedia models that use fate modelling approach,

In combination with

- Toxicological effect information's.

Following this methodological approach a generic definition of the characterisation factor (CF) is:

$$CF_i = FF_i x XF_i x EF_i \quad (5.1)$$

Where CF_i is the ecotoxicological characterization factor of chemical *i*. Fate factors (FF, that defines the residence time of the substance in an environmental compartment) and (XF) exposure factor (e.g. the intake fraction of contaminant *i* by its ingestion as exposure route for human toxicity impact; or the bioavailable fraction of contaminant *i* for ecotoxicity impacts) are calculated by fate, transport and exposure model, while the effect factor (EF) is derived from toxicity data as the EC50 value.

5.2 USEtox model

5.2.1 Historical context

Several LCIA methodologies has been developed, such as CML 2002 (Guinée *et al.,* 2002), Eco-Indicator 99 (Goedkoop and Spriensma, 2000) , EDIP (1997-2003) (Wenzel *et al.,*1997, Hauschild and Wenzel, 1998, Hauschild and Potting, 2005, Potting and Hauschild, 2005) ,EPS2000 (Steen, 1999a,b), Impact 2002+ (Crettaz *et al.,* 2002, Jolliet *et al.,* 2004, Payet, 2004, Pennington *et al.,* 2005, Pennington *et al.,* 2006, Rochat *et al.,* 2006, Rosenbaum, 2006, Rosenbaum *et al.,* 2007a), LUCAS (Toffoletto *et al.,* 2007) and others.

In order to assess non-toxicity impacts the various LCIA methodologies apply very similar characterisation models which can vary their characterisation factor on geographical base. It is possible to observe that characterisation factors are similar for non-toxic global impacts (such as climate change and ozone depletion), while can be different for non-toxic regional impacts (such as acidification). In fact, LCA typically has a global scale scope, as the supply chain behind products tends to be global in nature crossing national and geographic border. Moreover, the location of emission sources or resources is not known. For this reason, multimedia fate models and associated characterisation models on a global level are recommended. However, models that represent large heterogeneous regions or continental or national scales are widely applied (ILCD Handbook, 2011). For instance, Ecoindicator 99 has global impact categories for climate change, and ozone depletion but for other impact categories emissions are assumed to take place in Europe and European model is applied. The LIME´s region validity is Japan, except for global impacts like climate change and stratospheric ozone depletion. LUCAS´s characterisation model has region validity in Canada for regional impact categories, for which spatially differentiated characterisation factors are calculated.

On the contrary, for toxic impact categories the characterization models have been developed with differences in scope, modelling principles, and therefore in the characterization factors they provide. The widespread use of several characterisation models creates criticism when the use of LCA gives different result depending on the methodology chosen (Fig.5.3).

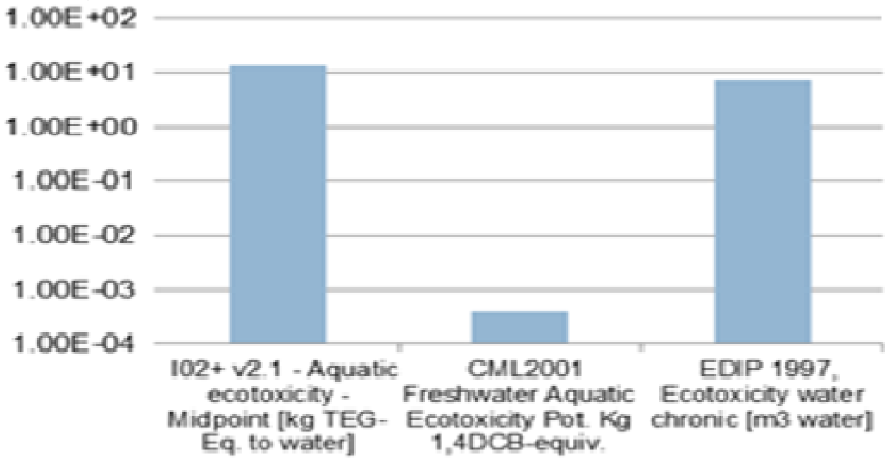

Fig.5.3: LCIA results for the impact category of freshwater ecotoxicity evaluated on a generic process of "biomass cogeneration" available in the software Gabi 4.3; the figure aims to show the different results obtained when different LCIA methods are applied.

In response to large variation in available methods, in 2003 an international model comparison/consensus process focused on characterization of human- and ecosystem-health impacts was initiated. This process was under the umbrella of the Life Cycle Initiative, a joint effort of the United Nations Environment Program (UNEP) and the Society of Environmental Toxicology and Chemistry (SETAC). The process involved an international group of model developers responsible for the most commonly used LCIA characterization models; it also involved disciplinary experts in fate and transport, exposure assessment, health risk assessment, and ecotoxicology (Hauschild et al., 2008). The model comparison/consensus process joined by the Task Force of UNEP-SETAC involved the developers of models commonly used in current practice in an LCA context: CalTOX, IMPACT 2002, USES-LCA, BETR, EDIP, WATSON, and EcoSense.

Therefore, the Task Force on Toxic Impacts under the UNEP-SETAC Life Cycle Initiative aimed to a comparison and harmonization of existing characterization models in order to (Huijbregts et al., 2010):

- Identify which differences (both in model results and structure) in the old characterization models cause the observed differences in their characterization factors;
- Develop a scientific consensus about good modelling practice based on the identified influential differences;
- Harmonise the old characterisation models removing unintended differences; and
- Develop a scientific consensus model based on the learnings from the comparison of the characterization models with the following characteristics:
    A. parsimonious (as simple a s possible, as complex as needed) containing only the model elements which were identified as the most influential in the comparison of the existing characterisation models;
    B. transparent and well documented;
    C. falling within the range of the existing characterisation models, i.e. not differing more from the old characterisation models than these differ among themselves;
    D. endorsed by the modellers behind all participating models.

The model comparison/consensus process was carried out to identify specific sources of differences (both in model results and structure) and the indispensable model components. This led to the development of **USEtox**, "*a scientific consensus model that is parsimonious and contains only the most influential model elements based on current best practice in the context of LCA"* (ILCD Handbook, 2010).

5.2.2 The characterisation of freshwater toxic impact

The USEtox model is a fate, transport, and exposure model widely applied for Life Cycle Impact Assessment. The potential toxic impacts of a substance are characterized in terms of Comparative Toxic Unit (CTU) and the characterization factor CF [PAF m^3day/kg emitted] represents the freshwater ecotoxicological impacts of chemicals per mass unit of chemical emitted, where the impact is quantified as the potentially affected fraction (PAF) of species (Rosenbaum et al.,2008)

The characterisation factor for toxic impact category is calculated following the framework proposed in Fig.5.4.

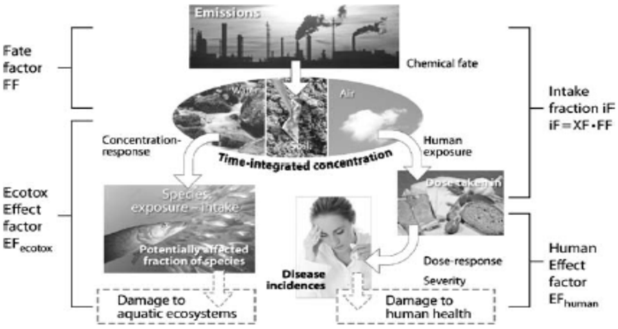

Fig.5.4: Framework proposed by the USEtox model to calculate the CF for toxic impact (ecotoxicity, left side and human toxicity right side)

Following the framework illustrated in Fig.5.4 the toxic impacts are calculated linking the cause-effect chain mechanism (Fig.5.5)

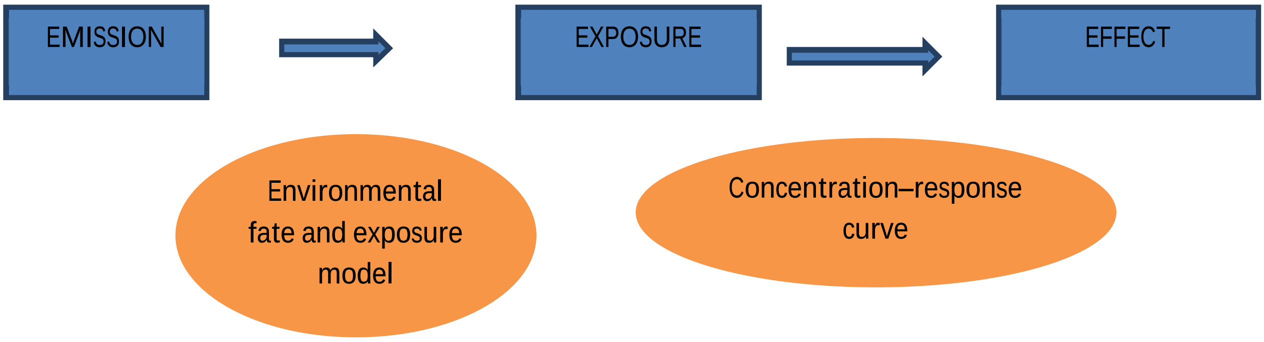

Fig.5.5: Environmental pathway of a substance and the tools to assess its potential toxic impacts

Thus, the characterization factor for **freshwater ecotoxicity** is calculated as (Henderson et al., 2011):

$$\mathbf{CF_w = f_{i,w} * FF_{w,w} * XF_w * EF_w \ (5.2)}$$

Where:

- $f_{i,w}$ is the fraction of contaminant transferred from the source compartment *i* to freshwater compartment
- $FF_{w,w}$ (day):is the fraction factor in freshwater and represents the mass increase of a substance (mass, kg) in the compartment *w*($[kg_{inwater}/(kg_{emitted}day^{-1}) = day]$). It is referred as the persistence of a substance in water, for an emission to the water compartment;
- the XF [-] describes the exposure factor indicating the dissolved and bioavailable fraction
- EF_w ($[PAF\ m^3/kg_{in\ water}]$) is the freshwater ecotoxicological impact.

USEtox is structured with a matrix framework composed by a series of matrices representing the cause-effect chains that occurs when a chemical is released into the environment.
Thus, each factor presented in the equation 1 is expressed and as matrix (Rosenmbaum et al., 2008)

5.3 The fate model

- **Fate matrix ($\overline{FF}$):** Links the quantity released into the environment (e.g. compartment of emission *m*) to the chemical masses in a given compartment (e.g. receiving compartment *i*). It accounts for multimedia and spatial transport between the environmental media (e.g. air, water soil, etc.).In the fate matrix a column denotes the emission compartment *m* and a row the destination environmental compartment *i*. The size of the FF matrix is determined by the number of environmental compartments n_i considered and the number of source compartments n_m are equal, since every destination compartment can also be a source compartment, hence $n_m=n_i$), and thus be ($n_i \times n_i$). The fate factor $FF_{i,m}$ [day] can be interpreted as the increase of chemical mass in compartment *i* [kg] due to an emission in compartment *m* [kg/day].

$$\overline{FF} = \underbrace{\begin{pmatrix} FF_{1,1} & \cdots & FF_{1,m} \\ \cdots & \cdots & \cdots \\ FF_{i,1} & \cdots & FF_{n_i,n_m} \end{pmatrix}}_{\text{emission compartment } m} \left.\vphantom{\begin{matrix} a \\ a \\ a \end{matrix}}\right\} \begin{array}{c} \text{final} \\ \text{compartment } i, \\ \text{i.e. where the} \\ \text{chemical is} \\ \text{transferred into} \end{array}$$

The elements of the fate matrix are fate factors with unit of day. The fate matrix can be interpreted as:

- **Residence time:** The diagonal elements $FF_{m,m}$ describes the residence time of the substance in the respective compartment *m*.
- **Mass in the environment:** A column of $\overrightarrow{FF}$ matrix describes the mass in the environmental resulting from a unit emission flow in the corresponding compartment. Then, dividing each element of the column by the sum of all elements of the respective column the results indicates the repartition of the resulting mass between all destination compartments due to an emission in the compartment represented by the column
- **Inter-compartment transfer fractions**: dividing each element in a row by the residence time (diagonal element) provides the transferred fractions from media *i* to *j*: $f_{i,j} = FF_{i,j} / F_{ii}$.

USEtox spans two spatial scales (Fig.5.5). The continental scale consists of six environmental compartments: urban air, rural air, and agricultural soil, and industrial soil, freshwater and coastal marine water. The global scale has the same structure as the continental scale, but without the urban air, and accounts for impacts outside the continental scale. The fate model calculates the mass increase (kg) in a given medium due to an emission flow (kg/day). The unit of the fate factor is in days. "*It is equivalent to the time-integrated concentration* × *volume over the infinite of a pulse emission*" (For more details see: Rosenbaum et al.,2008).

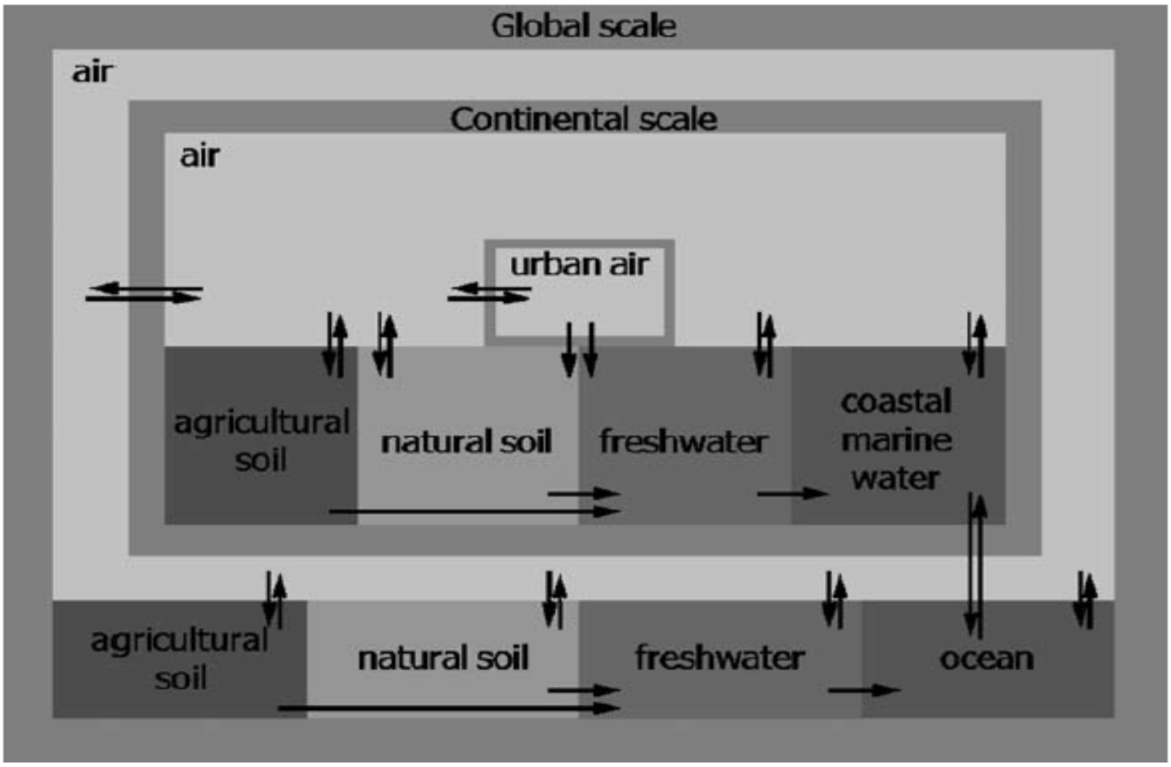

Figure 5.5:USEtox geographical scale and compartments setup (Source: Rosenbaum et al., 2008).

The emission scenarios are continental emission to urban air, rural air, freshwater and agricultural soil (Rosenbaum et al., 2008) The USEtox model is based on the principles of multimedia mass balance modeling (Mackay, 2002). Inter-media transport and removal processes at the two spatial scales are used to calculate the element (e.g. $FF_{i,j)}$ of the $\overline{FF}$ matrix. The transport of the substances is evaluated as intermediate processes along the geographical scales; thus, the USEtox is a "nested" multimedia model.

As argued, the fate factor is equal to the compartment-specific residence time (days) of a chemical. Where, the residence time of a chemical depends on (i) the chemical properties of the chemical, (ii) the selected emission compartment (e.g. urban air), and (iii) the selected receiving compartment (e.g. fresh water at the continental scale).

The fate model of USEtox accounts for:

- ✓ **Removal processes**: for example (bio) degradation by micro-organisms, transport of the chemical to the sediment, leaching to the groundwater and escape to the stratosphere;

- ✓ **Intermedia processes**: movement of chemicals from one compartment to the other (and back); such as advective and diffusive transport;
- ✓ **Fate and exposure in water**: In the model, the bioavailable mass of chemical dissolved in water per unit of emission (expressed as the residence time of chemical dissolved in water) is the product of the fate factor in water for an emission to water, $FF_{w,w,}$ and the dissolved fraction, XF_w. Four removal processes affect the dissolved mass of a chemical in water: adsorption/sedimentation, volatilization, degradation, and advective transport out of the water compartment;
- ✓ **Fate in soil and transfer to water:** The extent of transfer from soil to surface water is the net result of competition between the four main removal mechanisms from soil: degradation, volatilization, leaching to deeper layers of soil, and runoff to surface water. For surface water, only the chemical mass dissolved in (pore) water is modeled as available for taking part in physical and chemical processes
- ✓ **Fate in air and transfer to soil and water** The extent of transfer from air to soil is determined by a competition between three main removal mechanisms in the air compartment: degradation in air, advection to the air in the global box (where the soil surface is limited), and deposition either to soil or to surface water and oceanic water bodies. The transfer rate between air and soil primarily depends on deposition and degradation in air

The resolving mass balance equation at the steady state for multi-compartment environmental model has been proposed and can be expressed also, as a matrix algebra equation:

$$\overline{\frac{dM}{dt}} = \overline{S} + \overline{k}x\overline{M} = 0 \rightarrow \overline{M} = \overrightarrow{k^{-1}}x\overline{S} \quad (5.3)$$

Where

- $\overline{S}$[kg/ day] is the vector of the emission rate S_i in each compartment $_i$,
- $\overline{M}$ [kg] is the vector of the chemical masses with elements M_i, and
- $\overline{k}$ is the bulk rate coefficient matrix [1/day] or in terms of half-life of the substance, $\tau_{1/2}$ = ln (2)/k.

Per definition, the fate factor $\overline{FF}$ *matrix equal the negative inverse of rate coefficient matrix* thus, $\overline{FF} = -\overline{k^{-1}}$

$$\overline{k} = \begin{pmatrix} -k_{a,tot} & k_{w,a} & k_{s,a} & \cdots & k_{i,a} \\ k_{a,w} & -k_{w,tot} & k_{s,w} & \cdots & k_{i,w} \\ k_{a,s} & 0 & -k_{s,tot} & \cdots & k_{i,s} \\ \cdots & \cdots & \cdots & \cdots & \cdots \\ k_{a,j} & k_{w,j} & k_{s,j} & \cdots & -k_{i,tot} \end{pmatrix}$$

Fig.5.6: Rate coefficient matrix , k. (Source: Rosenbaum et al.,2008).

The elements of the rate coefficient matrix $k_{i,\ j}$ are the inverse of the residence time [$\tau_{½} = \ln(2)/k$; (1/day)]. In the multimedia model matrix[8] the first index of an element represent the column and the second index the row; the column represents the emission compartment and the row the receiving compartment. The indices *a, w, s* describe the environmental compartment (air, water, soil) and the indices *i,j* any possible compartment. The elements on the diagonal ($-k_{i,\ tot}$) represent the negative of the total removal rate coefficient for the compartment *i*. For instance, ($-k_{a,tot}$) is the negative total remove rate coefficient for the compartment air including abiot/biotic degradation, advection and intermedia removal processes. The elements off - diagonal ($k_{i,j}$) represent the advection or intermedia transport processes from the compartment *i* to *j*.

The $k_{i,j}$ elements are calculated on the basis of chemical properties of the substance and of the environmental compartments, evaluating the fate and transport processes occurring in the media (e.g. degradation/ sorption/ advection/ convection). Following the principles of the multimedia mass balance modeling the partitioning properties of the substances is applied to evaluate the concentration at the steady state. The partition coefficients are widely available and used for systems of air-water, octanol-water, lipid water, fat-water, hexane-water, “organic carbon”-water, aerosol-air. Estimation Programs Interface (EPI) Suite, Version 4.0, has been selected as the default database for calculating the USEtox fate of organic substances. Differently, for inorganic substances the physical-chemical properties are based on the IAEA (2009) and US-EPA (2002) databases. The physical-chemical properties required for USEtox fate calculation for organic and inorganic substances are reported in Tab.5.1

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[8] Differently from related mathematical conventions, where the first index describes the row and the second index the column.

USEtox requirements			**Organics (source**	**Inorganics (source)**
Key property	**Abbrevation**	**Unit**		
Molecular weight	MW	g.mol-1		Periodic table
Partioning coefficient octanol-water	Kow		—	—
Partioning coefficient organic carbon -water	Koc	L.kg^{-1}	US-EPA	–
Partioning coefficient dissolved organic carbon-water	Kdoc	L.kg^{-1}	US-EPA	–
Henry law coefficient (at 25°C)	K_H25C	Pa.m^3.mol^{-1}	US-EPA	IAEA set ot: 1.10^{-20}
Vapour pressure	Pvap25	Pa	US-EPA	–
Solubility	Sol25	mg.L-^{1}	US-EPA	–
Degradation rate in air	KdegA	s^{-1}	US-EPA	IAEA set ot: 1.10^{-20}
Degradation rate in water	kdegW	s^{-1}	US-EPA	IAEA set ot: 1.10^{-20}
Degradation rate in sediment	kdegSd	s^{-1}	US-EPA	IAEA set ot: 1.10^{-20}
Degradation rate in soil	kdegSl	s^{-1}	US-EPA	IAEA set ot: 1.10^{-20}
Bioaccumulation factor in fish/biota	BAFfish	L/kg	US-EPA	IAEA

Table 5.1: Substance key properties for USEtox requirements

5.4 Exposure and effect model

The ecotoxicological effect factor EF quantifies the fraction of species in an ecosystem which are affected by a given level of exposure. The size of the matrix ($\overline{\text{EF}}$) is determined by the number of ecosystem n_{es} and the number of the environmental compartments n_i considered, and thus (n_{es} x n_i). The ecotoxicological effect factor $EF_{es,i}$ [PAF m^3 kg-^{1}] can be interpreted as the time and volume integrated increase in affected fraction of species in an ecosystem *es*, per unit of chemical mass increase in compartment *i* [kg].

$$\overline{EF} = \begin{pmatrix} 0 & EF_{w,aqu} & 0 & \dots & 0 & \dots & 0 \\ EF_{a,ter} & 0 & EF_{s,ter} & \dots & 0 & \dots & 0 \\ \dots & \dots & \dots & \dots & \dots & \dots & \dots \\ 0 & 0 & 0 & \dots & EF_{o,mar} & \dots & 0 \end{pmatrix}$$

In ecotoxicological effect matrix a row entry denotes the affected ecosystem *es* (e.g. aquatic, marine or terrestrial) and a column entry denotes a final compartment *i*. The size of $\overline{EF}$will be determined by the number of ecosystems n_{es} and the number of environmental compartments n_i considered, and thus be (n_{es} × n_i). In the EF matrix the off-diagonal elements) are set to zero. To date the $\overline{EF}$matrix has only one ecosystem (freshwater) and .5 environmental compartments: air, freshwater, seawater, natural soil, agricultural soil.

The USEtox model, applies a PAF approach ($HC50_{EC50}$ based approach), where the PAF curve is based on the EC50 chronic value and the effect factor is evaluated as (Rosenmbaum et al.,2008; Huijbregts et al., 2010, Henderson et al., 20010:

$$EF = \frac{0.5(PAF)}{HC50_{EC50}} \quad (5.4)$$

HC50 is the hazardous concentration at which 50% of the species are exposed above their EC50. The EC50represent the effective concentration at which 50% of population displays an effect. Where, 0.5 represent the working point on the PAF curve corresponding to HC50 value which indicates that the potentially affected species is 50%.

The effect factor is calculated assuming the linearity in concentration-response which results in a slope of 0.5/HC50. The HC50 is based on the geometric means of all species-specific EC50 data available and collected on the organism representative of three trophic levels: crustaceans, algae and fish. For aquatic system the XF represent the bioavailability of the chemicals to aquatic species and is calculated as the truly dissolved fraction of a substance. (Henderson et al., 2011)

The USEtox model evaluates the $XF_{w,w}$as:

$$XFw, w = \frac{1}{1+(K_p * SUSP + K_{doc} * DOC + BCF_{fish} * BIOmass)/1*10^6} \quad (5.5)$$

Where Kp is the partition coefficient between water and suspended solids (L/kg), SUSP the suspended matter concentration in freshwater K_{doc} the partitioning coefficient between dissolved organic carbon and water, DOC the dissolved organic carbon concentration in freshwater, BCFfish the bio concentration factor in fish (L/kg) and BIOmass the concentration of biota in water (Huijbregts et al.,2010)

5.5 The characterisation factor

After multiplication of the scale-specific fate factors, exposure factors, and effect factors the final characterization factor for aquatic ecotoxicity is calculated by summation of the characterization factors from the continental and the global scale assessments. The characterization factor for aquatic ecotoxicity (Ecotoxicity Potential) is expressed in Comparative Toxic Units (CTUe) and provides an estimate of the potentially affected fraction of species (PAF) integrated over time and volume per unit mass of a chemical emitted (PAF m3.day.kg^{-1}).

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6 The characterisation of freshwater ecotoxicity for n-TiO_2: an open issue

6.1 A framework to evaluate the fate factor for n-TiO_2 in freshwater in accordance with USEtox requirements

6.1.1 Introduction

As argued in the Chapter III, the fate processes governing the behaviour of ENPs in aquatic environment are: aggregation, dissolution, binding to natural organic matter (NOM) or to suspended particle matter (SPM), and degradation (hydrolysis, photolysis) (Farrè et al., 2009; Lead et al., 2010). Focusing on the water and sediment compartment, the environmental behaviour of a chemical substance is mainly affected by 1) transformation and degradation 2) interaction with suspended particulate matter and 3) transport.

Moreover, the environmental behavior of ENPs is closely related to their intrinsic properties such as shape, surface charge, chemical composition and coating (Crane et al., 2008; Petosa et al., 2010) as well as to the physical-chemical properties of the aquatic environment: ionic strength, natural organic matter, pH, and ionic composition (Handy et al., 2008; Petosa et al., 2010). As discussed in the previous Chapter IV, the field of LCIA, fate, transport and effect model to predict the environmental concentrations and the exposure to a substance are applied. Whereas, the fate models are based on the well-known Mackay's model which, in general, follows an approach to the portioning coefficient.

Due to the evidence that the environmental behaviour of ENPs in water ecosystem may be compared to that of colloidal particles and also that the aggregation processes will determine the bioavailability and fate (for instance affecting the sedimentation) (Levard et al., 2012; Craneet al., 2008), the proposed framework to derive ~~at~~ a fate factor for n-TiO_2 intends to:

1) Describe the main fate processes ENPs are subject to in freshwater (aggregation and dissolution);
2) Be expressed in size range class;
3) Be applicable in the USEtox characterization model.

As argued by several authors (Mackay et al., 2006; Praetorius et al., 2012) the evaluation of the main processes affecting the ENPs behaviour differs from that of the organic substances. In fact the ENPs share similarities both with colloidal chemistry and metal chemistry (Handy et al., 2008; Shaw et al., 2011) and tend to form a thermodynamically unstable[9] suspension of particles. In contrast to organic substances, for

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[9] In colloid chemistry, a "stabilized" dispersion (kinetically stable when dispersed i.e., over long time scales, but still thermodynamically unstable) describes a liquid where the particles may collide by Brownian motion or shear flow,

which the partition coefficients (e.g. K_{ow}, K_d etc.) are widely applied in multimedia mass balance models, a different scenario is shown for ENPs (Table 6.1).

For ENPs the partition coefficients are not available in ~~the~~ literature. Further considerations have to be accounted for. Since that when ENPs are dispersed in solution they tend to form a thermodynamically unstable suspension (Handy et al., 2008), it is not possible to assume an equilibrium between two phases and therefore, several issues on the evaluation of the partition coefficients have to be accounted for. In the Table 6.1 a comparison between the key properties for organic substances and for ENPs to evaluate the processes affecting the ENPs environmental behaviour is listed.

Transformation and degradation processes can alter the environmental behaviour of a chemical substance and determine its persistence. For organic chemicals the dominant loss process is characterized by their degradation rate constant, k_{deg}, which is generally calculated from the half-life ($t_{1/2}$) of the chemical substance in the environmental medium of concern: $k_{deg} = \ln 2/t_{1/2}$.

The interaction of organic chemicals with other pollutants or suspended particulate matter (SPM) is evaluated by the sorption coefficient, K_d which is calculated from the octanol-water partitioning coefficient, K_{ow}.

Transport processes of pollutants in environmental media have to be characterized in order to predict mobility and transport pathways within and between environmental compartments. Organic chemicals are affected by transport processes such as advective transport k_{adv} and processes of sedimentation (k_{sed}), sediment resuspension (k_{resusp}), horizontal bed load transfer ($k_{sed,transfer}$) or burial in the deep sediment (k_{burial}).

Table 6.1:Fate modelling, a comparison organic substances vs ENPs (Reference: (1) Crane et al.,2008 (2) Praetorius et al.2012

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but do not stick together after the collision. A colloidal dispersion is thermodynamically unstable and will always tend to aggregate and separate; however, the process may be slow (hours-days), so that the dispersion appears to be virtually stable (Handy et al., 2008).

Env. Processe	**Organic substance**	**Chemical property/portioning coefficient**	**Nanoparticle**	**Chemical property**
Transformation and degradation	Degradation:	Half-life time ($\tau_{1/2}$) $K_{deg} = \frac{ln2}{\tau_{1/2}}$	Transformation: ✓ Aggregation ✓ Dissolution	-High surface energy ✓ K_{agg} = α*Kcoll ✓ K_{diss}
Interaction with NOM, SPM, pollutants	• Adsorption Absorption:	• Sorption coefficient K_d • Octanol-water partition-coefficient K_{ow} ➢ K_{oc}	Sorption on contaminant (1) Best described as aggregation (2) due to the collision event	-Composition, size purity, structure and solution condition (ionic strength, pH) K_{agg} = α*Kcoll
Inter media exchange and transport	Volatilization Advection	Vapor pressure Kflow	Advection	Water/air outflow
	Aria-water Soil- water	K_{aw} K_{sw}	Soil-water(particle resuspension) Water-soil	K_{resusp} K_{sed}

6.1.2 Material and method

The framework proposed in this work adopts the principles of the multimedia mass balance modeling and it includes two environmental compartments: freshwater and sediment at the continental geographical scale. The environmental compartments are described as "box" homogenous and well mixed.

The landscape parameters at the continental scale (such as the depth of sediment, height of column of water etc.) derived from the USEtox model. It is assumed that uncoated n-TiO2 are directly released to freshwater compartments from wastewaters. Therefore, it is reasonable to assume that the n-TiO2 reach the rivers as particle assemblies. In our study we adopt the size distribution of the aggregated applied by Praetorius et al., 2012. Where, n-TiO2 dispersed in freshwater were set to be log-normally distributed with a mode at 300 nm (particle diameter) and the particles in the distribution are assigned to 5 size classes assigned. To date no accurate information available on the form and size of n-TiO2 in freshwater are available, therefore it was impossible to set-up a size distribution of the aggregated at the continental geographical scale. Moreover, the environmental fate and the transport of ENPs are strongly influenced by the suspended particulate matter (SPM) present in natural waters (Lin et al., 2010). This is because ENPs are likely to attach to SPM by heteroaggregation and therefore to be transported or deposited as the bound SPM concentration E' UN SUGGERIMENTO!. In this framework a particle concentration of 3.7 E+10 m^{-3} of SPM with a density of 2 g/cm^3 and a particle concentration of 1.1 E+10 m^{-3} with a density of 2 g/cm^3 have been accounted for. Furthermore, for each particle concentration of SPM two scenarios have been developed assigning to the collision efficiency (α) the value 1 and 0.001.

The landscape parameters and the parameters of n-TiO_2 and SPM applied are reported in Table 6.2-6.2.1. The evaluated environmental processes are:

Transformation and degradation: ENPs undergo transformation processes such as aggregation and dissolution. ENPs have the tendency to aggregate with themselves, therefore they undergoing a homoaggregation process. A homoaggregation rate constant, $k_{homo\text{-}agg}$, can be calculated by multiplying the ENPs' collision rate, k_{coll}, by the collision efficiency for homoaggregation, α. Dissolution can occur for some ENPs and is described by the dissolution rate constant, k_{diss}, specific to the ENP and the characteristics of the environmental medium. Surface transformations of the ENPs are likely to occur in natural environments and can alter the ENPs properties and environmental behaviour. Aggregation and dissolution will not lead to the ENPs' disappearing, but rather to their being transformed into a new species such as the "aggregates of particles". Abiotic degradation processes that may occur include hydrolysis and photocatalysis. Near to the surface ENPs are exposed to sunlight. It is likely that light-induced photoreactions can account for the removal of certain ENPs and for changing the chemical properties of others.

Interaction with suspended particulate matter: The interaction with SPM is described by the aggregation process which is expressed as the second order rate coefficient of heteroaggregation. The heteroaggregation rate constant, $k_{het\text{-}agg}$, is calculated by multiplying the collision rate constant, k_{coll}, by the attachment efficiency for heteroaggregation (α).

Transport: ENPs can undergo transport processes such sedimentation and advection. Therefore, the following processes are taken into consideration: advection (k_{adv}), sedimentation of ENPs to the sediment compartment by gravitational settling, (k_{sed}), sediment resuspension (k_{resusp}), horizontal bed load transfer ($k_{sed,transfer}$) or burial in the deep sediment (k_{burial}).

Following the USEtox framework the fate factor has been calculated with a matrix approach. Thus, with the aim to obtain a $FF_{w,w}$ more environmentally realistic as possible, for each size of class of n-$Ti0_2$ (for i=1..n_{size}^{TiO2}) a fate factor matrix has been developed, thus leading to a $F_{w,w,i}$ for each of the size classes. Then, a single $F_{w,w}$ has been calculated as the average weighted of $F_{w,w,i}$. In this way we aim to calculate the average residence time of n-TiO_2 in freshwater.

6.1.2.1 Resolution equation

The water model (Quik et al., 2010) can be written as:

$$\frac{dM}{dt}(water) = E - \sum kM \quad (6.1)$$

Where E (kg/day or kg/s) describes the total ENPs mass flow into the water box and represents the input to the system, M (kg) represents the concentration of ENPs in water, and k (s^{-1} or day^{-1}) represents the total removal rate coefficient. The product of M and the total removal rate coefficient represents the output from the system. The removal processes of aggregation, dissolution, interaction with suspended particulate matter and sedimentation are taken into consideration.(Fig.6.1).

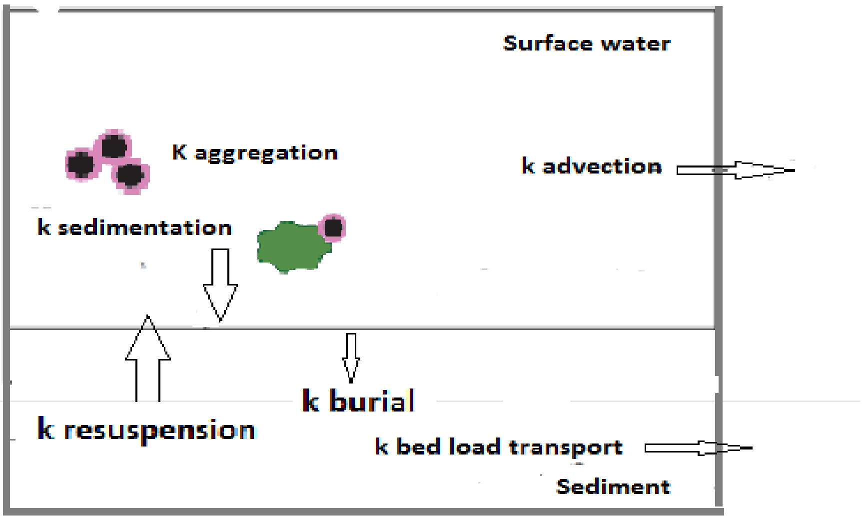

Figure 6.1: The “box” model and the fate processes accounted for..

Following the USEtox requirements a matrix algebra is applied to evaluate the fate of n-TiO_2 in freshwater and sediment. As described in the Chapter V the fate factor of a substance is represented by the $\overline{FF}$ matrix that is described as the negative and inverse of the rate coefficient matrix ($\bar{k}$).

$$\overline{FF_\iota} = \overline{-k_\iota^{-1}} \quad (6.2)$$

In the rate coefficient matrix the components are expressed as residence time (s^{-1}). **Under the hypothesis that the bioavailability of the metal oxide nanoparticles may be influenced by the size distribution of aggregated n-TiO2**, the $\overline{FF}$ components will be calculated in function of n-TiO_2 in a size range (for i = 1…,n_{size}^{TiO2}) and thus the fate matrix will be represented as:

$$\bar{k} = \begin{pmatrix} -k_{w,w,i} & k_{sed,w,i} \\ k_{w,sed,i} & -k_{sed,sed} \end{pmatrix} \quad (6.3)$$

Where:

the rate coefficients $K_{w,w,i}$, $K_{sed,sed}$ represent the total removal rate coefficient in the water column (advection, hetero-aggregation, dissolution, sedimentation) and in sediment (burial, resuspension, bed load transfer), respectively.

And so, $K_{w,w,i}$ is:

$$k_{w,w,i} = -(k_{w,adv,i} + k_{sed,i} + k_{dis} + k_{w,het-aggregation}) for\ i = 1 \ldots, n_{size}^{ENP} \quad (6.4)$$

And $K_{sed,sed}$ is

$$k_{sed,sed} = -(k_{burial} + k_{resusp} + k_{sed,transf}) (6.5)$$

$K_{w,sed,i}$ and $K_{sed,w,i}$ are the inter-media exchange between water and sediment and are described by the rate coefficient of sedimentation from freshwater to sediment and by the rate coefficient of resuspension from sediment to freshwater respectively.

6.1.2.2 *Model equations*

Water compartment

The fate processes are described by a set-up a of equations aiming to calculate the rate coefficients for n-TiO_2 distributed as size class (for i = 1…,n_{size}^{TiO2}). The model equations have been extrapolated from Praetorius et. al (2012). As reported in Chapter III, the aggregation processes are governed by (1) perikinetic agglomeration caused by Brownian motion; (2) orthokinetic agglomeration caused by shear flows; and (3) differential settling caused by sedimentation. Also, not all the collision are successful, so a collision efficiency α is included. The collision efficiency cannot be determined easily from the classic DLVO theory, which explains the colloidal stability (see Chapter III).Currently, it is assumed ranging from 1 to 0.001**. In our study the collision efficiency is set equal to 1 and 0.001.**

To calculate the aggregation of n-TiO_2 the kinetic equations described in Chapter III have been applied. The homo-aggregation (aggregation among n-TiO_2 themselves) seems to be irrelevant, due to the lower concentration of n-TiO_2 in comparison with higher concentration of SPM. Therefore, we assume only the heteroaggregation process (Praetorius et al., 2012).

When n-TiO_2 interact with SPM, the interaction is described as hetero-aggregation process and the hetero-aggregation rate coefficient ($k_{het-agg,i}$) is calculated as the product of the K_{coll} (m^3/s), the collision efficiency α and the particle concentration of C_{SPM} (m^{-3}):

$$k_{het-agg,i} = \alpha * k_{coll,i,j} * C_{particle}^{SPM} \quad (6.6)$$

$$k_{coll,i} = \alpha * \frac{2TK_B}{3\mu} * \frac{\left(r_{TiO2,i} + r_{SPM,}\right)^2}{r_{TiO2,i}\, r_{SPM,}} * \frac{4}{3} G * (r_{TiO2,i} + r_{SPM})^3 + \pi * (r_{TiO2,i} + r_{SPM})^2 * \left|v_{set}^{TiO2,i} - v_{set}^{SPM}\right|$$

$$\text{for i= 1.. } n_{size}^{TiO2} \quad (6.6.1)$$

where μ is the absolute viscosity of the water, α is the collision efficiency, T temperature (K), K_B Boltzman's constant, r_{Tio2i} is the radius of n-TiO_2 in size class *i* and r_{SPM} the radius of SPM.

The rate constant of aggregation $k_{het-agg,i}$ is a second order rate constant (m^3/s), here it is assumed as being as first order therefore it is multiplied by the particle concentration of C_{SPM} *(m^{-3})* (Praetorius et al.,2012*).*

The dissolution of ENPs can be described as a surface controlled process where the first order dissolution rate constant k_{diss} reflects the local hydrodynamic conditions near the nanoparticle–water interface. Currently, little is known about the dissolution of ENPs and the dissolution rate constant should be extrapolated only from experimental data performed on specific metal oxide nanoparticles. Therefore, Quick et al. (2010) referred a dissolution rate constant for ENPs in the range of $0\text{-}10^{-5}$ (s^{-1}).

ENPs are affected by transport processes such as advective transport with moving water. Thus, the evaluation of the rate constant of advection requires the evaluation of the water outflow from the boundary of environmental box model. Thus, the framework here proposed evaluates the water outflow (*adv,flow;* m^3/s) from freshwater at the continental geographic scale to continental sea water, as proposed by USEtox model proposed. The rate constant of advection (s^{-1}) is calculated dividing the water outflow by the volume of freshwater (m^3) and thus, as:

$$k_{adv} = \frac{adv,flow}{V_w} \quad (6.7)$$

Sediment:

As aggregation proceeds, particle aggregates grow in size and become prone to settling by gravity. The settling rate $v_{sed,i}$ (m/ s) which follows Stock's law is expressed as :

$$v_{sed,i} = \frac{2}{9} * \frac{\rho_{TiO2,i} - \rho_{aq}}{9\mu} * g * r^2_{Tio2,i} \quad \text{for i= 1..}n_{size} \ (6.8)$$

Where $r^2_{TiO2,i}$ is the radius of the n-TiO_2 aggregated for each of the size classes *and* $\rho_{TiO2,i}$ is the specific density of the n-TiO_2 aggregated for each size classes*. The* specific density of n-TiO_2 aggregated for each one of the size classes is calculated as:

$$\rho_{TiO2,i} = \frac{\rho_p * V_{solid,i} + \rho_{water} * (V_{total,i} - V_{solid,i})}{V_{total,i}} \quad (6.9)$$

Where ρ_p is bulk density of TiO_2 and $V_{solid,i}$ (m^3) is:

$$V_{solid,i} = \frac{4}{3} * \pi * \left(\frac{d_{TiO2,primary}}{2}\right)^{D_f} * \left(\frac{d_{Tio2,i}}{2}\right)^{3-D_f} \quad \text{for i= 1..}n_{size} \ (6.10)$$

Where $d_{TiO2,primary}$ is the primary diameter (nm) of the n-TiO_2, $d_{TiO2,i}(nm)$ is the diameter of n-TiO_2 in size class *I* and D_f is the fractal dimension of the aggregates.

Where, $V_{total,i}$ (m^3) is:

$$V_{total,i} = \frac{4}{3} * \pi * (\frac{d_{TiO2,i}}{2})^3 \text{ for i= 1..n}_{size} \ (6.11)$$

The aggregation processes increased the size of the NP with a subsequent settling of the n-TiO_2 into the sediment. Thus, the rate coefficient of sedimentation ($k_{sed,i}$; s^{-1}) is calculated by dividing the settling rate by the depth of the sediment compartment, h (m), and is expressed as:

$$k_{sed,i} = \frac{v_{sed,i}}{h} \ (6.12)$$

As organic substance, the ENPs are affected by transport process as resuspension, burial and bed load transport. Also, burial process in the sediment compartment is evaluated. The rate coefficients (s^{-1}) are respectively expressed as the ratio between the burial and resuspension flow (m^3/s) and the volume of sediment Vsed (m^3):

$$k_{burial} = \frac{Burial_{flow}}{V_{sed}} \ (6.13)$$

$$k_{resusp} = \frac{resus_{flow}}{V_{sed}} \ (6.14)$$

The rate constant of horizontal sediment transfer $k_{sed,\ transfer}$ is calculated as the ratio between the $V_{sed,transf}$ (kg/s) and the mass of sediment (kg), thus:

$$k_{sed,transf} = \frac{V_{sed,transf}}{m_{sed}} \ (6.15)$$

Where m_{sed} (kg):

$$m_{sed} = (1 - \varphi) * V_{sed} * \rho_{sed} (6.16)$$

Table 6.2: Parameters of n-TiO_2 and SPM applied.

Parameters	Symbol	Unit of measure	Value/Formula	Source
Number of ENP size classes in the model	n_{sizes}^{NP}	i=1…5		Praetorius et al.,2012
Primary ENP size	$d_{p,primary}$	nm	15	Praetorius et al.,2012
Fractal dimension of ENP aggregates	D_f	3		Praetorius et al.,2012
Diameter of ENP in size classes *i*=1…n	$d_{p,i}$	nm	[16; 212; 408;604;800]	Praetorius et al.,2012
Radius of NP in size class *i*=1…n	$r_{p,i}$	nm	[8;106;204;302;400]	Praetorius et al.,2012
ENP density (as bulk form)	ρ_p	g/cm^3	4.2	Praetorius et al.,2012
Density of ENP aggregates of size class *i*	$\rho_{p,i}$	g/cm^3	See eq. 6.9	Praetorius et al.,2012
Particle concentration SPM	C_{SPM}	$1/m^3$	3,70E+10	Praetorius et al.,2012
Density of SPM	ρ_{SPM}	g/cm^3	2	Praetorius et al.,2012

Table 6.2.1: Landscape data and rate coefficients applied

Parameters	Symbol	Unit of measure	Value/Formula	Source
Boltzmann constant	K_B	*J/K*	*1,38E-23*	USEtox 2008 (continental scale)
Volume freshwater	V_w	m^3	6,76E+11	USEtox 2008 (continental scale)
Volume sediment	V_{sed}	m^3	8,11E+09	USEtox 2008 (continental scale)
Density of water	ρ_w	g/m^3	1	Praetorius et al.,2012
Viscosity of water	µ	mPas	1	Praetorius et al.,2012
High of freshwater	h_w	m	2,5	USEtox 2008 (continental scale)
High of sediment	h_{sed}	cm	3	USEtox 2008 (continental scale)
Concentration of SPM	C_{SPM}^{mass}	mg/l	1,5	USEtox 2008 (continental scale)
Resuspension flow from freshwater sediment	Resusp,flow	m^3/s	7,19E+01	USEtox 2008 (continental scale)
Sediment burial flow	Burial,flow	m^3/s	2,33E+01	USEtox 2008 (continental scale)
Sediment burial rate coefficent	K burial	s^{-1}	Burial, flow/Vsed	
Flow of continental freshwater to continental seawater	Adv,flow	m^3/s	5,45E+04	USEtox 2008 (continental scale)
Porosity of sediment	ϕ	[-]	0,85	Praetorius et al.,2012
Flow of sediment transferred	Vsed,trasf	Kg/s	3	Praetorius et al.,2012
Bed load transport sediment	ksed, transfer	s^{-1}	$V_{sed,transfer}/m_{sed}$	Praetorius et al.,2012

6.1.3 Result

As proposed in Eq.6.2 the fate matrix of n-TiO_2 for each size class from *i* to *n* has been calculated. The evaluation of the fate matrix follows four scenarios where the collision efficiency **α** was set to be equal to 1 and to 0.001 and the particle concentration of SPM was set to be equal to 3.7 E+10 m^3 and 1.10 E+10m^3

In the following Tables the values of the fate matrix is proposed and:

- $FF_{w,w}$ expresses the residence time (day) in freshwater for each class size of aggregates.
- $FF_{w,sed}$ is calculated from the intermedia rate coefficient (sedimentation from water to sediment), it expresses the time to sediment; the $FF_{sed,w}$ represents the time of resuspension as well;
- $FF_{sed,sed}$ expresses the residence time (day) in freshwater for each class size of aggregates.

SCENARIO 1: α=1, particle concentration of SPM 3.40E+10 m^{-3} with ρ=2.0 g/cm^3

In Table 6.3 the components of the fate matrix, FF, for each size class of n-TiO_2 aggregated are reported:

Table 6.3: The components of the FF matrix (day) depending on the radius of the aggregates of n-TiO_2 (Scenario α=1)

raggio(nm)	8	106	204	302	400
$FF_{w,w,i}$	5.91E-01	5.28E-01	3.60E-02	4.72E-03	1.05E-03
$FF_{w,sed,i}$	4.47E-01	3.99E-01	2.72E-02	3.57E-03	7.95E-04
$FF_{sed,w,i}$	7.98E-03	1.04E+03	1.85E+03	1.73E+03	1.57E+03
$FF_{sed,tot,i}$	9.86E+02	1.77E+03	2.39E+03	2.29E+03	2.17E+03

The results show how the aggregates of n-TiO_2 tend to a strong sedimentation, leading to a low persistence (here referred to residence time) in freshwater in the order to 10^{-1} to 10^{-3} day.

The $FF_{w,w}$ has been calculated as weighted average of the FF $_{w,w,i}$ in the each size class (Table 6.4), where the weight is the frequency of each class within the distribution.

Table 6.4 : Fw,w as weighted average of FF $_{w,w,i}$ in each size class

radius	weight	FF $_{w,w,i\ weighted}$
106	0.372	1.97E-01
204	0.465	1.67E-02
302	0.140	6.59E-04
400	0.023	2.45E-05
sum	1	2.14E-01
FF $_{w,w}$		5.35E-02

Thus, the $F_{w,w}$ to be applied in the calculation of the characterization factor for n-TiO_2 is 5.32E-02 (day).

SCENARIO 2: α=0.001, particle concentration of SPM 3.40E+10 m-3 with ρ=2.0 g/cm^3

In Table 6.3 the components of the fate matrix, FF, for each size class of n-TiO_2 are reported:

Table 6.5: The components of the FF matrix (day) in dependence on the radius of the aggregates of n-TiO_2

radius(nm)	8	106	204	302	400
$FF_{w,w,i}$	1.15E+00	7.35E-01	7.32E-02	1.09E-02	2.70E-03
$FF_{w,sed,i}$	8.66E-01	5.55E-01	5.53E-02	8.24E-03	2.04E-03
$FF_{sed,w,i}$	1.55E-02	1.45E+03	3.77E+03	3.98E+03	4.01E+03
$FF_{sed,tot,i}$	9.86E+02	2.08E+03	3.83E+03	3.99E+03	4.02E+03

The results show how the aggregates in each of the size classes of n-TiO_2 have a low persistence (here referred to residence time) in freshwater in the order from 1.5 to 10^{-3} day.

As above the $F_{w,w}$ has been calculated as the weighted average of the $FF_{w,w,i}$.

Table 6.6: $F_{w,w}$ as weighted average of $FF_{w,w,i}$

radius(nm)	weight	FF $_{w,w,i\ weighted}$
106	0372	2.73E-01
204	0.465	3.40E-02
302	0.140	1.52E-03
400	0.023	6.27E-05
Sum	1	3.09E-01
FF $_{w,w}$		7.72E-02

Thus $F_{w,w}$ is 7.2E-02 (day).

SCENARIO 3: α=1, particle concentration of SPM 1.10E+10 m^3 with ρ=2.0 g/cm^3

In Table 6.7 the components of the fate matrix, FF, for each of the size classes of n-TiO_2 are reported:

Table 6.7: The components of the FF matrix (day) in dependence on the radius of the aggregates of n-TiO_2 (Scenario α=1)

radius (nm)	8	106	204	302	400
$FF_{w,w,i}$	8.99E-01	6.59E-01	5.62E-02	7.88E-03	1.85E-03
$FF_{w,sed}$	6.79E-01	4.98E-01	4.24E-02	5.95E-03	1.40E-03
$FF_{sed,w}$	1.21E-02	1.30E+03	2.89E+03	2.88E+03	2.75E+03
$FF_{sed,tot}$	9.86E+02	1.97E+03	3.17E+03	3.16E+03	3.06E+03

The results show as the aggregates in each of the size class of n-TiO_2 have low persistence (here referred to residence time) in freshwater in the order to 10^{-1} to 10^{-3} day.

As above the $F_{w,w}$ has been calculated as the weighted average of the $FF_{w,w,i}$.

Table 6.8: $F_{w,w}$ as weighted average of $FF_{w,w,i}$

radius(nm)	weight	$FF_{w,w,\ weighted}$
106	0,372	2,45E-01
204	0,465	2,61E-02
302	0,140	1,10E-03
400	0,023	4,30E-05
Sum	1	2,73E-01
FF w,w		6,81E-02

Thus $F_{w,w}$ for scenario 3 is 6.81E-02 (day).

SCENARIO 4: α=0.001, particle concentration of SPM 1.10E+10 m^{-3} with ρ=2.0 g/cm^3

In Table 6.9 the components of the fate matrix, FF, for each size class of n-TiO_2 are reported:

Table 6.9: The components of the FF matrix (day) in dependence on the radius of the aggregates of n-TiO_2

radius(nm)	8	106	204	302	400
$FF_{w,w,i}$	1.15E+00	7.35E-01	7.32E-02	1.09E-02	2.70E-03
$FF_{w,sed}$	8.67E-01	5.55E-01	5.53E-02	8.25E-03	2.04E-03
$FF_{sed,w}$	1.55E-02	1.45E+03	3.77E+03	3.99E+03	4.02E+03
$FF_{sed,tot}$	9.86E+02	2.08E+03	3.83E+03	4.00E+03	4.02E+03

The results show as the aggregates in each of the size classes of n-TiO_2 leading to a low persistence (here referred to residence time) in freshwater in the order to 1.5 to 10^{-3} day.

As above the $F_{w,w}$ has been calculated as the weighted average of the $FF_{w,w,i}$.

Table 6.10: $F_{w,w}$ as weighted average of $FF_{w,w,i}$

radius(nm)	weight	$FF_{w,w,I}$ weighted
106	0.372	2.73E-01
204	0.465	3.41E-02
302	0.140	1.52E-03
400	0.023	6.28E-05
Sum	1	3.09E-01
FF w,w		7.73E-02

Thus $F_{w,w}$ is 7.73E-02 (day)

6.1.5 Discussion and conclusion

The framework here proposed aims to show the challenges to calculate the fate factor for the characterization of freshwater ecotoxicity. In contrast to organic substances a new set of equations have been taken into consideration. Formulated in this way **the challenge** to evaluate a fate factor for ENPs is based on the application of kinetic equations and on the evaluation of the rate coefficients (k, s^{-1}) specific for each fate process (dissolution, aggregation etc.). In our framework the fate factor is calculated as dependent on the size distribution of the aggregated n-TiO_2 freshwater ecosystem. This approach is new and innovative in the LCIA scenario, leading to the next developments of characterization based on the distribution of the aggregates in the environment of concerns.

In each of the scenario performed the weighted **average of $F_{w,w}$ (day) is in the order of 10^{-2}.**

The non-significant difference among the scenarios is not in accordance with the case of study on the fate of n-TiO_2 in Rhine river conducted by Praetorius et al., 2012. The authors applied a fate and transport model in which the site-specific conditions were used. In their case study the collision efficiency and the hetero-aggregation processes have been observed as key parameters into the evaluation of the fate and transport of n-TiO_2 in the river system. We hypothesize that this difference is due to the evaluation of the $F_{w,w}$; in our case no site-specific conditions as the size distribution of SPM in freshwater, that may influence the differential settling (Eq. 6.6.1), have been considered. The hetero-aggregation rate coefficient, as product of collision efficiency, collision rate and particle concentration (Eq. 6.6.) has been calculated. Where, the collision rate is calculated by eq. 6..6.1 accounting for Brownian and orthokinetic transport and differential settling. **We calculated the collision rate with a fixed size, density and particle concentration of SPM.** Whereas, the case of study performed by Praetorius et al., 2012 has been performed accounting a size distribution of SPM, with several density and particle concentrations. The authors show how the parameter of SPM, such as the density, may influence the fate and transport of n-TiO_2 in freshwater (Rhine river). Lower density value of SPM decreases the sedimentation velocity of SPM (the equation 6.8 proposed to calculate the velocity of sedimentation of n-TiO_2 is applicable to SPM as well). Therefore, the hetero-aggregation rate coefficient of n-TiO_2 with SPM decreases, because the contribution from differential settling is reduced (eq.6.6). The authors show that when SPMs have low density the n-TiO_2 may be transported with the SPM in the water column over long distances from the emission source. In contrast, with large value hetero-aggregation rate coefficient, high value of SPM density and with high values of concentration (mg/L) of SPM, the n-TiO_2 particles are quickly removed from the water.

Several **limitations** have to be taken into consideration as well:

- Abiotic degradation processes that may occur include hydrolysis and photocatalysis near to the surface ENPs when they are exposed to sunlight are not accounted. Even if it is likely that light-induced photoreactions can account for the removal of certain ENPs and for changing the chemical properties of others;

- It was impossible to consider an average size distribution of n-TiO_2 in freshwater on a continental geographical scale; this may be leading to a bias evaluation of the fate factor;
- The landscape data are estimated as average on a continental scale. However the prediction of environmental fate of ENPs is best described when local conditions are considered (Chapter III).
- The results show how the sediment compartment is greatly affected by the release of n-TiO_2 into the aquatic ecosystem. Currently, this compartment is not taken into consideration in the USEtox model. Therefore, it should be added as an environmental compartment considered into the "fate and transport" model.
- The suspended particle matter is evaluated with a fixed 1) size, 2) density and 3) particle concentration. That is, without account for a size distribution of the SPM in the media.

Therefore, we suggest **further investigations** of the $FF_{w,w}$ in which the size distribution of SPM and site–specific conditions could be considered.

The requirements of site-specific conditions into the evaluation of the potential impacts in the filed LCIA is not new (Zamagni et al., 2008). Since emissions in a life cycle can occur in many different parts of the world, and since the location may influence impact, a first improvement could be the development of regional versions of USEtox model. An important issue is the determination of which level of spatial differentiation is relevant for the fate and the effect (Henderson et al., 2011). As well for ENPs, on metal compound several issues on the evaluation of the characterization factor were raised. Ghandy et al. (2010) proposed a CF (with USEtox model) for metals as Cu, Ni and Zn that express the relative hazard associated with their release into an evaluative freshwater environment for which the authors have specified 12 water chemistries termed as 12 EU water archetypes.

In this PhD research a different environmental behavior of n-TiO_2 in terms of aggregation (qualitatively evaluated as the repulsive force acting between n-TiO_2 themselves) has been observed when it has been evaluated on the basis of the DLVO theory and considering the water chemistry for 12 EU water archetypes (Chapter III). Aiming to the determination of a level of spatial differentiation, relevant for the fate and effect for ENPs, we suggest the 12 EU water archetypes as starting point for a site-dependent assessment.

6.2 The effect factor for freshwater toxic impact for n-TiO_2 in accordance with USEtox model

6.2.1 Introduction

This part of doctoral research aims to calculate the effect factor for n-TiO_2, adopting the USEtox framework. On the basis of the bibliographic review of the ecotoxicity of n-TiO_2 (Chapter IV) to aquatic organisms representative of three trophic levels, **the effect concentrations (EC50 values) have been collected**. Furthermore, due to the high variability of the EC50 data for which the experimental parameters (e.g. chemical composition of n-TiO_2, exposure mode) may be a source of variability, **criteria rules to choose the EC50 values involved into the EF calculation are proposed.**

6.2.2 Ecotoxicity effect indicator (EEI)

The ecotoxicity effect indicator (EEI) is defined as the effect part (effect factor) applied to calculate the characterisation factor in LCIA methodology (see Chapter V). The development of ecotoxicity effect indicator for life cycle impact assessment proposal has been going on only for about two decades yet and it must to be conform to the general framework of Life Cycle Impact Assessment thus:

- The indicator shall be a best estimate;
- LCA is site-independent (the point of release of the toxicants being unknown), thus site-specific models are not applied;
- The effect part has to be compatible with the fate part to calculate the characterisation factor.

The methods used for effect factor calculation (or EEI) within Life Cycle Impact Assessment can be grouped into two main groups: 1) Assessment Factor (AF) based approach (Predicted No Effects Concentrations, PNEC); 2) Species Sensitivity Distribution (SSD) based approach (Potentially Affected Fraction of species, PAF approach). The PNEC approach is applied in many LCIA methodologies e.g. USES-LCA (Huijbregts et al., 2000), EDIP 98 (Hauschild et al., 1998); whereas, in the USEtox model and others LCIA methodologies - as Eco-indicator 99 - a PAF approach is used.

A based PNEC approach has a low data demand with high data availability (i.e. only one acute value may be applied) but results in a conservative estimation aiming to protect the most sensitive species (Larsen and Hauschild, 2007). The PNEC approach is used in regulatory generic risk assessment to estimate a PNEC value which is combined with an estimated Predicted Environmental concentration resulting in a risk quotient (RQ=PEC/PNEC). The PNEC is estimated by dividing the NOEC value [10], no-observed-adverse-effect concentration, by an Assessment Factor (AF). The AFs are used conservatively and typically vary between 10 and 1000 depending on the data availability and quality of the ecotoxicity effect data.

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[10] No observed effect concentration. The highest concentration tested causing no statistically measurable effect to the test system.

However, also the chronic EC50 values can be applied; since the number of chronic EC50 values available is limited than the number of NOEC (especially in the older literature), then an estimation of EC50 value from NOEC values, thanks to appropriate factors of correction, is applied (Larsen and Hauschild, 2007; Eckelman et al., 2012). Commonly, in LCIA the PNEC approach is applied to model the impact up-to the midpoint impact categories. Many existing LCIA methods adopt the PNEC approach, e.g. USES-LCA (Huijbregts et al., 2000) and EDIP (Hauschild et al., 1998), CML method (Guineé J., 1996). The procedure used is in principle identic to the estimation of PNEC in generic risk assessment, as described above. For example in CML methodology the freshwater effect factor of a substance is calculated as the reciprocal of the PNEC (EF =1/PNEC). The PAF approach describes the fraction of species that is expected to be potentially affected above its no effect level. The PAF approaches are based on the principle of Species Sensitivity Distribution (SSD), which is a statistical distribution describing the variation among a set of species in toxicity of a certain substance or mixture adopted from Larsen and Hauschild (2007).

Since that the PAF approach is based on the concentrations of effect such as EC50 values, it results in non-conservative estimates and has a relative high demand of data (a set of species has to be covered) with low availability (in the past researches were focused more on the extrapolation of NOEC values than EC50 values) (Larsen and Hauschild, 2007; Pennington et al., 2004).

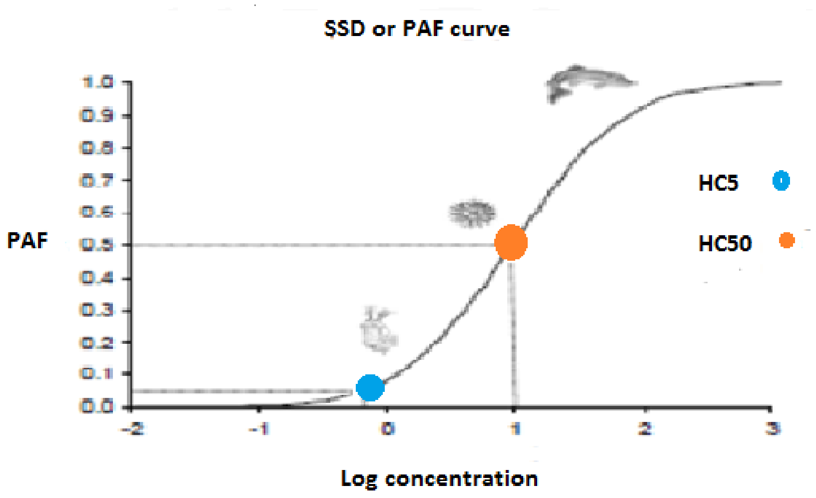

Fig.6.2:Example of a species sensitivity distribution (SSD) or PAF curve illustrating the relationship between the environmental concentration of a toxicant and PAF (Source: Huijbregts et al., 2010)

Several PAF approaches are used in LCIA: marginal PAF increase approach e.g. Eco-indicator 99, (Goedkoop and Spriensma, 2001a) or average PAF increase.

In the average PAF increase, the average gradient is a linear gradient between the origin of the PAF curve and the working point (HC5 or HC50) chosen on the curve. For an average PAF approach on HC5 based values, the working point on the curve is 0.05 assuming that the background impact level is below PAF=0.05.

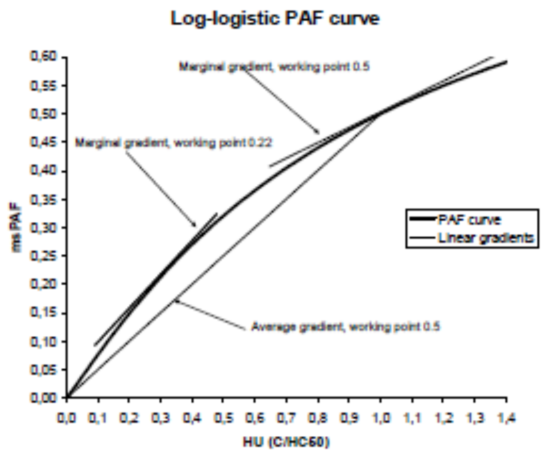

Figure 6.3: Example of "marginal PAF increase" approach and "Average PAF increase" approach. Whereas in the HC50 based approach the 0.5 as working point for the average gradient is chosen (Huijbregts et al., 2010)

PAF approaches are also modelled to the level of impact (midpoint categories) but attempt to combine up to a damage modelling; where a damage model is required to be able to transform midpoint indicator to endpoint indicator (e.g. the LCIA results are expressed in terms of changes of biodiversity). Henderson et al., (2011) with a case study on malathion (as insecticide) and using chronic ecotoxicity data (EC50s) from 16 species covering five different phyla, demonstrated that the PNEC approach is strongly dependent on the species tested. Depending on whether the most sensitive species or the second most sensitive species are included in the evaluation of PNEC; it can vary by 1 or 2 orders of magnitude. In contrast, the HC50 varies by a factor of 1.5. Thus, the HC50 leads to a robust derivation of freshwater ecotoxicological effect factor as it is less dependent on the species tested than the PNEC or on safety factor.

For this reason, in LCA methodology, within a comparative context and where best estimates are reached, an effect-based PAF approach has been recommended (Pennington et al., 2004; Larsen and Hauschild, 2007). The PAF approach based on the average toxicity of HC50 was found suitable for the evaluation of the EF:

$$EF = \frac{0.5}{HC50_{EC50}} \quad (6.15)$$

Where, $HC50_{EC50}$ representing the hazardous concentration at which 50% of species exposed above their chronic EC50 values and 0.5 is the working point (PAF=0.5) on the PAF curve. The $HC50_{EC50}$ is calculated as geometric mean of the EC50 values of the species or as trophic level.

Species selection for calculation of HC50s should in general aim to for the highest physiological variability, for as many species as possible, representing as many taxonomic groups as possible. In practice, (e.g. in

USEtox) at least three EC50s from three different phyla are required to reflect the variability of the physiology and to ensure a minimum diversity of biological responses (Henderson et al., 2011).

To calculate the $HC50_{EC50,}$ chronic or acute EC50 values are applied, where chronic values are preferred and the following trophic levels are recommended for the inclusion into the estimation of HC50: Primary producers (alga), Primary consumer(invertebrates) and secondary consumers (fish). To date, there is no consensus on which averaging principles the HC50 should be estimated: if on basis of trophic levels or single species.

6.2.2.1 The effect factor (EF) in the USEtox model

As argued in Chapter IV, the USEtox model applies a PAF approach where the EF is expressed as the ratio between 0.5 (PAF) and the $HC50_{EC50}$ value, as reported in eq. 6.15.

The $HC50_{EC50}$ is calculated as the geometric mean of the EC50 value of all species-specific data available on the organisms representative of three trophic levels: crustacean, alga and fish. Therefore, the USEtox model suggests to estimate the $HC50_{EC50}$ as the geometric means of the EC50-specie data.

Furthermore, to calculate the $HC50_{EC50}$ the following recommendations are given (Rosenbaum et al., 2008):

- HC50 has been calculated at least on three different EC_{50} values (species) from at least three different trophic levels; otherwise the estimated HC50 value is designated as “interim”;
- Chronic EC50s with relevant endpoint such as reproduction, growth and mortality are preferred;
- If a sufficient number of $EC_{50chronic}$ is available ($n \geq 3$) an $HC50_{chronic}$ is calculated and used directly in the EF;
- If the number of $EC50_{chronic}$ values are insufficient (<3) but if a sufficient $EC50_{acute}$ values ($n \geq 3$) are available an $HC50_{acute}$ is calculated. On the basis a $HC50_{chronic}$ is calculated by use of an assessment factor of 2.Thus, $HC50_{chronic} = HC50_{acute} /2$.

Also, in order to treat the chemical equally as possible, the USEtox model suggests to choose the EC50 values among standard ecotoxicity tests performed with standard organisms:

- Primary producers (algae): Time (72-120 hours), Endpoint (Inhibition of growth)
  Species: *Raphidocelia subcapitata, Scenedesmus subspicatus, Scenedesmus quadicrola, Chlorella vulgaris, Anabaena flos-aqua, Microcysstis areuginosa, Navicola seminulum, Navicula pelliculosa*
- Primary consumers (crustacean): Time (24-96 hours), Endpoint (Mortaility or Immobility)
  Species: *Daphnia magna, Daphnia pulex, Daphnis sp., Ceriodaphnia dubia, Neomysis mercedis*
- Secondary/tertiary consumer (fish): Time (96-336 hours), Endpoint (Mortality)
  Species: *Ambassis macleayi, Carassius auratus auratus, Cyprinus carpio carpio, Danio rerio, Ictalurus punctatus, Lepomis cyanellus, Lepomis macrochirus, Leuciscus idus, Melanotaenia*

splenidia inornata, Onchorhynchus kisutch, Onchorhynchus mykiss (Salmone gairdneri), Oeyzias latipes, Pimephales promelas, Poecelia reticulate and Salvelnius fontinalis.

Whereas, the effect concentration of a substance (EC50 data) can be obtained from the major data sources such as, ECOTOX: ECOTOXicology Database system US EPA: (http://cfpub.epa.gov/ecotox/), or ESIS, including IUCLID (http://ecb.jrc.ec.europa.eu/esis/).

6.2.3 Material and method

The effect factor has been calculated following the USEtox framework (Eq.6.15) and recommendations. The EC50 values for freshwater organisms representative of three trophic levels (algae, crustaceans and fish) have been collected from the previous bibliographic review reported in Chapter IV.

On the basis of the knowledge acquired by the bibliographic review and by the practical experience in laboratory a set of criteria on how to select the input data (EC50 values) to the EF calculation has been established. Further the algal species of *Pseudokirchineriella subcapitata, C. reinhardatii* have been added to the standard test species proposed by USEtox.

The criteria rules are listed below:

Chemical tested:

- Due to the lack of a n-TiO_2 as reference and the evidences that the anastase form is more toxic than rutile, toxicity tests performed with titanium dioxide nanoparticles composed mainly by anastase should be preferred to those conducted with titanium dioxide composed mainly of rutile.
- If a sufficient number of study (≥ 3) is available for each trophic level the toxicity results should be chosen from the test that uses the same type of ENPs (e.g. P-25).

Toxicity test:

To avoid, as much as possible, the possible difference in terms of toxic effect, determined by different treatments of n-TiO_2 in the test media the EC50 value should be extrapolated by toxicity tests in which similar treatments of ENP are applied. More, due the concerns about the use of chemical solvent (e.g. THF) toxicity tests in which solvents are used should be not applied into the calculation of EF.

Primary producers (algae):

- Time duration: 72 - 120 hours;
- End point: Inhibition of growth rate;
- Toxicity test in which the evaluation of photosynthesis activity by the measurement of chlorophyll should be preferred;

Crustaceans:

- Chronic tests should be preferred to acute toxicity test;
- Time duration: for acute toxicity tests the standard protocol defines an exposure duration of 24-h-48-h. However studies report that a prolonged acute exposure (up to 96-h) may affect the toxicity results. Thus, for acute toxicity test in which a prolonged exposure time is performed it should be chosen the EC50 with the highest exposure time.

Secondary/tertiary consumers (fish):

- Chronic test should be preferred to acute toxicity test;
- Toxicity tests performed with an exposure length of 21-days are preferred to those conducted with an exposure length of 96h. Thus accounting the life stage of the organism;
- Endpoint of mortality should be preferred.

The EC50 values have been selected among the EC50 values collected from the bibliographic review following the criteria above described.

The $HC50_{EC50}$ has been calculated as the geometric mean of EC50 values reported for each species (GM-species) and it has been calculated as the geometric mean of the three EC50 values, one from each trophic level represented by algae, invertebrates (crustaceans) and fish (GM- trophic) (Larsen and Hauschild, 2007).

Where, the GM trophic is claimed to be more representative for the true HC50 of an ecosystem (Larsen and Hauschild, 2007).

The average approach of GM requires a lognormal distribution of the data; due to the low number of data collected and the evidence that the EC50 values are positive physic entities, a lognormal distribution is assumed (Limpert et al., 2001). The calculation of the characterization factor requires the evaluation of the bioavailability; it is referred as the fraction of the stressor available for uptake by organisms of the substance. Since that the bioavailability of ENPs is still far to be known we assume that 100% of n-TiO_2 in the aquatic system is bioavailable.

6.2.4 Result

Based on the set of criteria described above the EC50 values to applied in the EF have been collected and reported in Tables 6.11-6.12-6.13.

Table 6.11 Toxicity values reported in literature for organism representative of the trophic level of crustaceans, criteria applied and toxic values selected for the calculation of EF

Specie	Primary size and crystal form	Method	Toxicity test	Toxic value reported	Criteria	Toxic value applied(mg/L)
D. magna	10-20 nm		Acute test 48h. 10 neonates/50 ml solution Endpoint: mortality	THF and filtred:LC50 5,5 ppm. Sonication LC50 was incalculable	**To treat the chemical as equally as possible the use of solvent as dispersant treatment has been excluded**	
D. magna	particle size of 25 nm and 100 nm	ISO 6341, OECD	Acute test performed in Petri dishes (Ø 55 mm):5 neonates/20 ml . Endpoint: immobility	No concentration-effect curves were determined;		
D. magna	25–70 nm	Daphtoxkit(1996)	Acute test 48h. Endpoint: immobility	EC50> 20000 mg/L	**Toxicity value refers ah the higher concentration tested**	
D. magna	7 and 20nm	(OECD, 1984, 1998)	Mortality, growth inhibition	EC50 not reported		
D. magna	6 nm	Feed before exposure; Daphtoxkit(OECD 202)	Acute test 48-h; Endpoint: mortality	LD50 not calculate		
D. magna	<40 nm (ratio rutile/anastase of 30/100)	US EPA(1993)	Test acute 48h. 25ml/5 neonates. Endpoint: mortality- Chronic test 21 days, Endpoint reproduction	LC50 impossible to calculate		
D. magna	20–30 nm; Ratio anatase/rutile of 70/30;TiO2-content (%) >99.5	Acute test:OECD202.	Acute 48h ,10 neonates/10 Ml; Endpoint immobility	EC50> 100 mg/ L		
D. magna	<20 nm;	Acute test OECD 202.	Acute 48h.10 neonate/30 ml in beacker. Endpoint: immobility, mortality	EC50= 35.306 mg/L (95%CI: 25.627-48.928). LC50=143.387 mg/L	**Acute; Lethal media concentration is preferred (USEtox)**	143
D. magna	7,5 nm	OECD 202	Acute test 48-h; 10 organism/20 ml Endpoint :immobility	LC50= 0,016mg/ml		16
D. magna	P25: 21 nm 20%rutile; 80% anastase)	OECD 202; OECD 204	Acute test :72-h. 10 neonates/30 ml in beacker. Endpoint: immobility and mortality Chronic test :21-days; Endpoint: total living offspring	Acute:EC50(48-h) and LC50(48-h) >100 mg/l; EC50(72-h)= 1.62 mg/L, LC50 (72-h)= 2.02 mg/L Chronic: EC50 0.46 mg/l LC50 2.62 mg/l	**Chronic values are preferred and lethal median concentration is preferred (USEtox)**	2.62
D. magna	18 nm (96%wt of anastase, 4%wt rutile)	OECD 202; exposure time prolonged up t 96h	Acute test (96-h) 10 organism/20 ml. Endpoint immobility ; test media ELENDT M7	EC50= 32 mg/L	**Acute**	32
D. magna	18 nm (96%wt of anastase, 4%wt rutile)	OECD 202; exposure time prolonged up t 96h	Acute test (96-h) Endpoint immobility; test media ISO water	EC50= 33 mg/L	**Acute**	33
D. magna	18 nm (96%wt of anastase, 4%wt rutile)	OECD 202; exposure time prolonged up t 96h	Acute test (96-h) Endpoint immobility; test media bottle water	EC50= 82 mg/L	**Acute**	82
D. pulex	P25:30 nm (20%rutile; 80% anastase)	ASTM	48-h static renewal; Endpoint Mortality; 5 adults /200 ml of test solution in filtered (0.45 µm) test water	LC50 > 10 mg/L	**Acute value higher than the higher nominal concentration tested**	
D. pulex	10 nm (99% TiO2)	U.S. EPA (2002 a,b)	Acute (48-h)-Mortality;	LC50= 6.5 mg/L	**Acute**	6,5
D. pulex	10 nm (99% TiO2)	U.S. EPA (2002 a,b)	Acute (48-h)-Mortality	LC50= 13.0 mg/L	**Acute**	13
C.dubia	10 nm (99% TiO2)	U.S. EPA (2002 a,b)	Acute(48h)and chronic (7 day)-reproduction	LC50 (acute) 3.0 mg/l; IC25 (chronic)=2.5-9.4-26.4 mg/L	**IC25 value are not applicable to USEtox model**	3
C.dubia	10 nm (99% TiO2)	U.S. EPA (2002 a,b)	Acute(48h)and chronic (7 day)-reproduction	LC50 (acute) 13.4 mg/L ;IC25 (chronic)=2.5-9.4-26.4 mg/l	**IC25 value are not applicable to USEtox model**	13.4
C.dubia	10 nm (99% TiO2)	U.S. EPA (2002 a,b)	Acute(48h)and chronic (7 day)-reproduction	LC50 (acute)11.0 mg/L; IC25 (chronic)=2.5-9.4-26.4 mg/L	**IC25 value are not applicable to USEtox model**	11
C.dubia	10 nm (99% TiO2)	U.S. EPA (2002 a,b)	Acute(48h)and chronic (7 day) reproduction	LC50 (acute) 3.6 mg/L-; IC25 (chronic)=2.5-9.4-26.4 mg/L	**IC25 value are not applicable to USEtox model**	3,6
C.dubia	10 nm (99% TiO2)	U.S. EPA (2002 a,b)	Acute(48h)and chronic (7 day;reproduction;	LC50 (acute) 15.9 ; IC25 (chronic)=2.5-9.4-26.4 mg/L	**IC25 value are not applicable to USEtox model**	15.9
C.dubia	P25:30 nm (20%rutile; 80% anastase)		Acute 48h. Death/immobilization	LC50 >10 mg/L	**Acute value higher than the higher nominal concentration tested**	

Table 6.12: Toxicity values reported in literature for organisms representative of the trophic level of algae criteria of selection applied and toxic values selected for the calculation of EF.

Specie	Primary size and crystal phase	Treatment of NPs	Method	Toxicity test	Toxic value (mg/L)	Criteria	Toxic value applied (mg/L)
P. subcapitata	20.5	Stock suspension: 10 mg powder to ml of Milli-Q water, sonicated with a probe sonicator	U.S EPA	Chronic 96-h;Algal growth	Not measured		
P. subcapitata	<100	filtered	/	inhibition of growth rate(72 h)	IC25> 100	IC25 value are not applied	
P. subcapitata	25-70	Stock suspensions were prepared in algal medium immediately before each experiment then ultrasonicated for 30 min	OECD 201	inhibition of growth rate(72 h) assessed by Fluorescence analysis	EC50=5,83	chlorophyll content measured	**5,83**
P. subcapitata	10 nm	NPs dispersed in hard water(USEPA), stirring for 30 min. The test solutions were frequent agitated to mantain the NPs in suspension	U.S EPA	Chronic (96-h)-Cell production	IC25=1.0-2.0	IC25 value are not applied	
P. subcapitata	<10 nm,67,2%anatase;	Stock solutions were prepared by suspending TiO2 particles in algal test medium; 10 min sonication in a water bath.	ISO 8692	inhibition of growth rate (72 h) assessed by fluorescence analysis	EC50= 241	chlorophyll content measured	**241**
P. subcapitata	30 nm: 72,6%anatase; 18,4rutile; 9%amorph				EC50=7.1		**7.1**
P. subcapitata	300 nm; anatase+ amorph				EC50=145		**145**
P. subcapitata	8 nm	The dispersion was prepared in according to OECD test guideline no. 201. The nanomaterials were suspended by stirring and/or ultrasonication in a bath sonicator;	OECD test guideline no.201	inhibition of growth rate	EC20 values reported	EC20 value are not applied	
	150 nm						
P. subcapitata	15 nm	Stock solution in MilliQ-water (pH=4) ultrasonication in a bath sonicator(30 min	ISO 8692:2004	growth inhibition rate (72-h)	2.2 (cell counting by optical microscope) 3.5 (fluorescence measurement)	chlorophyll content measured	3.5
Scenedesmus sp.	<25nm	Stock suspensions were prepared in algal medium before each experiment. Before to use the the dispersion were sonicated for 30 min	OECD 1984	algal growth inhibition (72-h); chlorophyll content at 24,48,72-h	EC50(72h)=21.2	chlorophyll content measured	21
D. subspicatus	25 nm; anatase	ENPs dispersed in test medium and dispersion was achieved by ultrasonication. dispersionwas continuously stirred on a magnetic stirrer. During the incubation time the plates were shaken.	ISO 6341, OECD	inhibition of growth rate	44	chlorophyll content is preferred	**44**
D. subspicatus	100 nm mainly anatase		202, DIN 38412-30.	(72 h)assessed by fluorescence	EC50could be not calculated		
Chlorella sp.	<25nm	Stock suspensions of nano-and bulk titanium dioxide were prepared in algal medium before each experiment The suspension were sonicated for 30 min before use	OECD 1984	algal growth inhibition (72-h); chlorophyll content	EC50(72h)=16.1	chlorophyll content measured	**16.1**
Chlorella sp.	5-10 nm; mainly anatase	sonication 30 min	/	inhibition of growth rate (144 h) assessed by algal cells count under microscope	EC50(144h) =120	Even if the chlorophyll content was not measured a Longer time of exposure was applied	**120**
Chlorella sp.	50 nm; mainly rutile	sonication 30 min	/		not reported		
Chlorella sp.	20 nm; TiO2purity >99%	sonication 30 min	/		EC_{50}(144h) =20		**20**

Table 6.13 Toxicity values reported in literature for the organisms representative of the trophic level of fish, criteria of selection and applied toxic values selected for the calculation of EF

Specie	NP	Primary size	Treatment of NPs	Method	Toxicity test	Toxic value	Criteria	Toxic value chosen (mg/L)
Oncorhynchus mykiss	79 %rutile, 21 % anatase	Refers as median particle size in water (DLS)		OECD 203	48-h and 96-h; endpoint immobilization	LC50 >100 mg/L	Toxic value higher than the higher concentration tested	
Danio renio	TiO2-P25	20.5	Stock suspension: 10 mg powder to mL of MilliQ water, sonicated with a probe sonicator	ASTM	48-h static renewal; Endpoint: survival; organisms exposed in 12-well plates with 4 mL of solution	LC50 48h > 10 mg/L	Toxic value higher than the higher concentration tested	
Pimephales promalas	TiO2 (99%)	10 nm	NPs dispersed in hard water (USEPA), stirring for 30 min. The test solutions were agitated to maintain the NPs in suspension	OECD 203	USEPA protocol; Acute (96h-growth as dry weight); Chronic (7-days) two replicate of 10/exposure; feed	Acute (96h) LC50>1000 mg/L; Chronic IC25 =342-597 mg/L	Toxic value higher than the higher concentration tested; IC25 are not applied	
Zebrafish	TiO2 (purity 99%); Anastase	30 nm	Dispersed with a bath sonicator for 20 min instead of using stabilizing agents.	OECD 203	Acute test (96-h); unfed Endpoint :Lethality and oxidative stress and damage	96h LC50 of 124.5 mg/L.		124.4

The geometric means such as the GM-species and GM-trophic are listed in Table 6.14.

Table 6.14: GM species and GM-trophic

Taxon	Specie	Criteria	Toxic value applied (mg/L)	GM species level	GM trophic level
Crustaceans	*Daphnia. magna*	Acute; Letahl media cocnetration is preferred (USEtox)	143	28	13
	Daphnia. magna		16		
	Daphnia. magna	Chronic values are preferred and Lethal median cocnetration is preferred (USEtox)	2.62		
	Daphnia. magna	Acute	32		
			33		
			82		
	Daphnia pulex	Acute	6.5	9	
		Acute	13		
	Ceriodaphnia.dubia	LC50 Acute (IC25 value are not applicable to USEtox model)	3	8	
			13.4		
			11		
			3.6		
			15.9		
Algae	*Pseudokirchneriella subcapitata*	chlorophyll content measured	5.83	22	29
		chlorophyll content measured	3.36		
		chlorophyll content measured	241		
			7.1		
			145		
	Scenedesmus sp.	chlorophyll content measured	21	21	
	Desmodesmus subspicatus	chlorophyll content is preferres	44	44	
	Chlorella sp.	chlorophyll content measured	16.1	34	
	Chlorella sp.	Even if the chlorophyll content was not measured a Longer time of exposure was applied	120		
	Chlorella sp.		20		
Fish	*Zebrafish*		124.5	124	124
		Geometric mean	**20**	**25**	**36**

The geometric mean based on the average of a trophic level **(GM-trophic) is 36 mg/L**, is on the same order of magnitude of geometric mean based on the average of species level **(GM-species) of 25 mg/L**.

To calculate the $HC50_{EC50}$ acute EC50 values have been applied thus, the $HC50_{EC50}$ is referred to $HC50_{acute}$. The effect factor is calculated as the ratio between 0.5 (PAF) and the $HC50_{EC50}$ .The USEtox guidelines prioritize chronic toxicity values ($EC50_{chronic}$) and when chronic data are unavailable, the guidelines suggest an acute-to-chronic ratio (ACR) of 2. Thus, $HC50_{EC50chronic}$ is the ratio of $HC50_{acute}$ and ACR: $HC50_{EC50chronic} = HC50_{EC50acute}/2$.

Then, the effect factor(Table 6.15) is calculated as the ratio between 0.5 (PAF) and the $HC50_{EC50chronic}$

Tab. 6.15: Effect factor (EF) values for n-TiO_2

	Toxic value applied	Species level	Trophic level
$HC50_{EC50acute}$(mg/L)	20	25	36
EF (PAF m^3 kg^{-1})	**46**	**20**	**28**

6.2.5 Discussion and conclusion

The large variation in the toxicities of nanoparticles of n-TiO_2 is a major impediment for making robust estimations of their ecotoxicity. Given the multiple differences among the studies and the lack of standard procedures to testing metal oxide ENPs, a set of criteria has been proposed to select the EC50 values. Similar $HC50_{EC50}$ values are obtained at GM species-level (25 mg/ L) and GM trophic (36 mg/L).

As argued, the USEtox model suggests to calculate the $HC50_{EC50}$ on the species-level. However, Larsen and Hauschild (2007) indicate an average approach based on the GM-trophic as the best practice to calculate the $HC50_{EC50}$. In our case study an average approach on the EC50 specie-values (GM-species) will lead to bias (putting a weight on the trophic levels with many measured values), since that there is an unequal distribution of the EC50 values among the trophic level.

Therefore, we suggest to calculate the $HC5_{EC50}$ as the GM-trophic in which each trophic level is represented by one EC50 value.

Thus, an EF of 28 (PAF m^3 kg^{-1}) for n-TiO_2 is proposed.

6.3 How to calculate the characterisation factor for n-TiO_2?

As argued in the Chapter V, the characterization factor for freshwater ecotoxicity is calculated as:

$$CF_w = f_{i,w} * FF_{w,w} * XF_w * EF_w \quad (6.16)$$

If a direct emission to freshwater by a wastewater stream (thus $f_{i,w}$ is equal to 1) and also a full bioavailability of n-TiO_2 aggregated dispersed in water (XF equal to 1) is supposed, the characterisation factor may be expressed as:

$$CF_w = FF_{w,w} * EF_w \quad (6.16)$$

Where:

- **EF_w of 28 (PAF m^3 kg^{-1}) for n-TiO_2 is proposed.**

The $FF_{w,w}$ has been calculated as the average weighted of the $FF_{w,w,i}$, that represents the average residence time of a size distribution of the n-TiO_2 aggregated. Where the $F_{w,w}$ is 10^{-2} (day).

This approach aims to be more environmentally realistic as possible and it hypothesize that the bioavailability of the ENPs depends on the size range. This approach leads to a **CF_w of 0.28 PAF day m^3 kg^{-1}.**

However, it cannot be overlooked that a different approach to calculate the CF_w may be followed. In fact, to date, the ecotoxicity of n-TiO_2 is far to be known. As argued in the Chapter IV, the mechanism of toxicity of ENPs to freshwater organisms is still not clear. Some authors refer that the toxicity may be confers by the small size of ENPs, other authors suggest that the aggregate particle assemblies may be toxic as well.

Therefore, the CF_w may be evaluated following a precautionary approach in which the environmental hazard of ENPs is governed by the "small size of particles". Thus, the CFw is calculated as the product of the $FF_{w,w,i}$ evaluated for the smallest size of ENPs (n-TiO_2) and with the lower efficiency of collision (Table 6.9). This scenario leads to $CF_{w,i}$ of 32 PAF day m^3 kg^{-1}.

6.4 Conclusion

In our framework the fate factor is calculated as dependent by the size distribution of the n-TiO_2 aggregated of in freshwater ecosystem. This approach is innovative in the LCIA scenario. In fact the calculation of a fate factor in dependence of the size of aggregated of ENPs (e.g $F_{ww,i}$), will permits to obtain a characterisation factor (CFw) dependent on the size distribution of aggregated of n-TiO_2.

Following this approach two aspects may be covered: 1) the fate factor may be express as dependent on a size class of ENPs: if the effect factor on the basis of the size of particle is also known, the exposure scenario may be correctly assessed 2) the framework allows to apply the size distribution of ENPs or SPM evaluated for site-dependent condition.

Very recently, Eckelman et al. (2012) following the USEtox model estimated the EF of CNTs for freshwater ecotoxicity equal to 200 PAF m^3 kg^{-1} but, to my knowledge, the EF for metal oxide nanoparticle such TiO_2 is for its first time calculated.

Thus, to date not comparison with EF of other metal oxide nanoparticles is possible to perform. The criteria applied to choose the EC50 values aims to reduce the high variability of toxic values and to consider the experimental parameters that could influence the tests result the EF has been calculated with a criteria based approach. However, it cannot be overlooked that for practical reason the concentration of effect reported being higher than the highest concentration tested (e.g. EC50 > 100 mg/L) have not be include into EF calculation. This, leading to an underestimation of the EF and therefore to an overestimation of n-TiO_2 ecotoxicity.

Currently no characterisation factors for metal oxide ENPs are available. This is due to the lack of characterisation models, as consequences of the scarce knowledge on the toxicity of ENPs and of the lack of environmental models for ENPs.

The framework here proposed is based on the well-establish multimedia box model, usually applied for organic substances. In the fate model here proposed the fate behaviour of ENPs in freshwater has been described following the colloidal science. Therefore, in contrast to organic chemical for which portion coefficient are usually applied, kinetic equations has been applied. Formulated in this way the framework account for the specific fate processes of ENPs such aggregation.

Furthermore, since that the toxicity of ENPs may dependent by the size of ENPs to which the organism are exposed , we focused on the development of a CF in which the size distribution of ENPs in freshwater has been considered.

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CONCLUSION

This thesis has highlighted the complexity of assessing the ecotoxicity impacts of metal oxide nanoparticles as n-TiO_2 in the field of LCA. To date, to conducted a “cradle-to-grave” LCA study on nanotechnology is itself a challenge because the characterisation factors associated to the release of ENPs into the environment are not yet introduced in LCA database. Several limitations have been shown that can be traced to two main themes: 1) the lack of knowledge on ENP’s environmental behaviour and the lack of nano-specific environmental fate models 2) the high variability of the toxic results and the uncertainty of the toxicity of ENPs to aquatic organisms.

The overview about the fate processes of metal ENPs allows to draw general conclusions about the assessment of the toxic impact (e.g. freshwater ecotoxicity) with the Life Cycle Impact Assessment.
The environmental fate models usually applied in LCIA require to be adapted to the specific behaviour of ENPs (e.g. aggregation and dissolution). The PhD research has highlighted the requirements to implement the environmental fate model for ENPs such as n-TiO_2 with a different approach based on the colloidal science. Therefore, using the USEtox model as starting point and applying the colloidal science a framework for the calculation of the fate factor has been suggested. Thus, The mathematical model behind the derivation of the fate factor in USEtox was modified incorporating the kinetic equations describing environmental fate processes such the aggregation and the sedimentation. Next efforts should be focused on the evaluation of the abiotic degradation and on the evaluation of the dissolution. Furthermore, more efforts should be directed towards the introducing of spatial differentiation in regional impact categories such as ecotoxicity. In ordinary LCAs the location of the processes which release toxicants to the environment is usually not precisely known and, therefore, site-specific models cannot easily be used. Most often large-average landscape data and environment conditions are assumed. To date, **the site-independent approach seems a limitation of the assessment of toxic potential impact of ENPs in Life Cycle Assessment**. In fact a strong correlation among the environmental parameters and the fate of ENPs in the environment (e.g. freshwater) has been pointed out. Furthermore, the emission of a toxicant listed in a life-cycle inventory (LCI) is regarded as a single pulse without time duration and, therefore, time and space are integrated in the assessment posing further restrictions to the modelling. This is in contrast with the environmental behaviour of the ENPs for which the environmental fate and behaviour (stability and persistence) have been observed as concentration-dependent. More during the time, the physical form and intrinsic property of ENPs (e.g. size distribution of aggregates, surface charge) may be subject to transformations, therefore leading to a different bioavailability and route of exposure.

Thus, the **next challenge could be to develop a site-dependent approach**. A first step should be the assessment of toxic impacts for “environmental archetypes” (e.g. freshwaters, soils archetype) and on regional scale. Formulated in this way (i) the fate of ENPs on the physical-chemical water parameter (e.g. IS, pH, content of TOC) will be evaluated and therefore, (ii) different behaviors, and bioavailability based on archetype will be assessed.

The conceptual framework adopted by the LCIA’s characterisation model for the evaluation of the effect factor can be applied for this new class of contaminants as ENPs. However, it cannot be overlooked that the toxicity of ENPs is still poorly understood and more scientific researches have to be carried out to understand the source of variability of the toxic results reported in literature. Furthermore, the bibliographic review has highlighted a low number of toxicity study on the organism representative of the trophic level of fish. Based on the experimental work and the scientific literature it can be concluded that the establishment of the concentrations of effect (e.g. EC50) is influenced by the treatments of the sample to test, by the method of testing followed, by the lack of engineered nanoparticles of reference and of standard testing procedures as well. For this reason decision criteria were suggested on how to select input data for the effect factor.

Moreover, the effect mechanisms relating to nanoparticles are still not clearly understood. The understanding of the underlying mechanisms relating to the potentially adverse effects of nanoparticles on aquatic organisms is a prerequisite for determining appropriate hazard assessment strategies. Furthermore, the lack of acknowledge in what the toxicity is size-dependent (e.g. aggregates or small particles or their mixture) leading to an impossible characterization of the ENP's bioavailability.

The assessment of toxic impact within aquatic environment should be also focused on the sediment compartment that seems to be affected by the sedimentation of metal oxide nanoparticles. Thus, the characterization models such as the USEtox model should be include the “sediment” in their framework.