Fate and Biological Effects of Titanium Dioxide Nanoparticles in the Aquatic Environment

Ling Li

Laboratory Centre
Unit of Ecotoxicology and Risk Assessment
Finnish Environment Institute (SYKE)

Department of Environmental Sciences
Faculty of Biological and Environmental Sciences
University of Helsinki
Finland

Academic dissertation in Environmental Ecology

To be presented for public examination with the permission of the Faculty of Biological and Environmental Sciences of the University of Helsinki in the Auditorium of Lahti Science and Business Park, Niemenkatu 73, Lahti,
on May 26th 2017, at 12 o'clock noon.

Lahti 2017

Supervisors: Dr. Markus Sillanpää
Laboratory centre
Finnish Environment Institute, Finland

Dr. Eija Schultz
Laboratory Centre, Ecotoxicology and Risk Assessment
Finnish Environment Institute, Finland

Dr. Olli-Pekka Penttinen
Department of Environmental Sciences
University of Helsinki, Finland

Thesis advisory committee: Dr. Anna-Lea Rantalainen
Department of Environmental Sciences
University of Helsinki, Finland

Prof. Mika Sillanpää
Laboratory of Green Chemistry
Lapeenranta University of Technology, Finland

Pre-examiners: Prof. Jussi Kukkonen
Department of Biological and Environmental Science
University of Jyväskylä, Finland

Associate Prof. Anita Jemec
Biotechnical Faculty
University of Ljubljana, Slovenia

Opponent: Adjunct Prof. Jarkko Akkanen
Department of Environmental and Biological Sciences
University of the Eastern Finland, Finland

Custos: Prof. Marko Virta
Department of Environmental Sciences
University of Helsinki, Finland

ISBN 978-951-51-3175-1 (Paperback)
ISBN 978-951-51-3176-8 (PDF, http://ethesis.helsinki.fi)
ISSN 1799-0580
Unigrafia, Helsinki 2017

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ABSTRACT

The increasing use of titanium dioxide nanoparticles (TiO_2 NPs) and their consequent release into the environment, make it important to understand the behaviour, fate and potential adverse effects that TiO_2 NPs may have upon organisms. After being introduced into the aquatic environment, TiO_2 NPs will undergo transformation processes, which will affect their properties, including, chemical composition, size, surface charge and coating, and these may also have substantial implications on the fate and toxicity of the NPs. This highlights the importance of physiochemical characterization of the NPs in conjunction with a toxicological assessment. The transformation processes are dependent both on the physicochemical properties of the NPs and on the physicochemical properties of the ambient environment. However, these properties have been seldom studied in complex natural waters. Therefore, the research done for this PhD dissertation aimed to investigate the behaviour of TiO_2 NPs in natural waters, and to associate the NPs colloidal stability with various water properties. The toxicity tests of TiO_2 NPs were performed on two aquatic organisms each of which represent different trophic levels: *Lemna minor (L. minor)* and *Daphnia magna (D. magna)* according to ISO 20079 and OECD test 202, respectively. The specific aim was to assess the possible toxic effects of TiO_2 NPs with maintained colloidal stability in an optimized ecotoxicological test medium. Given the potential of TiO_2 NPs to carry other pollutants, the mixture toxicity of TiO_2 NPs with cadmium (Cd) and lead (Pb) on *D. magna* were also investigated.

TiO_2 NPs exhibited different colloidal stability, which depend on the physicochemical properties of the natural waters. Total phosphorus, total nitrogen, alkalinity, pH, electrical conductivity and turbidity were shown by experimental and linear regression analysis to be the main water properties that negatively influence NPs stability among 14 water properties that were compared. All six of these physicochemical properties of water had positive correlations on the deposition of TiO_2 NPs in lake waters. TiO_2 NPs showed low colloidal stability in standard ecotoxicological test media, but modification of the current standard medium enabled the steady exposure of *L. minor* and *D. magna*. TiO_2 NPs showed no adverse effect on the growth rate or chlorophyll *a* content of *L. minor*, or immobility of *D. magna.* However, TiO_2 NPs were found to become attached to the cell wall of *L. minor* and to the body surface of *D. magna*, both of which may cause the potential transfer of TiO_2 NPs into the aquatic food chains. Such a transfer would lead to an exposure of other organisms and contribute to the environmental fate of NPs. The presence of TiO_2 NPs also influenced the bioaccumulation and toxicity of Cd and Pb in *D. magna*. TiO_2 NPs increased the bioaccumulation and toxicity of Cd in daphnia, whereas it reduced the toxicity of Pb regardless of the 3- to 4-fold increase of bioaccumulation found. Surface attached TiO_2 NPs combined with adsorbed heavy metals, caused adverse effects on *D. magna* swimming and moulting behaviour, which was assumed to lead to chronic toxicity. The research findings presented in this thesis elucidate the water physicochemical properties that control the aggregation and deposition of TiO_2 NPs in complex natural waters, and offer an insight into their biological effects and accumulation potential upon aquatic organisms.

TIIVISTELMÄ

Titaanidioksidi-nanohiukkasten käytön lisääntymisen myötä niitä päätyy ympäristöön entistä enemmän. Tämän vuoksi on tärkeää ymmärtää metallinanohiukkasten käyttäytyminen, ympäristökohtalo ja haitalliset vaikutukset luonnoneliöihin. Vesiympäristöön joutuessaan reaktiiviset titaanidioksidihiukkaset ovat alttiita muutoksille jotka vaikuttavat nanohiukkasten kemialliseen rakenteeseen, kokoon ja pintakemiaan tavalla, joka heijastuu sekä nanopartikkelin ympäristökohtaloon että ekotoksisuuteen. Tämä korostaa nanomateriaalin fysikaaliskemiallisen karakterisoinnin tärkeyttä toksisuuden testauksen yhteydessä.

Nanohiukkasten muuttuminen vedessä on riippuvainen sekä hiukkasten että ympäristön fysikaaliskemiallisista ominaisuuksista. Tätä asiaa on tutkittu kuitenkin hyvin vähän ominaisuuksiltaan erilaisissa luonnonvesissä. Tämän väitöskirjatyön tavoitteena onkin tutkia titaanidioksidinanohiukkasten käyttäytymistä luonnonvesissä ja yhdistää tarkasteluun titaaninanohiukkasten kolloidinen vakaus eri vedenlaatuominaisuuksia suhteen. Nanohiukkasten välitöntä myrkyllisyyttä vesieliöille testattiin kahdella eri trofiatasolla toksisuusstandardeissa mainittuja testilajeja käyttäen: vesikirppu *Daphnia magna* (OECD 202) ja pikkulimaska *Lemna minor* (ISO 20079). Nanohiukkasten välitöntä toksisuutta pyrittiin testaamaan erityisesti olosuhteissa missä testiväliainetta muokattiin nanohiukkasten kolloidisuuden optimoimiseksi. Lisäksi seostoksisuuskokeiden avulla selvitettiin voiko titaanidioksidihiukkasiin kiinnittyä myös muita haitallisia metalleja käyttämällä kadmiumia ja lyijyä malliaineina. Tulosten mukaan titaaninanohiukkasten kolloidinen vakaus riippui luonnonvesien fysikaaliskemiallisista ominaisuuksista. Regressioanalyysien perusteella 14:stä mitatusta veden ominaisuudesta erityisesti kokonaisfosfori ja –typpi, alkaliniteetti, pH, sähkönjohtavuus ja sameus vaikuttivat kaikkien negatiivisimin titaaninanohiukkasten kolloidiseen tilan säilymiseen. Myös järviveden fysikaaliskemiallisten ominaisuuksien ja nanotitaanihiukkasten laskeuman välillä oli positiivinen korrelaatio.

Toksisuustestien aikana standardoidussa testiliuoksessa titaanidioksidi-nanohiukkaset pyrkivät aggregoitumaan mutta standardikoeveden muokkaaminen mahdollisti vakaat altistusolosuhteet molemmille testilajeille. Altistuminen nanohiukkasille ei vaikuttanut vesikirpun liikkuvuuteen eikä pikkulimaskan kasvunopeuteen tai klorofylli a–

pitoisuuteen. Nanotitaanihiukkaset kuitenkin kiinnittyivät limaskan soluseinän pintaan ja vesikirpun kitiinikuoreen mikä mahdollistaa titaanidioksidihiukkasten siirtymisen akvaattiseen ravintoketjuun altistaen uusia eliölajeja nanotitaanille sekä vaikuttaen samalla nanohiukkasten ympäristökäyttäytymiseen. Lisäksi titaaninanohiukkaset lisäsivät kadmiumin bioakkumulaatiota ja toksisuutta vesikirpulla *D. magna* mutta lyijyllä kasvanut bioakkumulaatio ei vaikuttanut toksisuuteen. Kuitenkin nanohiukkasten pintaan kiinnittymisen ja metallien akkumuloitumisen aiheuttama ympäristöstressi voi aiheuttaa kroonista myrkyllisyyttä vesikirpuilla. Väitöskirjan tulokset korostavat vesiympäristön fysikaaliskemiallisten ominaisuuksien merkitystä ja monimutkaisia vuorovaikutussuhteita tarkasteltaessa titaaninanohiukkasten ympäristökohtaloa erilaisissa vesiympäristöissä. Tuloksilla on merkitystä myös nanomateriaalien bioakkumulaatiopotentiaalin ja biologisten vaikutusten ymmärtämiselle.

LIST OF ORIGINAL PAPERS

This thesis is based on the following original publications and manuscripts, which are henceforth referred to in the text by Roman numerals.

I. Li L, Sillanpää M and Risto M. 2016. Influences of water properties on the aggregation and deposition of engineered titanium dioxide nanoparticles in natural waters. Environmental Pollution 219: 132-138.

II. Li L, Sillanpää M, Tuominen M, Lounatmaa K and Schultz E. 2013. Behaviour of titanium dioxide nanoparticles in *Lemna minor* growth test conditions. Ecotoxicology and Environmental Safety 88: 89-94.

III. Li L, Sillanpää M and Schultz E. Influence of titanium dioxide nanoparticles on Cadmium and Lead bioaccumulations and toxicities to *Daphnia magna*. Submitted manuscript.

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THE AUTHOR'S CONTRIBUTION

I. LL took part in planning the experiments, performed most of the experimental laboratory work and all the data analyses, and was the main person responsible for the manuscript preparation.

II. LL participated in the designing of the study, conducted most of the experimental laboratory work and all the data analyses, and was the main person responsible for the manuscript preparation.

III. LL designed the study with MS and ES, performed most of the experimental laboratory work and all the data analyses, and was the main person responsible for the manuscript preparation.

ABBREVIATIONS

List of most common abbreviations used in this thesis

CCC	Critical coagulation concentration
COD	Chemical oxygen demand
DLS	Dynamic light scattering
DLVO	Derjaguin-Landau-Verwey-Overbeek
EC	Electrical conductivity
EC_{50}	Median effective concentration
EDL	Electrical double layer
EDTA	Ethylenediaminetetraacetic acid
ENP	Engineered nanoparticle
HDD	Hydrodynamic diameter
ICP-OES	Inductively coupled plasma-optical emission spectrometer
IS	Ionic strength
ISO	International Organization for Standardization
LDV	Laser Doppler velocimetry
NNMs	Natural nanomaterials
NOM	Natural organic matter
NP	Nanoparticle
OECD	Organization for Economic Co-operation and Development
pH_{pzc}	Point of zero charge
SEM	Scanning electron microscopy
TEM	Transmission electron microscopy
UV-vis	UV-visible spectroscopy

1. INTRODUCTION

1.1 Nanotechnology and nanomaterials

Nanotechnology is a broad interdisciplinary area of research, development and industrial activity. It involves the design, characterization, production and application of structures, devices and systems by controlling their shape and size at the nanometer scale (Royal Society and Royal Academy of Engineering, 2004). Nanotechnology has been widely applied in all fields of science and technology such as material science, mechanics, electronics, optics, medicine, plastics, energy and aerospace.

A nanomaterial is a material with at least one dimension between 1 and 100 nm (Nowack and Bucheli, 2007). Nanomaterials are not a homogenous group of materials but encompass various types and forms of materials, including nanofilms which has one dimension on the nanoscale, nanowires and nanotubes, which have two dimensions on the nanoscale, and nanoparticles (NPs), all three spatial dimensions on the nanoscale. Nanomaterials are classified into three groups on the basis of their origin as: natural nanomaterials (NNMs), anthropogenic incidentally produced nanomaterials, and engineered nanomaterials (Oberdörster et al., 2005). In general, the engineered nanoparticles (ENPs) can be categorised into carbon-based nanomaterials (carbon nanotubes and fullerenes), inorganic NPs including the one based on metal oxides (zinc oxide, iron oxide and titanium dioxide), metals (gold, silver and iron) and quantum dots (cadmium sulphide and cadmium selenide).

There are two basic approaches to manufacture ENPs: top-down (subtractive process) and bottom-up (additive process). The top-down method entails the ENPs being synthesized by breaking down a bulk starting material gradually into smaller sizes. It includes mechanical-energy, thermal, chemical, lithographic, and natural methods. The most common method of synthesis for fullerenes is based on the top-down method, which uses a carbon electric arc. The bottom-up method assumes more importance in nanotechnology nowadays. The bottom-up method entails the ENP being started from the atomic or molecular precursors and gradually assembled and aggregate until the desired ENP is formed. The production of metallic NPs is based on bottom-up methods and especially liquid phase synthesis. Carbon nanotubes are usually obtained by the chemical vapour deposition method. Metal oxides ENPs can be synthesized by catalyst-free and catalyst-assisted procedures that can be further distinguished between vapour and solution phase growth (Lu et al., 2006). In addition, surface modification and functionalization such as a coating is often performed for ENPs in order to improve the biological, chemical, and mechanical properties.

1.2 Production and emission of engineered nanoparticles

Metal oxide NPs have attracted considerable interest due to their variety of properties and potential technological applications in medicine, information technology, catalysis, energy storage and sensing (O'Brien, 2012). Titanium dioxide (titania, TiO_2) NPs are one of the most extensively used metal oxide NPs. There are three forms of crystal structure: rutile, anatase, and brookite, of which the first two play a significant role in industrial applications and therefore are the most important TiO_2 in the environment (Diebold, 2003). Each of these forms present different chemical properties and activities, and hence different industrial uses. Rutile TiO_2 NPs are the less reactive NPs and have the ability to absorb ultraviolet radiation, and are used in paints, lacquers, sunscreens, and cosmetics (Nowack and Bucheli, 2007). Anatase TiO_2 NPs are able to produce reactive oxygen species under ultraviolet irradiation, and are hence used as a photocatalyst in environmental remediation such as water treatment and benzothiophene removal from diesel fuel, and also in the degradation of air pollutants and anti-fouling (Ju-Nam and Lead, 2008; Vucko et al., 2013). Kunhikrishnan et al. (2015) estimated that the production of TiO_2 NPs to be 50,000 tons per year and Robichaud et al. (2009) forecast that the quantity TiO_2 NPs will increase to 2.5 million tons per year by 2025.

With the large and increasing production and usage, ENPs are inevitably released into the environment (Conway et al., 2015; Lettino et al., 2015; Shandilya et al., 2015). ENPs can enter the environment through intentional release such as the use of ENPs in various industrial applications, ground water remediation, drug delivery, diagnostics, biomedical imaging. ENPs can also be unintentionally released during various industrial and mechanical processes such as vehicle exhaust, coal/oil/gas boiler, fuel cells. Emissions may also occur by accidental spills during production or transportation. There have been significant concerns about the release of ENPs into the aquatic environment via municipal wastewater and urban runoff. For example, the release of TiO_2 NPs could pose an exposure risk in the aquatic environment, particularly in freshwater (Salieri et al., 2015). A study reported that modelled TiO_2 NPs concentrations in rivers ranged from 3 ng L^{-1} to 1.6 μg L^{-1}, but the proportion of engineered TiO_2 NPs could not be quantified (Gottschalk et al., 2013).

1.3 Fate of TiO_2 nanoparticles in the aquatic environment

Upon emission into the aquatic environment, the ENPs with high reactivity will readily be subjected to transformation processes. These transformation processes include the following: physical transformations (homo-aggregation, agglomeration, and sedimentation/deposition), interaction with surfaces/substances (natural organic matter adsorption, sorption onto other surfaces and desorption), chemical transformation (photochemical transformation, redox and dissolution) and biological transformation (biodegradation and bio-modification) Transformations will affect the properties of the ENPs such as chemical composition, size, surface charge and coating. The changes in these properties may therefore have substantial implications on ENPs fate and toxicity. The main transformation processes for TiO_2 NPs include: homo-aggregation, agglomeration, sedimentation, hetero-aggregation, interaction with surfaces/substances, photochemical transformation and bio-modification. A brief overview of these transformation processes will be provided in the following subsections.

1.3.1 Colloidal stability

The fate of ENPs in the aquatic environment is strongly determined by their colloidal stability and their tendency to form aggregates/agglomerates. Aggregates are particles held together by strong chemical bonds, whereas agglomerates are primary particles held together by weak van der Walls forces. Aggregation and agglomeration of NPs could not be distinguished in this study, so the term of aggregation is henceforth used to describe the formation of large particles. The colloidal stability of ENPs suspended in aqueous environments can be considered in the context of the traditional Derjaguin-Landau-Verwey-Overbeek (DLVO) theory (Derjaguin and Landau, 1941; Verwey and Overbeek, 1948), and non-DLVO theory (Petosa et al., 2010). DLVO theory was developed for colloids with a spherical shape, but it is applicable to nanoparticle aggregates that can be characterized as minor colloids (Godinez and Darnault, 2011). Most particles will develop a surface charge when dispersed within an aqueous system. An electric double layer (EDL) will be formed around the charged particle as the effect of the surface charges (Figure 1).

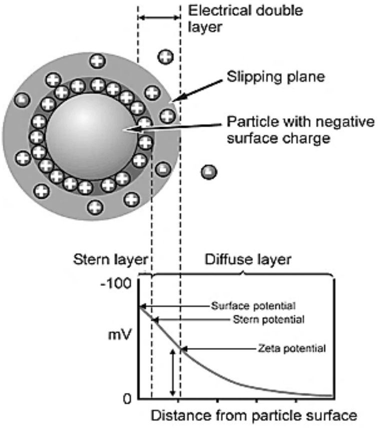

Figure 1. Schematic representation of electrical double layer and zeta potential (Shaw, 2003).

According to the DLVO theory, the stability of NPs suspension is determined by the sum of the van der Waals force and the electrostatic EDL force. The van der Waals force between two spherical particles with identical particle radius can be theoretically calculated according to Equation 1 (Elimelech et al., 1998):

$$V_{VDW} = \frac{-A \cdot R}{12 \cdot h} \tag{1}$$

Where V_{VDW} is the van der Waals interaction energy between two particles with radius R, the A is the Hamaker constant, h is the minimum distance between particles. This equation is only valid when h is much smaller than R.

EDL is important when the particles are charged. The thickness of the EDL is called the Debye length. The interaction between two EDLs leads to an electrostatic repulsive force and is calculated by the following equation:

$$V_{EDL} = 2\pi \cdot \varepsilon_r \cdot \varepsilon_0 \cdot R \cdot \zeta^2 \cdot \ln(1 + e^{-hk}) \tag{2}$$

Where V_{EDL} is the EDL repulsive interaction energy between two particles with radius R, ε_r is the dielectric constant of the liquid (2.5 for water), ε_0 is the permittivity of vacuum (8.85×10^{-12} C $V^{-1}m^{-1}$) (Hiemenz and Rajagopalan, 1997), ζ is the experimental zeta potential, h is the minimum distance between particles and k is the inverse Debye length that is calculated using Equation 3:

$$k = 2.32 \cdot 10^9 \cdot \sqrt{\sum C_i \cdot {z_i}^2} \tag{3}$$

Where c_i is the ion molar concentration and z_i is the charge of the ion.

The zeta potential and the Debye length are the two important properties of the EDL (Morrison and Ross, 2002). One can manipulate these two parameters to optimize stability and electrochemical properties of a colloidal dispersion. The surface charge is controlled by several mechanisms, including surface ionization, ion adsorption and ion dissolution (Stumm and Morgan 1996), whereas the Debye length is inversely proportional to the ionic strength (IS) of the solution.

In addition to the classical DLVO interactions, non-DLVO interactions including steric hindrance, magnetic forces (for iron-based ENPs) and hydration forces, can also influence the colloidal stability of ENPs (Petosa et al., 2010). When particles are either coated by polymers, or adsorb natural organic matter (NOM) in natural aqueous environment, a steric repulsive force between particles must be considered (Peijnenburg et al., 2015). The colloidal stability of ENPs and their aggregation in the aquatic environment are usually controlled by both DLVO and non-DLVO interactions in complex ways.

Aggregation is a key process that governs the fate of ENPs in the aquatic environment. There are two types of aggregation: homo-aggregation between the same NPs and hetero-aggregation between different NPs. It has been shown that the aggregation of NPs is controlled both by the physicochemical properties of the NPs such as size, structure, composition and surface chemistry, and by the water properties such as pH, IS, and NOM. Furthermore, water temperature and flow velocity may also play important roles in the aggregation and sedimentation behaviour of TiO_2 NPs (Lv et al., 2016). The aggregation can be explained by the particle-particle collision, which is dependent on attachment efficiency and collision frequency. The attachment efficiency is controlled by the interaction forces (attractive van der Waals force and electrostatic repulsion force) as described by the traditional DLVO theory. The collision frequency is influenced by the Brownian motion, fluid motion and particle number concentration.

Some water properties have shown important roles in the aggregation of NPs (Baalousha et al., 2013; Peijnenburg et al., 2015). For example, an increase in the IS compresses the EDL, which decreases the electrostatic repulsion between two particles that have the same charge. The energy barrier will then decrease and the attachment probability becomes closer to unity. As a consequence, particles can collide and form aggregates. In general, aggregation and deposition is favoured under conditions where pH values are near the point of zero charge i.e. the pH at which the net surface charge is zero (pH_{pzc}), and IS is high in the presence of a number of multivalent counter-ions (French et al., 2009; Guzman et al., 2006).

Hetero-aggregation of ENPs and natural colloids is relevant for the fate of ENPs in natural waters, as the number concentration of natural colloids is expected to be much higher than those of ENPs (Hotze et al., 2010; Klaine et al., 2008; Lead and Wilkinson, 2006). Earlier reports have shown the importance of hetero-aggregation of TiO_2 NPs in the presence of clay (Adam et al., 2016; Labille et al., 2015). Kaolin clay can hetero-aggregate with TiO_2 NPs and alter their colloidal stability at low pH and high IS. However, humic acid has a significant impact on the stability of the hetero-aggregates (Wang et al., 2015). Another study found that the hetero-aggregation was reduced at higher dissolved organic matter concentrations (Quik et al., 2014). It is a combination of NOM, IS and pH that influences the hetero-aggregation of ENPs with natural colloids in aquatic environments.

NOM, including humic substances (such as humic acid and fulvic acid) and natural biopolymers (such as polysaccharides and proteins), has been shown to interact strongly with ENPs, which modifies the surfaces of ENPs and affects their colloidal stability. At low concentration, NOM may destabilize TiO_2 NPs in solution through charge neutralization and bridging mechanisms (Christian et al., 2008). In contrast, an increase of the concentration of NOM might enhance the TiO_2 NPs stability via NOM coating, which would provide an efficient electrostatic and/or steric stabilization (Erhayem and Sohn, 2014; Thio et al., 2011). In addition, the same type of NOM may lead to either the stabilization or destabilization of ENPs depending on the pH and IS of the solution, especially in the presence of multivalent cations. For example, at low cation concentration where IS was lower than the critical coagulation concentration (CCC), humic acid generally increased colloidal stability of TiO_2 NPs; whereas at conditions IS $>$ CCC, destabilization was occurred via IS-induced entanglement and NOM/IS bridging mechanisms (Luo et al., 2016).

Apart from the interactions with natural colloids and NOM, TiO_2 NPs may also hetero-aggregate with suspended large particles, and then transport or deposit along with the suspended large particle (Lin et al., 2010; Praetorius et al., 2012). Moreover, TiO_2 NPs can also attach to aquatic organisms such as algae and crustaceans. These actions alter the dispersion stability of NPs and resulting in possible bioaccumulation (Aruoja et al., 2009; Dabrunz et al., 2011). Such process changes the mobility, activity, transport and fate of ENPs in the aquatic environment.

1.3.2 Photochemical transformation and bio-modification

As TiO_2 NPs are photo-inducible and redox active ENPs, they may undergo transformation when subjected to UV light. UV irradiation of TiO_2 suspensions were found to increase the surface concentrations of hydroxyl groups, reduced TiO_2 NPs surface charge, and consequently promoted particle aggregation (Sun et al., 2014). UV irradiation can also excite the photocatalytic activity of TiO_2 NPs. After UV irradiation, TiO_2 NPs can produce reactive oxygen species (Brunet et al., 2009; Menard et al., 2011), and can cause oxidative stress to cells in the ambient environment (Okupnik and Pflugmacher, 2016). Such photo-activity is usually eliminated in sunscreens by using a TiO_2 core, coated with an $Al(OH)_3$ layer and then a hydrophobic layer. The $Al(OH)_3$ coating could reduce reactive oxygen species production, and the hydrophobic coating could provide an efficient protection against dispersion in water (Nowack et al., 2012).

However, upon their release into the environment, these surface coatings will be subject to transformations and degradation, which change the properties of ENPs, and in turn may influence the behaviour and fate of ENPs such as TiO_2 NPs. A study using clay colloids conducted by Labille et al. (2010), found that a TiO_2 nanocomposite underwent rapid alteration and desorption of its hydrophobic coating layer when exposed to aqueous media, which subsequently promoted the dispersion of these materials. The $Al(OH)_3$ mineral layer remained on the TiO_2 surface, which suggested that inert TiO_2 by-products had formed under the studied conditions.

Bio-modification may be relevant for TiO_2 NPs due to their interaction with living organisms. Bio-modification in this context refers to the biologically mediated transformation that occurs as a result of uptake into organisms and/or the indirectly mediated modification by an organism. It was found by Della Torre et al. (2015) that organic particles released by mussels could bind to the TiO_2 NPs and changed their agglomeration/adsorption process. As a result, a reduction on the suspended TiO_2 NPs concentration was observed. However, current knowledge indicates that in comparison to other transformation processes, bio-modification is thus considered to have less importance for ENPs fate and behaviour (Hartmann et al., 2014).

1.3.3 Analyses methods

Accurate characterization of ENPs is an essential prerequisite to assess the fate and toxicity of ENPs in the environment. The particle size, size distribution and surface charge are often valuable measurements (Zänker and Schierz, 2012). The detection and characterization of ENPs in the aquatic environment is complicated by two issues: (1) ENPs normally enter into the environment at low concentrations that range from ng L^{-1} to the low µg L^{-1} (Boxall et al., 2007) and (2) the concentration of NNMs in the environment is much higher than that of ENPs. These NNMs arise from various biological and abiotic processes in the environment, and some of them have similar sizes, shapes, and in some cases composition, to the ENPs (Hochella et al., 2008). The accurate determination and characterization of ENPs therefore have to overcome the hurdles of ENPs low concentration and minimize the confounding caused the NNMs. Sample enrichment methods that concentrate the ENPs, and pre-fractionation methods that separate ENPs from natural background materials must still be improved in order to facilitate the detection and characterization of ENPs. There are some reviews on the analyses of ENPs and ENP characterization methodologies (Liu et al., 2012; Lopez-Serrano et al., 2014; Schaumann et al., 2015). Table 1 summaries current methods that are available for the analyses of ENPs in environmental samples.

Transmission electron microscopy (TEM) and scanning electron microscopy (SEM) are high-resolution microscopic methods. They enable the identification of size, morphology of single NP, and are successfully used to determine the aggregation, dispersion of NPs. However, both of these approaches require sample pre-treatment e.g. drying, whereas drying of a sample may induce unexpected aggregation processes and change the morphology of the ENPs. Supercritical drying and its advanced modifications are the commonly used drying methods that reduce the drying artefacts (Gehrke et al., 2011). TEM and SEM can be performed in conjunction with elemental analyses such as X-ray energy dispersive spectroscopy (EDS), which can provide particle-specific information about the elemental composition.

Spectroscopic methods such as fluorescence spectroscopy, Surface enhanced Raman spectroscopy and UV-visible spectroscopy (UV-vis) provide information on the ENP surface chemistry. Fluorescence spectroscopy has promising potential for the investigation of fluorescent ENPs and ENP interaction with fluorescent NOM. Two-dimensional Fourier transformation infrared correlation spectroscopy assisted by the fluorescence excitation-emission matrix method have been successfully used to investigate the interaction mechanism of humic acid with TiO_2 NPs at a molecular level (Chen et al., 2014).

Inductively coupled plasma-mass spectrometry (ICP-MS) and inductively coupled plasma-optic emission (ICP-OES) can be used to determine the total elemental concentration in a sample, with a detection limit in the range of ng L^{-1}. They are thus attractive for environmental samples and were coupled to fractionation techniques such as field flow fractionation (FFF) or hydrodynamic chromatography (HDC) (Kim et al., 2013; Rakcheev et al., 2013). Single particle ICP-MS is more suitable for detection and quantification of ENPs at very low concentrations (Gondikas et al., 2014). It is possible to characterize ENPs in hetero-aggregates in complex media and at low concentrations by coupling single particle ICP-MS with HDC (Rakcheev et al., 2013). The number concentration, size and elemental composition of ENPs can be determined in the range of expected environmental concentrations by using these methods.

Table 1. Methods commonly used for the characterization and separation of ENPs. Collected from Liu et al., 2012; Lopez-Serrano et al., 2014 and Schaumann et al., 2015.

	Characterization methods		**Information provided**
Microscopic methods	AFM	Atomic force microscopy	Size, morphology, surface texture, electrical and mechanical properties
	ESEM	Environmental scanning electron microscopy	Surface, size, morphology, elemental composition
	TEM	Transmission electron microscopy	Size, morphology, elemental composition
Light scattering	DLS	Dynamic light scattering	Particle size, size distribution
Spectroscopic methods	EDX	Energy dispersive X-ray spectroscopy	Surface chemistry
	-	Fluorescence spectroscopy	Surface chemistry
	LC-MS/MS	Liquid chromatography-tandem mass spectrometry	Elemental composition, mass
	SERS	Surface enhanced Raman spectroscopy	Surface chemistry
	UV-vis	UV-visible spectroscopy	Size, aggregation, structure, surface chemistry
Plasma methods	ICP-MS	Inductively coupled plasma-mass spectrometry	Elemental composition, mass
	ICP-OES	Inductively coupled plasma-optic emission spectrometry	Elemental composition, mass
	EDS	X-ray energy dispersive spectroscopy	Elemental composition
	Fractionation methods		**Principle of separation**
Filtrationmethods	FFF	Field flow fractionation	Interaction with an external and perpendicular field
Chromatographic methods	HDC	Hydrodynamic chromatography	Routes formed by open capillaries packed with non-porous materials
	SEC	Size exclusion chromatography	Interaction with the stationary phase

1.4 Ecotoxicology of TiO_2 nanoparticles in the aquatic environment

1.4.1 Ecotoxicology test principles

Aquatic ecotoxicology is the study of the toxic effects of introducing and exposing aquatic organisms to un-naturally occurring chemicals (contamination) or to excessive quantities of naturally occurring chemicals (pollution). Toxic effects include lethality (mortality) and sub-lethal effects such as changes in growth, development, reproduction and behaviour. Such toxic effects are often correlated to a dose effect of the introduced chemical. Therefore, aquatic ecotoxicological tests are usually carried out by exposing an organism to a series of concentrations of a sample that contains the substances or chemicals. Observations of the organism's response (such as growth, mortality and reproduction) are made at specific time intervals, and are compared to an unexposed set-up (control). This allows for the construction of a dose-response curve graph and calculation of effective concentration. A dose-response curve can show a number of categories of concentrations of a chemical that indicate the effect of that chemical including: the 'no observed effective concentration', the 'lowest observed effective concentration', and the 'effective concentration'. The no observed effective concentration is the highest concentration at which no adverse effect observed in the test, whereas the lowest observed effective concentration is the lowest tested concentration of the chemical that causes an adverse effect. The concentration at which 50% of the exposed test organisms have been affected is usually used as a measure of the toxicity of the chemical. The median lethal concentration (LC_{50}) is used to determine the lethal effect i.e. the concentration at which 50% of the test organism dies, and median effective concentration (EC_{50}) is used for other effects including: behavioural or physiological effects. In ecotoxicological tests, positive controls are often used to ascertain the validity of the particular test occasion. A positive result from the positive control will ensure the applicability of the test.

Toxic effects are often studied on the laboratory-scale by acute or chronic exposure to chemicals. Different endpoints can be used for the quantification of toxic effects. Acute toxicity tests are short-term exposure tests and generally use lethality as an endpoint. If a chemical does not have adverse effects on aquatic organisms in acute toxicity tests, it does not necessarily indicate that it is not toxic to these species. Chronic toxicity tests enable the evaluation of the possible adverse effects of the chemical under long term exposure. Endpoints for these tests could either be lethal or sub-lethal. Sub-lethal effects include behavioural changes (e.g., swimming and prey-predator relationships), physiological changes (e.g., growth, reproduction and development), biochemical changes (e.g., blood enzyme and ion levels), and histological changes (Rand, 1995). Furthermore, some effects may occur when an organism is simultaneously exposed to two or more chemicals. Such mixture-toxic effects include additive effects, synergistic effects, potentiation effects and antagonistic effects. In addition, bioaccumulation a chemical with low solubility in water is of major environmental concern. Bioaccumulation occurs when there is an increase in the concentration of a chemical in a biological organism over time, compared to the chemical's concentration in the ambient environment. The storage of the chemical within an organism may lead to the possibility of affects being carried along the food chain and exacerbating its general effect in an ecosystem.

There are several groups of organisms have been used for the aquatic toxicology test, and the most commonly used organisms include: bacteria (e.g., *Vibrio fischeri* and *Escherichia coli*), algae (e.g., *Pseudokirchneriella subcapitata* and *Phaeodactylum tricornutum*), duckweed (*L. minor*), rotifers, annelids, molluscs, crustaceans (*D. magna*), echinoderms, and fishes (e.g., *Oncorhynchus mykiss* and *Oryzias latipes*). Different species differ in their susceptibility to chemicals. The selection of the test organisms is therefore important in assessing the toxicity of a chemical. Generally, they are selected on the basis of availability, commercial, recreational, ecological importance, past successful use, regulatory use and bioremedial use (Rand and Petrocelli, 1985). There are different sets standards and guidelines that can be performed on various test species. Some of the more widely accepted agencies that publish guidelines include the Organization for U.S. Environmental Protection Agency, American Society for Testing and Materials, Organization for Economic Co-operation and Development (OECD) and International Organization for Standardization (ISO). Standardized tests allow the comparison of results between laboratories.

The ecotoxicological tests in the context of this study were performed on two organisms from different trophic levels and environments. The *D. magna* organism is a small crustacean that belongs to the zooplankton group of organisms, and zooplankton is generally the food and energy link between algae and fish. Therefore, it is an important model organism in ecotoxicology. *D. magna* is exposed to the test chemicals under defined test conditions. Toxic effects of the test chemical are assessed based on different endpoints, such as immobility in acute toxicity test (24 or 48 hours), and reproduction and survival in chronic toxicity test (3 weeks). In addition to animal species the duckweed species, *L. minor* are also widely used in the aquatic toxicological investigations. *L. minor* plants are one of the main autotrophs in the ecosystems of small lakes and rivers, and are representative of vascular plants. The plants possess physiological properties (small size, relative structural simplicity, rapid growth and vegetative propagation), which make them advantageous for use in laboratory toxicity tests and assessments of freshwater systems. The endpoints examined during the ecotoxicological tests are the number of fronds, frond area, chlorophyll content, and fresh or dry weight.

1.4.2 Ecotoxicity of TiO_2 nanoparticles

There is emerging literature on the ecotoxicity of TiO_2 NPs when exposed to various test organisms that range from bacteria, algae, crustaceans to fish and higher plants. Minetto et al. (2014) reviewed a large number of articles (n=129), about the ecotoxicity effects of TiO_2 NPs toward aquatic organisms. Among these publications, 74% of the toxicity studies were carried out using freshwater species, of which crustaceans, bacteria, fishes and algae are the most studied organisms. Table 2 gives some examples of the toxicity tests for TiO_2 NPs.

The toxicity mechanisms of TiO_2 NPs have been elucidated to some extent. TiO_2 NPs were shown to cause the production of reactive oxygen species, which led to cell membrane damage and protein oxidation (Cherchi and Gu, 2010; Neal, 2008). In addition, oxidative stress was the main reason for adverse effects found in fish exposed to TiO_2 NPs (Federici et al., 2007; Xiong et al., 2011). Federici et al. (2007) reported that TiO_2 NPs caused respiratory distress and disturbed the metabolism of some trace elements in rainbow trout. The whole-body concentrations were much higher than concentrations in the internal organs, which suggested particle attachment on the outer surface of the fish. Huang et al. (2005) also showed that algae *Pseudokirchneriella subcapitata* adsorbed TiO_2 NPs on the cell surface. According to Aruoja et al. (2009)

large TiO_2 aggregates could entrap algal cells, which may contribute to the inhibition of algae growth. Furthermore, *D. magna* accumulated a significant amount of TiO_2 NPs from the test environment and showed abnormal food intake, which ultimately affected growth and reproduction (Zhu et al., 2010).

Table 2. Review of toxicity studies on TiO_2 nanoparticles and the toxic effects on organisms.

Group of organisms	**Species**	**Materials**	**Toxic effects**	**References**
Bacteria	*Escherichia coli*	Synthesized rutile TiO_2; P25 TiO_2 (21 nm; 81% anatase, 19% rutile)	30% reduction in the growth by 10 mg L^{-1} TiO_2; anatase TiO_2 NPs are more toxic than rutile NPs by inducing greater oxidative stress; the toxicity was found to be lower at pH 5 than at pH 8.	Planchon et al., 2013
		Anatase TiO_2 of 10, 25 and 50 nm; rutile TiO_2 of 50 nm; mixed anatase and rutile TiO_2 of 25 nm	LC_{50} increasing with lower particle size; Rutile TiO_2 NPs almost non-toxic.	Lin et al., 2014
Alage	*Pseudokirchneriella subcapitata*	25−70 nm	EC_{50} = 5.83 mg Ti L^{-1}; large TiO_2 aggregates could entrap algal cells, which may contribute to the inhibition of algae growth.	Aruoja et al., 2009
	Phaeodactylum tricornutum	Anatase TiO_2 at 15, 25 and 32 nm	Anatase is more toxic than the rutile form; EC_{50} is 10.91, 11.30 and 14.3 mg L^{-1} respectively for TiO_2 NPs of 15, 25 and 32 nm; cell growth stimulation at very low con.	Clément et al., 2013
Crustaceans	*Daphnia magna*	P25 TiO_2 (~70% anatase and ~30% rutile) and A-100 TiO_2 (99% anatase) of primary particle size of 55, 100 and 140 nm	Smaller initial particle sizes (i.e. 55 nm) and anatase form particles showed an approximately 90% lower EC_{50} value compared to the larger ones.	Seitz et al., 2014
		P25 TiO_2 (21 nm; 81% anatase, 19% rutile)	$LC_{50} = 118$ mg L^{-1}.	Li et al., 2014
Fishes	*Oryzias latipes*	P25 TiO_2 (21 nm; 81% anatase, 19% rutile)	$LC_{50} > 500$ mg L^{-1} under dark conditions; $LC_{50} = 8.5$ mg L^{-1} under simulated solar radiation.	Li et al., 2014
	Rainbow trout (*Oncorhynchus mykiss*)	P25 TiO_2, 75% rutile and 25% anatase, 21 nm	Respiratory toxicity; Oxidative stress occurred. Pathologies are reported in the gills and other internal organs.	Federici et al., 2007
	Zebrafish	Anatase TiO_2 of 30 nm	96-h $LC_{50} = 124.5$ mg L^{-1}; OH• generated by TiO_2 NPs may result in oxidative stress and damage to the cell membrane(s).	Xiong et al., 2011

The ecotoxicity of ENPs is closely related to their physicochemical properties, e.g., size, shape, surface area and surface chemistry. Large discrepancies in the toxicity of TiO_2 NPs in the same organism are reported, and this variation depended on the different physicochemical properties of TiO_2 NPs. A size-dependency of inhibition on algal growth rate caused by TiO_2 NPs has been observed by Aruoja et al. (2009). Smaller initial particle sizes (55 nm) and anatase structural form of TiO_2 NPs showed an approximately 90% lower EC_{50} value compared to the larger forms (100 nm and 140 nm) (Seitz et al., 2014). Moreover, the TiO_2 NPs in anatase crystal structure are more toxic than rutile form for *cladocerans*, algae, rotifers and plants (Clément et al., 2013).

In addition, the physicochemical properties of the exposure conditions can also influence the toxicity via altering the fate and behaviour of ENPs. For example, the EC_{50} values of TiO_2 NPs on green algae *P. subcapitata* vary widely, in the range of $2-70$ mg L^{-1} , for the same TiO_2 NPs (P25) (Nur et al., 2015). There are several broad reasons that have been attributed to this variation: differences in the exposure protocols, differences in the ENPs suspension preparation methods, and differences in the ENPs behaviour in the test media. Depending on the colloidal stability of TiO_2 NPs dispersions in toxicity testing, large different or even contradictory TiO_2 NPs toxicity were presented (Cupi et al., 2016).

Regardless of the direct biological effects of ENPs themselves, they often co-exist with other pollutants in natural environments. The highly reactive nature of ENPs enables one ENP to influence the speciation and bioavailability of the other polluting chemicals and *vice versa* (Sun et al., 2007). Moreover, the large surface areas of ENPs gives ENPs the potential to carry other pollutants (Dalai et al., 2014; Rosenfeldt et al., 2014; Tian et al., 2014), and subsequently alter the bioavailability of co-pollutants. For instance, Rosenfeldt et al. (2014) have found that the body burden of copper (Cu) encountered by *D. magna* was significantly decreased by the presence of TiO_2 NPs. In another study, the presence of TiO_2 NPs significantly increased the accumulation of cadmium (Cd) by 146% in carp after 25 days of exposure (Zhang et al., 2007). Therefore, mixture toxicity should be taken into consideration when evaluating the aquatic toxicity of ENPs.

1.4.3 Challenges in the evaluation of ecotoxicity

Most standard and guideline test protocols by organizations such as the OECD and the ISO have shown some shortcomings in the testing of ENPs, because ENPs were originally developed for the soluble chemicals. The ‘OECD Working Party for

Manufactured Nanomaterials' has therefore been established to address these shortcomings and thus a major function is to improve test guidelines for ENPs (OECD, 2010). Several projects and numerous studies on the Series of Safety of Manufactured Nanomaterials have been published, including a guidance document on testing the ecotoxicity and environmental fate of manufactured nanomaterials (OECD, 2014).

Studying the potential ecotoxicological risks of TiO_2 NPs has numerous challenges that primarily arise from the differences in the fate and behaviour of ENPs that have been released into the environment. The problem is much more complex in aquatic environments, because ENPs will interact with the ambient environment and undergo transformation processes that may have significant impacts on their mobility, bioavailability and toxicity. Therefore, in toxicological studies, a certain set of potentially important properties including: size, shape, chemical composition and surface chemistry, must be addressed or be measured in the toxicity tests. In addition, the water exposure conditions (such as pH and IS) should also be considered as they may also affect these processes.

2. OBJECTIVES OF THE STUDY

The general objective of this thesis was to investigate the fate and biological effects of TiO_2 NPs in aquatic environments. The more specific aims of the thesis were:

- to investigate the relationship between water properties and the TiO_2 NPs behaviour in complex natural waters with experimental and statistical tools (Paper I)
- to analyse the colloidal stability of TiO_2 NPs in two ecotoxicological test media (Paper II and III)
- to assess the toxicity of TiO_2 NPs with maintained colloidal stability on two aquatic organisms: *L. minor* and *D. magna* (Paper II and III)
- to investigate the mixture toxicity of TiO_2 NPs and Cd and lead (Pb) on *D. magna* (Paper III).

3. MATERIALS AND METHODS

3.1 Preparation of TiO_2 suspension

The titanium dioxide nanoparticles (TiO_2-P25) were obtained from Evonik industries AG (Essen, Germany). The information provided by the manufacturer stated that TiO_2-P25 is a mixture of anatase (81%) and rutile (19%) forms with a nominal primary diameter of 21 nm, and a specific surface area of 50 ± 15 m^2 g^{-1}. A stock suspension of 1 g L^{-1} was prepared by dispersing TiO_2-P25 powder into deionized water followed by a 30-min sonication in an ultrasonic bath (Ultrasonic Cleaners, Bronson, USA). The stock suspension was stored in the dark at 4ºC and sonicated for another 30 min prior to further dilution and exposure experiments. The average aggregate size of the TiO_2 NPs in stock suspension was 215 ± 21.5 nm. The measured pH_{pzc} was approximately 6.1. The zeta potential of the TiO_2 NPs in the stock suspension was 22.5 ± 0.9 mV at a pH of 5.5.

3.2 Sampling and analyses of natural waters

A total of 39 samples: 34 lake water samples from 24 lakes and 5 brackish water samples in the coastal area of Uusimaa were taken along with those for the national monitoring programme (Figure 2 and Table 3). All the samples were collected to a depth of 1 meter below the water surface during summer and autumn, 2013. The samples were stored in the dark at 4ºC. The analyses of the water properties were carried out at the SYKE Environmental Chemistry Research group. Lake waters were analysed for the following properties: alkalinity, colour, chemical oxygen demand (COD_{Mn}), electrical conductivity (EC), turbidity, pH, Fe, Mn, NH_4, NO_x (NO_2^- and NO_3^-, nitrogen from both nitrite and nitrate), total nitrogen, dissolved oxygen, and total phosphorus. Brackish waters were analysed for the following properties: NH_4, NO_x, total nitrogen, pH, dissolved oxygen, total phosphorus, salinity and turbidity. Alkalinity, COD_{Mn}, EC, DO, and pH were measured by a Titrator and Robotic sample processor; colour was measured by a Comparator; Fe and Mn were extracted by microwave digestion and then measured by an ICP-OES analyser; total nitrogen, NH_4, NO_x and total phosphorus were measured by continuous flow analyser; salinity was measured by Salinometer; turbidity was measured by Turbidimeter.

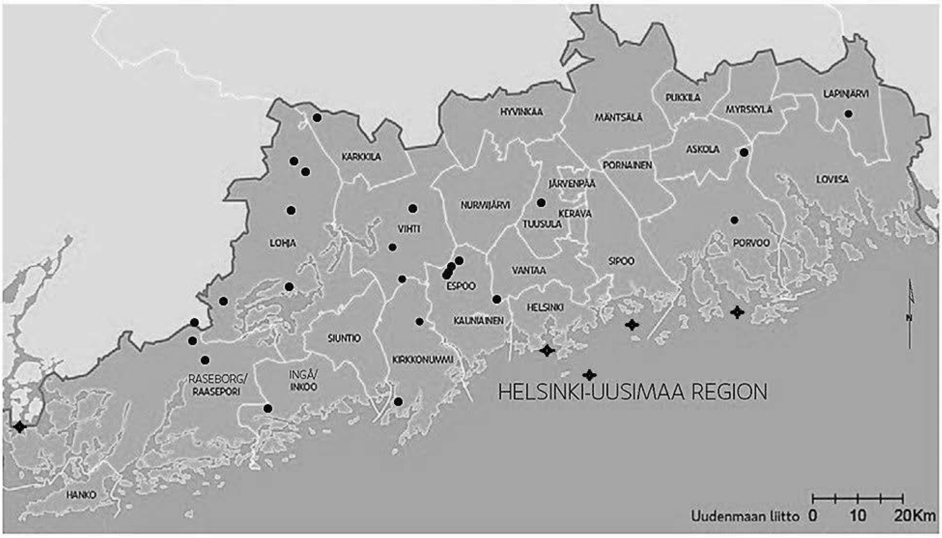

Figure 2. Map of the studied sites of water samples in the coastal area of Uusimaa on the south coast of Finland. The sampling sites of lake are marked with dots and brackish waters are marked with stars. Map was obtained from http://www.uudenmaanliitto.fi/en/helsinki-uusimaa_region/helsinki-uusimaa_region_facts.

3.3 Preparation of ecotoxicological test media

The ecotoxicological test media used in this study include: Steinberg medium used in freshwater *L. minor* growth inhibition test (ISO 20079, 2005), and Elendt M7 medium used in *Daphnia sp.* acute immobilisation test (OECD 2004). A series of dilutions of the media were prepared as followed: Steinberg growth medium was diluted in volume ratios of 1:0 (undiluted), 1:1 and 1:9 with deionized water whereas the corresponding ratios for the Elendt M7 medium were1:0 (undiluted), 1:9 and 1:19.

Table 3. Sampling information on natural waters.

Nutrient-rich lake		**Humus-poor lake**		**Humic lake**		**Brackish water**	
Waters	**Date**	**Waters**	**Date**	**Waters**	**Date**	**Waters**	**Date**
Palojärvi	4.7	Simijärvi	30.5	Niittylampi	15.7	Eekholmanselkä	15.8
Kilpijärvi	8.8	Simijärvi	21.8	Saarijärvi	17.7	Längden	3.9
Lapinkylänjärvi	9.7	Degersjön	18.6	Rausjärvi	22.7	Sipoonselkä	30.9
Enäjärvi	17.6	Ruuhijärvi	31.7	Onkimaanjärvi	22.7	Porvoo	26.8
Enäjärvi	24.7	Kattilajärvi	9.7	Onkimaanjärvi	1.10	Länsi-Tonttu	23.9
Enäjärvi	24.9	Kattilajärvi	31.10				
Veckjärvi	2.9	Orajärvi	31.7				
Lapinjärvi	2.9	Puujärvi	22.8				
Pitkäjärvi	11.7	Orijärvi	29.8				
Pitkäjärvi	25.9	Orijärvi	16.10				
Siippo	8.10	Vahermanjärvi	1.10				
Tuusulanjärvi	29.7	Vitsjön	21.10				
Tuusulanjärvi	12.8	Hormajärvi	6.11				
Tuusulanjärvi	16.9	Marsjön	18.6				
Tuusulanjärvi	14.10						

3.4 Ecotoxicological tests

3.4.1 The *Lemna minor* growth inhibition test

The cultivation and exposure tests were carried out by following the modified standard of duckweed growth inhibition test (ISO 20079): temperature 20ºC (24 ± 2ºC in standard), light intensity 67.5 µE m^{-2} s^{-1} (85−135 µE m^{-2} s^{-1} in standard), and light cycle of 16 h light/ 8 h dark (continuous lighting in standard). A 10-fold diluted Steinberg growth medium was used for the exposure test because TiO_2 NPs showed the highest colloidal stability in this medium as shown in 4.2. The pre-cultivation and the exposure tests were all performed in a Versatile Environmental Test Chamber (MLR-350H, Sanyo, Japan). The *L. minor* was exposed to a concentration range from 0.01 to 5 mg L^{-1} of TiO_2 NPs and one control. The nutrient solution was renewed every 48 or 72 hours during the test period. New test suspensions were prepared from the freshly sonicated TiO_2 NPs stock suspension. Plants were then moved from the old vessels to the new vessels. The test medium was gently stirred with a magnetic stirrer throughout the test period.

3.4.2 The *Daphnia magna* acute immobilisation test

The toxicity test was carried out according to OECD guideline 202 (2004) with some modifications. Ethylenediaminetetraacetic acid (EDTA) is a chelator that can strongly bind with various metal ions and results in a reduction on metal ion concentration in solution (Sorvari and Sillanpää, 1996; Tan et al., 2012). Therefore, Elendt M7 medium that excluded EDTA was used as the cultivation solution in this study. A 20-fold diluted Elendt M7 medium without EDTA was used for the exposure test because TiO_2 NPs showed the highest colloidal stability in this medium. TiO_2 NPs were spiked into the test medium just before the toxicity test was started. Each test involved six test concentrations and one control group and each was carried out with four replicates. For each replicate, five *D. magna* neonates with an age of less than 24 h were transferred into a 25-mL glass test vessel containing 10 mL test solution. The number of immobile organisms was counted after 24-h exposure. The dose-effect relationship was calculated by using Microsoft Excel 2003. From this the EC_{50} related to the mobility of the *D. magna* after exposure was calculated as a final result.

3.4.3 Bioaccumulation and toxicity of Cd and Pb in *Daphnia magna*

The toxicity and bioaccumulations of Cd and Pb were determined both in the absence and presence of 2 mg L^{-1} TiO_2 NPs. Cadmium chloride ($CdCl_2$, 99.99%) was purchased from Acros Organics (Geel, Belgium). Lead (II) nitrate ($Pb(NO_3)_2$, 99.999%) was purchased from Merck KGaA (Darmstadt, Germany). Cd stock solution (100 mg L^{-1}) and Pb stock solution (100 mg L^{-1}) was prepared by dissolving $CdCl_2$ and $Pb(NO_3)_2$ in deionized water. The test concentration of Cd and Pb ranged from 66−200 μg L^{-1} and 98−300 μg L^{-1}, respectively. All the dilutions were prepared by diluting the stock solution using a 20-fold diluted Elendt M7 medium without EDTA. Each test involved six test concentrations and one control group each with four replicates.

3.5 Measurement techniques and sample preparation

3.5.1 Dynamic light scattering

The size and size distribution of TiO_2 NPs were determined by using an instrument based on dynamic light scattering (DLS), (Malvern Zetasizer Nano ZS, Malvern Instruments Inc, UK). A typical DLS system consists of six main components: laser source, light attenuator, sample cell, detector, digital processing board called a correlator and computer with the appropriate software. The small particles in suspension undergo Brownian motion. When a laser beam is shone through a liquid with suspended particles, the light scattered by the particles is collected by a detector using a suitable optical arrangement. The intensity of the scattered light fluctuates at a rate which relates to the size of particles. Smaller particles move more quickly than larger particles, thus the intensity of the scattered light by small particles also fluctuates more rapidly than those of the large particles. After the correlator analysis of the fluctuations in light intensity, it is possible to get information about the velocity of the Brownian motion that can be converted into hydrodynamic diameter (HDD) by using the Stokes-Einstein equation (Einstein, 1956) thus:

$$HDD = \frac{k_B \cdot T}{3 \cdot \pi \cdot \eta \cdot D} \tag{4}$$

Where k_B is the Boltzmann's constant, T is the absolute temperature, η is the viscosity of the liquid, and D is the translational diffusion coefficient of the spherical colloidal particles.

The HDD of TiO_2 NPs spiked in different natural waters was determined by using DLS over a 3-h period. The instrument was operated with a He-Ne laser light at a wavelength of 633 nm, and light scattering was detected at an angle of 173° at 25°C. DLS measurement was supposed to be interfered by the suspended particles present in natural waters. However, preliminary experiments showed that the 10 mg L^{-1} TiO_2 NPs spiked into natural waters accounted for 99.8% of signal intensity. This strongly suggests that the TiO_2 particles and their homogenic and heterogenic aggregates are the principal fraction in the test systems. Therefore, the measured sizes after spiking of TiO_2 in natural waters were regarded as those of TiO_2 NPs.

The increase of particle size indicates the formation of the aggregates. The aggregation rate (k_{agg}) was calculated according to Equation (5):

$$k_{agg} = \frac{\Delta \mathrm{HDD}}{\Delta \mathrm{t}} \tag{5}$$

Where *HDD* is the measured hydrodynamic diameter, and *t* is the aggregation time.

3.5.2 Laser Doppler velocimetry

The surface charge of TiO_2 NPs was measured using the same Malvern Zetasizer Nano ZS instrument that utilizes a combination of LDV and phase analysis light scattering (M3-PALS). An electric field was applied to a dispersion of particles, which then move with a velocity related to their zeta potential. This velocity was measured using the M3-PALS. This enabled the calculation of electrophoretic mobility, and from this, the zeta potential and the distribution of the zeta potential to be calculated. The zeta potential was obtained by using the Henry equation with the Smoluchowski approximation (Delgado et al., 2005; Hunter, 1988; Smoluchowski, 1921):

$$U_E = \frac{2 \cdot \varepsilon \cdot \zeta \cdot f(ka)}{3 \cdot \eta} \tag{6}$$

Where U_E is the electrophoretic mobility, ε is the dielectric constant of the solvent, ζ is the zeta potential, $f(ka)$ is the Henry's function and η is the viscosity of the solvent. For particles in aqueous solutions of moderate electrolyte concentration, a value of 1.5 was used for Henry's function (the Smoluchowski approximation); for particles in non-polar media, a value of 1.0 is used (the Hückel approximation).

3.5.3 UV-visible spectroscopy

UV-vis spectroscopy (Shimadzu UV-1601 PC, Shimadzu Corporation, Japan) was used to study the deposition kinetics of TiO_2 NPs dispersed in natural waters. The basic principle of the UV-vis method is based on the Beer-Lambert law. Thus, when a beam of monochromatic light is passed through a solution, the absorbance of the light by a substance in the sample solution is proportional to the thickness of the solution and also the concentration of the substance in the entire volume. The absorbance of the TiO_2 NPs suspensions was measured at a wavelength of 378 nm every 5 min over a 3-h period. The test samples were prepared in 20 mm quartz cuvettes. The detection limit of the method was about 1 mg L^{-1}. All experiments were conducted in duplicates.

The deposition rate constant (k_{dep}) was proposed as a parameter to compare the stability of TiO_2 NPs in various natural waters with different water properties, and it was calculated according to Equation (7):

$$k_{dep} = -\frac{ln\frac{A}{A_0}}{t} \tag{7}$$

Where A is the absorbance of tested suspensions at a certain time, A_0 is the absorbance of tested suspensions at the beginning of test, and t is the deposition time.

3.5.4 Inductively coupled plasma-optical emission spectrometer

The elemental analysis in papers II and III was accomplished by using an ICP-OES (Varian Vista PRO Radial, Varian, Australia). The ICP-OES consists of two parts: the ICP and the optical spectrometer. The liquid samples are introduced into the plasma, which desolvates the solvent and ionizes and excites the solute elements. The excited atom emits a unique radiation signature when it returns to the ground state. The emitted characteristic radiations and intensities are measured as optical spectra by a detector. Each element has its own characteristic emission spectrum, and ICP-OES therefore could use the emission spectra to identify and quantify the elements present.

The samples were prepared for ICP-OES analysis of the *L. minor* growth inhibition test as follows: (1) Medium samples were collected at the beginning of the test, on the day of the medium change and at the end of the test (one combined sample per concentration and one sample from the control). After removing the plant, the test medium was allowed to stand without mixing for about 1 min, and then a 9 mL volume was pipetted from the middle of the test medium into a test tube containing 1 mL of concentrated sulphuric acid. Recovery of Ti analyzed by ICP-OES was 82 ± 5% when calculated from the nominal TiO_2 NP concentrations added to the medium and incubated in the presence of the plants under the test conditions for 2 days. (2) The burden of TiO_2 in the *L. minor* plant was obtained from the plants in each test vessel at the end of exposure test. The plants were collected and rinsed for 1 min in deionized water. They were then dried by gently dabbing them with soft paper towels to remove excess water and weighed to obtain their fresh weight. The plant samples were then heated in 2 mL of pure nitric acid (67−69%, Romil Ltd., Cambridge, UK) at 60°C for 30 min and then 2 mL of hydrogen peroxide (30%, Merck KGaA, Darmstadt, Germany) was added and the mixture was further heated to 90°C for 60 min. The whole process was repeated until all the plant material was completely digested. The solution containing plant tissues was evaporated until almost dry, which was followed by the addition of 2 mL of sulphuric acid (concentrated acid diluted 1:1 with deionized water) and metal-free water to a final volume of 10 mL. Reagent blanks were prepared similarly but without the plants.

In the *D. magna* acute immobilisation test, the samples were prepared for ICP-OES analysis as follows: (1) Medium samples were collected at the end of the test (one sample per concentration and one sample from the control). After removing the daphnia, the test medium was allowed to stand without mixing for 1 hour, and then a 4 ml volume was pipetted from the middle of the test medium into a test tube containing 1 mL of sulphuric acid diluted 1:1 with deionized water. (2) The daphnia from each exposure concentration were combined and rinsed with a 20-fold diluted Elendt M7 medium without EDTA and this was repeated one more time. The daphnia samples were then transferred into small basin and dried at room temperature to remove excess water. After drying, concentrated nitric acid (HNO_3, 0.25 mL) and water (0.75 mL) were added to the reaction vessel containing the daphnia, which were subsequently subjected to a microwave digestion at 240ºC and 45 bar for 1 hour (Milestone Ultrawave MA149-003, Milestone srl, Italy). After digestion, 50 µL of Rhodium (1 mg L^{-1}) was added as an internal standard, and the resulting solution was diluted to a total volume of 5 mL with deionized water.

3.5.5 The LemnaTec Scananalyzer

A LemnaTec Scanalyzer (LemnaTec GmbH, Wuerselen, Germany) was used to carry out the *L. minor* image acquisition. The instrument has a robotic arm that holds a camera (Sony DFW-SX900). The distance from the camera is fixed and calibrated to measure the actual surface area of the leaves in mm^2. Calibration was done for the vessels containing 100 ml test solution. Information on parameters such as frond number and frond area could be extracted with the Duckweed software from the high-resolution images. Growth rate per day (r) was then calculated using the following equation:

$$r = \frac{\ln x_{t_1} - \ln x_{t_0}}{t_1 - t_0} \tag{8}$$

Where x is the frond number or frond area at the measured time point (t_1) and at the start of the test (t_0).

3.5.6 Spectrophotometer

A spectrophotometer (Hitachi 2000, Tokyo, Japan) was used for the determination of Chlorophyll *a* concentration. The fronds were removed from the exposure vessels at the end of the test and rinsed in pure water. The fronds were dried by gently dabbing them with soft paper and then they put into pre-weighed test tubes that had been placed in ice. The fresh weight was recorded and 6 mL of methanol was added to the tubes. The tubes were stored at -20°C overnight before homogenization. The plant tissue was homogenized manually with a glass homogenizer and the absorbance of the clear extract was measured using the spectrophotometer set at 666 and 653 nm wavelengths. The concentration of chlorophyll *a* (mg L^{-1}) was calculated from the equation (Wellburn, 1994):

$$Chl - a(mg \cdot L^{-1}) = 15.65 \cdot Abs_{666nm} - 7.34 \cdot Abs_{653nm} \tag{9}$$

Where *Chl-a* $(mg\ L^{-1})$ is the chlorophyll content expressed as mg Chl-*a* per gram plant fresh weight, Abs_{666nm} and Abs_{653nm} are the absorbance at 666 nm and 653 nm, respectively.

3.5.7 Scanning and transmission electron microscopy

A field emission scanning electron microscope (FESEM; JSM-6335F, Jeol, Japan) was used for the imaging of *L. minor* after its exposure to TiO_2 NPs. In SEM, a beam of electrons is generated by a field emission gun and accelerated by the anode. The beam travels through electromagnetic lenses to produce a thin beam of electrons. Then the electron beam scans the surface of the sample by means of scan coils. When the beam strikes the sample, electrons and x-rays are emitted from the sample, and are collected by a suitably-positioned detector. The detector converts these electrons into a signal that reveals information about the sample including surface topography and composition. The samples were fixed with glutaraldehyde and osmium tetroxide for the SEM imaging. After washing and dehydration the samples were critical point dried, mounted on a metal base and sputter coated with chromium. The SEM was operated at 15 kV.

The selected post-exposure samples of the plant leaves and roots of *L. minor* were also imaged by a TEM (JEM-1200EX, Jeol, Japan). TEM also uses an electron beam, but instead of scanning the sample surface, the beam passes through the sample and interacts with the sample. An image is formed from the electron-sample interactions and provides information on the element and compound structure. The samples were prefixed in a phosphate buffer at pH 7.2 in a 2.5% glutaraldehyde matrix. The fixed plant tissues were washed three times with the phosphate buffer. All samples were post-fixed with phosphate-buffered 1% osmium tetroxide matrix and dehydrated in an acetone series and then embedded in Taab resin. The thin-sectioned tissues were post-stained with uranyl acetate and lead citrate and then examined with the TEM at 60 kV.

3.6 Data analysis

The association between TiO_2 NPs stability behaviour and lake water properties were studied by linear regression analysis. The statistical analysis was performed using SPSS version 22.0 software (IBM Corp.) software. Stability parameters, i.e. zeta potential, initial hydrodynamic diameter (HDD_i), aggregation rate, and deposition rate constant, of suspended TiO_2 NPs in lake waters were used as the dependent variables, whereas the 14 water properties were taken as independent variables. The statistical analysis was performed only for the lake waters as there were sufficient numbers of samples.

In *D. magna* acute immobilisation test, after gentile stirring, the number of daphnia immobilised was recorded after 24 and 48 h of exposure. The percentages immobilised are plotted against test concentrations, and the EC_{50} values were calculated from the regression curves.

4. RESULTS AND DISCUSSIONS

4.1 Behaviour and characteristics of TiO_2 in natural waters

The behaviour of TiO_2 NPs was investigated in three types of lake waters and also coastal brackish waters. There was a large range in colour (15−500 mg Pt L^{-1}) and turbidity (0.5−76 FNU) of the lake waters, but the turbidity of the brackish waters varied only between 0.5−2 FNU. The brackish waters were all basic (pH >7.9), whereas lake waters ranged from acidic to basic (pH 5.8−8.7). In addition to the highest concentration of total nitrogen and total phosphorus, nutrient-rich lakes had the highest value of EC, alkalinity, pH and turbidity, whereas humic lakes had the highest value of colour and COD, but the lowest value of EC, alkalinity and pH. Humus-poor lakes had the lowest concentration of Fe, Mn, and lowest value of colour, COD and turbidity. These water properties reflect different water physicochemical characteristics. The alkalinity is the capacity of aqueous solution to neutralize acid. The EC is a quantitative value for the solutions ability to conduct current and it is related to ionic strength, temperature, and the mobility of ions. The colour indicates the amounts of dissolved colouring compounds in water, including natural metallic ions (Fe and Mn), coloured dissolved organic matter, plankton, weeds, and industrial wastes. The COD_{Mn} value refers to the amounts of organic matter that can be chemically oxidized, and it is more suitable to indicate the amount of NOM than water colour. Turbidity indicates the amounts of the suspended particles, i.e., organic matter and inorganic colloids. Water properties such as pH, IS and NOM are supposed to influence the TiO_2 NPs behaviour. The colloidal stability of TiO_2 NPs in different water samples and its relationship with water properties will be presented and discussed in the following text.

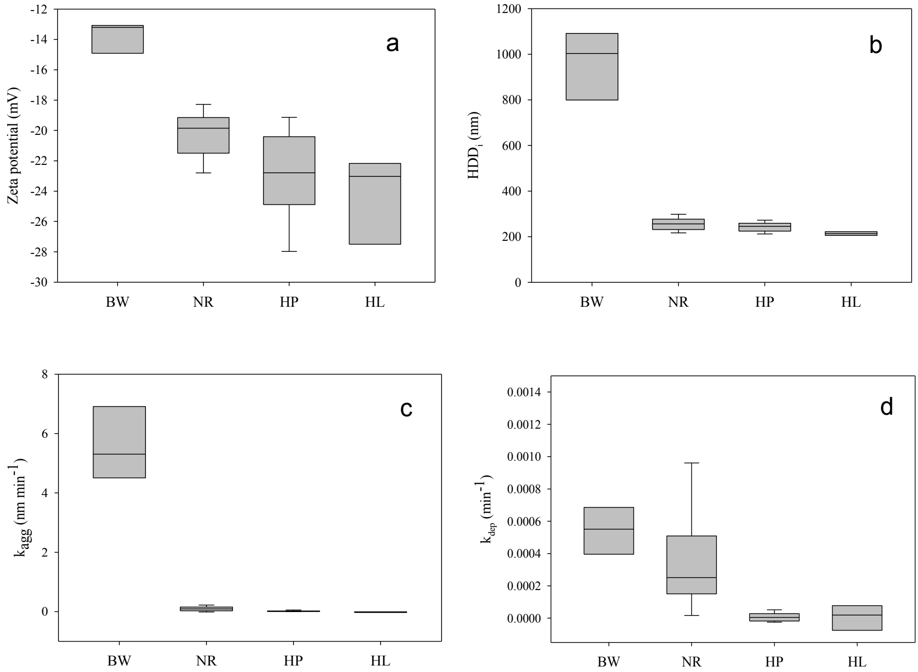

Figure 3. (a) Zeta potential, (b) initial hydrodynamic diameter (HDD$_i$), (c) aggregation rate, and (d) deposition rate constant of engineered TiO_2 nanoparticles in different natural water types at a concentration of 10 mg L^{-1}. BW is brackish water; NR is nutrient-rich lake; HP is humus-poor lake; HL is humic lake. The boxes indicate the 5th and 95th percentiles, the whiskers indicate the minimun and maxium values, and the central lines indicate the medians (from Paper I).

Surface ionization controls the surface charge of mineral oxides dispersed in deionized water (Morrison and Ross, 2002). At pH below pH_{pzc}, NPs surface is positively charged due to the presence of $[M–OH_2]^+$ groups (Jolivet et al., 2000). Then an increase in pH leads to deprotonation and the formation of negatively charged $[M–O]^-$ groups on the NPs surface. At pH above pH_{pzc}, the surface of the NP therefore has a negative charge. The zeta potential data (Figure 3a) indicate that the surface charge of TiO_2 NPs changed immediately from positive (22.5 mV) to negative after spiking TiO_2 NPs into all the water samples with a pH range of 5.8−8.7, which indicated pH_{pzc} lower than 5.8 under the studied conditions. This difference is most likely due to the presence of negatively charged NOM and polyvalent anions in natural water. When positively charged TiO_2 NPs were spiked into the natural waters, the adsorption of the negatively charged NOM and/or natural colloids onto the surface of the TiO_2 NPs immediately occur due to the electrostatic attraction on the surface, which results in a rapid charge reverse. Similarly, Adam et al. (2016) also found that the TiO_2 NPs presented negative surface charges in river water over the tested pH values that ranged from 2 to 11. In another study by Oriekhova and Stoll (2016), negatively charged TiO_2 NPs were used as the test NPs. After spiking into lake water and sea water, the surface charge of TiO_2 NPs became less negative compared to that of the stock solution. Thus, cations were more likely to adsorb onto this negatively charged surface instead of the negatively charged NOM, and this reaction decreased the surface charge on the TiO_2 NPs.

TiO_2 NPs exhibited different colloidal stability, which depended on the physicochemical properties of the water samples (Figure 3). In general, TiO_2 NPs suspension was more stable when suspended in lake waters than in brackish waters. Different lake water types could either stabilize or destabilize the TiO_2 NPs: aggregation and deposition probably occurred in nutrient-rich lakes which have high pH, EC, alkalinity, and turbidity values. In contrast, the spiked TiO_2 NPs remained stable in humus-poor and humic lake waters that had low pH, EC, alkalinity and turbidity values.

The linear regression analysis (Table 3, Paper I) revealed that total phosphorus, total nitrogen, alkalinity, pH, EC and turbidity were shown to be the main water properties that influence the colloidal ENPs stability out of 14 water properties tested. All the six of these water physicochemical properties correlated positively with the deposition of TiO_2 NPs in lake waters. A high EC value indicates a high IS, and high alkalinity indicates a high concentration of calcium and magnesium in the water. According to DLVO theory, the Debye length is inversely proportional to the IS (Elimelech, 1992). An increase in IS could therefore compress the EDL, which manifest as a decreased zeta potential. The reduced strength of repulsive forces between particles leads to the aggregation of ENPs when steric hindrances are absent (French et al., 2009; Shih et al., 2012). In addition, at pHs higher than the pH_{pzc}, the presence of divalent cations such as Ca^{2+} could effectively decrease the Debye length and the electrostatic repulsion barrier, which results enhanced aggregation and sedimentation (Lee et al., 2016). Therefore, when the alkalinity and EC increased from humic lake waters to humus-poor lake waters and nutrient-rich lake waters, the respective zeta potential values became less negative from -24.5 mV (humic lake) to -22.9 mV (humus-poor lake) and to -20.2 mV (nutrient-rich lake). Moreover, the brackish waters contained higher concentrations of ions than the lake waters, which resulted in larger aggregations and deposition rates than those found in lake waters.

Although the colloidal stability of TiO_2 NPs was supposed to be highly dependent upon the NOM, no correlation was found between COD_{Mn} and stability parameters. NOM in natural waters is a complex mixture of humic substances, fibrillary polysaccharides and peptidoglycans, all of which have different physicochemical properties and reactivities, which may exert different influences on the stability of ENPs. Moreover, the adsorption of NOM onto ENPs is strongly influenced by water properties such as IS and pH (Mwaanga et al., 2014). Our study suggests that the combined effect of NOM along with other water properties played an important role in the behaviour of TiO_2 NPs in the test waters.

The calculated deposition rate constants of TiO_2 NPs were significantly correlated with turbidity, which implies that the deposition increased with the increase of suspended particles. Turbidity was 10 times higher in nutrient-rich lake waters than that in humic lake waters, whereas the COD_{Mn} values were comparable. This suggested that nutrient-rich lake waters contained a large fraction of natural colloids and large particles in turbidity. Earlier reports have shown the importance of hetero-aggregation of TiO_2 NPs in the presence of clays (Adam et al., 2016; Labille et al., 2015). The affinity of TiO_2 NPs for clay was driven by electrostatic interactions but only at higher IS values as TiO_2 NPs was shown to hetero-aggregate with clay. The relatively low IS values in the lake waters studied indicated that TiO_2 NPs would not be expected to hetero-aggregate with natural colloids in lake waters, and this explained why the aggregation rates in these lakes were not correlated with turbidity. In addition to natural colloids, TiO_2 NPs could attach to large particles and microorganisms present in the water, which would decrease the suspended TiO_2 mass concentration (Campos et al., 2013; Quik et al., 2014). Total nitrogen and total phosphorus were also important as high nitrogen and phosphorus concentrations are usually associated with high IS and high amounts of suspended particles (Yap et al., 1987), which result in the destabilization of NPs.

4.2 Behaviour and characteristics of TiO_2 in ecotoxicological test media

As discussed in section 4.1, the surface charge of ENPs is mainly affected by the ionization and ion adsorption in ecotoxicological test media, in which various electrolytes are present. multivalent anions such as PO_4^{3-} may be adsorbed by the positively charged $[M–OH_2]^+$ groups in the low pH of Steinberg medium (pH = 5.5 ± 0.2), which would lead to the neutralization of the ENPs surface charge (-0.04 ± 0.1 mV, Figure 4). Although a zeta potential value close to zero indicated unstable suspensions, large aggregates were formed at this pH (747 nm, Figure 5a). In comparison, the NPs surfaces were negative and had a zeta potential value -15.0 ± 2.2mV for Elendt M7 medium (Figure 4). This finding can be explained by the Elendt M7 medium having a high pH (7.8 ± 0.5) i.e. two pH units above the pH_{pzc} of TiO_2 NPs.

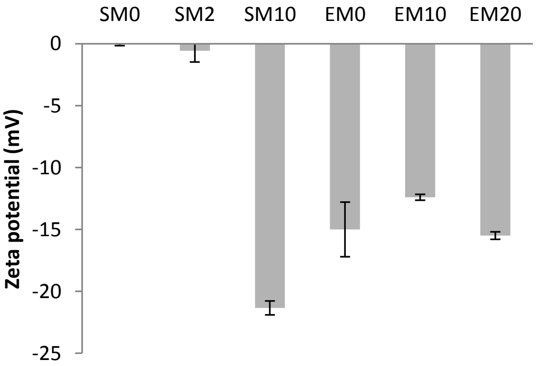

Figure 4. Zeta potential of TiO_2 nanoparticles (2 mg L^{-1}) in ecotoxicological test media: Steinberg medium (SM0), and diluted SM0 by a factor of 2 (SM2), and 10 (SM10); Elendt M7 medium (EM0), and diluted EM0 by a factor of 10 (EM10), and 20 (EM20).

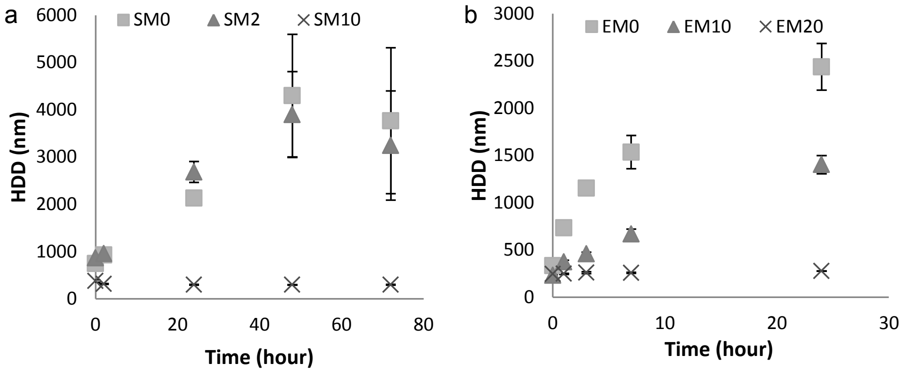

Figure 5. Hydrodynamic diameters (HDD) of TiO_2 nanoparticles (2 mg L^{-1}) in ecotoxicological test media. (a) Steinberg medium (SM0), and diluted SM0 by a factor of 2 (SM2), and 10 (SM10); (b) Elendt M7 medium (EM0), and diluted EM0 by a factor of 10 (EM10), and 20 (EM20).

TiO_2 NPs suspended in the undiluted media showed a tendency to aggregate, as indicated by the increased HDD (Figure 5). The general rule of the CCC is that the higher the number of valence electrons, the greater is the precipitating power. Thus, calcium with a 3-fold molar concentration to magnesium is probably the major regulator of particle aggregation as both calcium and magnesium are divalent. An increase in the colloidal stability of NPs, requires that one can either adjust the pH to increase the surface charge, or decrease the IS to avoid suppressing the EDL. The addition of surfactants and stabilizer agents is usually not recommended from the toxicological point of view, since they may result in toxic by-products or alter the properties and toxicity of the ENPs. In this study, dilution of the original medium was used to decrease the medium IS. The dilution decreased the calcium concentration clearly below the CCC level. These results showed that the dilution had effectively reduced the aggregation and deposition of TiO_2 NPs. The zeta potential of TiO_2 NPs suspended in diluted Steinberg medium became more negative with the increase of dilution factor and aggregation process slowed down (Figure 4 and 5). As shown in Figure 5, TiO_2 NPs remained stable in Steinberg medium with a dilution factor of 10 for 72 hours, and in *D. magna* test media with a dilution factor of 20 for 24 hours. No remarkable changes in particle size and concentration were observed in these diluted media over those times. Given that the toxicity of ENPs is correlated with their dose and size, the use of stable nanoparticle dispersions for which the exposure concentration and particle size is constant is required to assess accurately the biological effect of ENPs.

4.3 The bioaccumulation and effect of TiO_2 on *Lemna minor*

As discussed in 4.2, a 10-fold diluted Steinberg medium in which TiO_2 NPs showed the highest colloidal stability was used as the nutrient solution for the exposure experiments. The nutrient solution was renewed every 48 or 72 hours during the test periods in order to prevent the formation of large aggregates and also to maintain a sufficient nutrient supply for normal growth of *L. minor*.

Results of the growth inhibition test demonstrated that even at these high TiO_2 concentrations and long exposure times, no significant difference in the number of fronds produced or chlorophyll *a* concentrations was found between the control plants and the plants exposed to TiO_2 NPs (Figure 4, Paper II). Similarly, earlier reports showed that TiO_2 NPs have no effects on species of marine phytoplankton (*Thalassiosira pseudonana*, *Skeletonema marinoi*, *Dunaliella tertiolecta* and *Isochrysis galbana*), bacteria (*Vibrio fischeri*) and crustaceans (*Daphnia magna* and *Thamnocephalus platyurus*) (Heinlaan et al., 2008; Miller et al., 2010).

Although TiO_2 NPs showed no effect on growth rate, *L. minor* was found to accumulate TiO_2 NPs over the test concentration range (Figure 5 and 6, Paper II). Electron microscopic observations (Figure 6, Paper II) revealed that the accumulation was caused by surface attachment of the particles onto the cell walls of *L. minor*, but no uptake into the cells was observed. Given the rinsing treatment of the *L. minor* plant before electron microscopy and ICP-OES, the attached particles were clearly not removed by water, which suggests strong adhesion of TiO_2 NPs on the leaves. Most cells of plants, algae and fungi have cell walls that constitute a primary site for interaction with foreign particles, and it also acts as a barrier against their subsequent entrance into the cells. The plant cell wall is a complex structure formed by a mixture of polysaccharides, proteins, and other polymers that are assembled into a rigid, yet dynamically organized, network (Wolf et al., 2009). The reactive groups of these biomolecules offer a site to interact with NPs in the medium (Sadiq et al., 2011). Moreover, TiO_2 NPs did not penetrate into the plant tissue. This is probably due to the ‘sieving’ properties of the pores across the cell wall (Fleischer et al., 1999). Similar observations of particles bound to the cell surfaces have been reported for the algae *S. capricornutum* (currently named as *Pseudokirchneriella subcapitata*) and *Chlorella sp.* (Huang et al., 2005; Sadiq et al., 2011).

Although the mechanism of interaction between TiO_2 NPs and *L. minor* cell wall is poorly understood, the accumulation increased with increasing test concentration, and no saturation was observed under the conditions of this test. After 6 days of exposure to 0.01 mg L^{-1} of TiO_2 NPs, the Ti concentration of *L. minor* tissue was 0.22 mg kg^{-1}, which gives a ratio of tissue concentration to medium concentration of 83:1. Despite this high ratio there was no uptake into plant tissue. Since duckweed and algae are food sources of aquatic organisms such as water birds, plankton animals and fish, the transfer of TiO_2 NPs, and possibly also other ENPs to these higher organisms may occur. Therefore, the incorporation and accumulation of TiO_2 NPs into the aquatic food chain may become a mechanism of greater importance than previously considered. The results also indicated that along with the aggregation and deposition, the attachment to biological surfaces will occur. The ecological consequences of bioaccumulation/transfer become important regardless of the fact that TiO_2 NPs were not toxic to *L. minor* in this study, especially because TiO_2 NPs have been shown to be harmful to some other species such as Rainbow trout and zebrafish (Federici et al., 2007; Xiong et al., 2011).

4.4 The bioaccumulation and toxicity of TiO_2 in *Daphnia magna*

The findings presented in section 4.2 were that the 20-fold diluted Elendt M7 medium without EDTA was optimally used as a test solution for the exposure experiments. Additional experiments with neonates of *D. magna* cultured in this medium showed that no immobility was observed up to 72 h. This demonstrated that the medium was well tolerated by *D. magna*.

The calculated EC_{50} of TiO_2 NPs at 24 h was 8.0 ± 0.8 mg L^{-1}. TiO_2 NPs toxicity for different daphnia species has been investigated before (Hall et al., 2009; Hartmann et al., 2012; Lovern et al., 2007; Zhu et al., 2010). Reported 24-h EC_{50} values range from 7.6

to 143.4 mg L^{-1} depending on the different physicochemical properties of TiO_2 NPs (such as size, crystal composition and surface modification), and also variability in TiO_2 NPs dispersion methods and exposure protocols. The study by Cupi et al. (2016), used an unmodified OECD Elendt M7 medium, soft EPA medium and a very soft EPA medium to investigate the stability and toxicity of TiO_2 NPs on *D. magna*. Similar to the observation in our study, TiO_2 NPs were found to be unstable in M7 medium (pH 6−9) and formed large agglomerates in the micrometer range. No toxicity was found in this medium, 48-h EC_{50} >100 mg L^{-1}. A TiO_2 NPs suspension kept stable with small HDD (~200 nm) in very soft EPA medium (pH 7.0) caused a higher toxicity than that found in M7 medium, 48-h EC_{50} value of 13.7 mg L^{-1}. Study results indicate the imporant influence of media composition and IS levels on the stability of TiO_2 NPs suspensions and the immobilization of *D. magna*.

Although 2 mg L^{-1} of TiO_2 NPs showed no overt toxic effect on mobility, *D. magna* was found to accumulate TiO_2 NPs at this concentration over the test period. Microscopy (Figure 6) revealed that the accumulation was caused by two phenomena. First, was the phenomenon of the surface attachment of the particles onto the exoskeleton of *D. magna*, especially at the antennas and filtering apparatus (thoracic legs and abdominal claw). Large surface area and constant movement of these organs cause high numbers of encounters with NPs. This adhesion phenomenon has been previously described by Baun et al. (2008) and Dabrunz et al. (2011). Second, was the finding of the ingestion of the particles in the gastrointestinal tract that was also observed under the microscope. In this study, the TiO_2 NPs suspended in test medium had a particle size range of 202−404 nm, which falls into the lower end of the range 100−50000 nm that could be ingested by daphnia species (Geller and Müller, 1981). Seitz et al. (2015) also demonstrated that *D. magna* could ingest TiO_2 NPs that fell into the range of from ~90 nm to ~500 nm. Therefore, TiO_2 NPs suspended in the test medium could be readily ingested by daphnia. The surface attached TiO_2 NPs could be removed with the shedding exuviae when daphnia completed the moulting, but the suspended TiO_2 NPs will continuously attach to the new surface of daphnia.

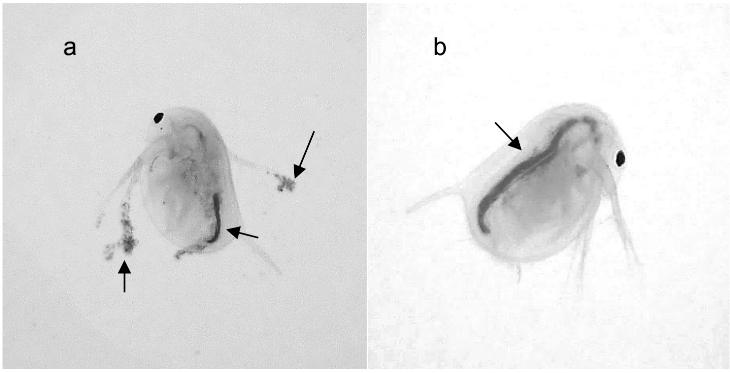

Figure 6. (a) Adhesion of TiO_2 nanoparticles to the exoskeleton and antennae of *Daphnia magna*, and (b) ingestion of TiO_2 nanoparticles in the gastrointestinal tract of *Daphnia magna*.

Though immobility was not observed within 48 h when exposed to 2 mg L^{-1} of TiO_2 NPs, *D. magna* showed abnormal behaviour such as slow and sporadic swimming during the exposure test. Such physical defects and a loss of mobility were also observed as result of the surface adhesion in earlier reports (Baun et al., 2008; Charde et al., 2014). Accumulation of NPs on the body surface could cause an increase in specific weight and physical resistance during swimming movements, and thus lead to an increase in energy consumption.

4.5 Mixture toxicity of TiO_2 NPs and Cd and Pb in *Daphnia magna*

In this study, *D. magna* was exposed to TiO_2 NPs at the ‘no observed effective concentration’ category of 2 mg L^{-1}, together with series of dilutions of Cd or Pb. The results revealed the influence of TiO_2 NPs on the bioaccumulation and toxicity of Cd and Pb to *D. magna*.

The 48-h EC_{50} value of Cd in the presence of TiO_2 NPs was 50 ± 9.3 μg L^{-1} which was much lower than the EC_{50} of Cd alone (97 ± 2.7 μg L^{-1}). When TiO_2 NPs was present, daphnia accumulated higher Cd than those exposed to the Cd only treatment (Figure 3b, Paper III). Strong sorption capacity of TiO_2 NPs for Cd has been reported in previous studies (Engates and Shipley, 2011; Zhang et al., 2007), and the bioaccumulation of Cd in carp was significantly enhanced by TiO_2 NPs. The mechanism of adsorption Cd was suggested to be the chemical sorption through chemical bonding and physical sorption through electrostatic force, of which the reversible physical sorption was dominant (Lin et al., 2016; Tan et al., 2012; Xie and Gao, 2009). The ingested Cd adsorbed onto TiO_2 NPs may have finally been released within the gastrointestinal tract of *D. magna*, as hypothesized by Tan and Wang (2014). Given that TiO_2 NPs was not toxic to *D. magna* at 2 mg L^{-1}, the accumulated Cd bound on NPs was still bioavailable, and could be assumed to be released from the NPs surface into free ions and eventually contributed to an increased toxicity of Cd.

The presence of TiO_2 NPs also caused changes in the bioaccumulation and toxicity of Pb for *D. magna.* At the end of the test, the accumulated Pb in daphnia exposed to the mixture of Pb and TiO_2 NPs was 3−4 fold higher than in daphnia exposed to Pb only (Figure 5b, Paper III). The results indicated that TiO_2 NPs had a strong sorption capacity for Pb, which was manifested by the bioaccumulation of Pb in the organism through nanoparticle uptake. Strong sorption capacity of TiO_2 NPs for Pb has been reported in previous reports (Engates and Shipley, 2011; Hu, et al., 2012; Liu et al., 2013), and the bioaccumulation of Pb in *Ceriodaphnia dubia* was significantly enhanced by TiO_2 NPs through nanoparticle uptake. Earlier studies also reported a higher sorption affinity of TiO_2 NPs for Pb than for Cd (Engates and Shipley, 2011; Liu et al., 2013). The stronger binding of Pd ions onto TiO_2 NPs relative to Cd may result in a much lower portion of Pb ions released from the NPs surface and subsequently decrease the bioavailability and toxicity of Pb bound on NPs. Moreover, adsorption onto the TiO_2 NPs surface and sedimentation of NPs may reduce the free Pb ion concentration in the medium, which decreases exposure of test organisms. In addition, once free Pb ion was adsorbed onto TiO_2 NPs, potential changes in Pb speciation may have occurred. Sun et al. (2009) previously reported the oxidation of As(III) to As(V) by TiO_2 NPs photocatalysis under sunlight. In the present study, when TiO_2 NPs was present, the Pb 24 h and 48 h EC_{50} values increased by 30% (144 ± 39.2 μg L^{-1}) and 47% (91 ± 2.2 μg L^{-1}) compared to Pb alone, respectively. Considering the lower toxicity of Pb when TiO_2 NPs was present, the adsorbed fraction of Pb on TiO_2 NP surfaces could be assumed to be less toxic or even non-toxic for *D. magna*, even though the bioaccumulation was enhanced through nanoparticle uptake and adhesion.

Interestingly, a delay and difficulty in moulting was found when *D. magna* was exposed to the mixture of TiO_2 NPs with Cd or Pb (Figure 4, Paper III). A juvenile *D. magna* kept at 20 ± 1ºC moults once within 48 h when starved (Smith, 1963). Daphnia moulted normally when exposed to 2 mg L^{-1} of TiO_2 NPs alone, whereas in the presence of a mixture of TiO_2 NPs and Cd, even the first moulting could not be completed entirely. The daphnia were observed to struggle with the exuvia, which were still tightly connected to the body. The mixture of TiO_2 NPs and Pb also caused a delay in moulting albeit the delay was not as long, but all the survived daphnia could complete the first moulting. The moult cycle has a large influence on the growth and reproduction. A delay or disturbance of moulting will ultimately lead to a reduction in reproduction rates (Buikema, 1973) and in long-term toxicity (Dabrunz et al., 2011).

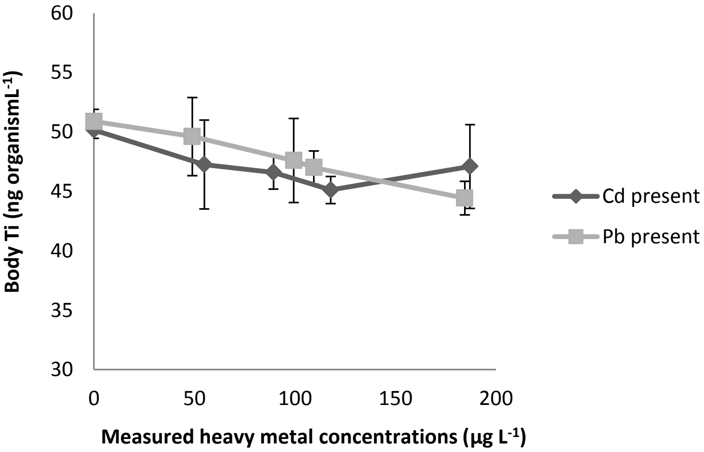

Figure 7. Bioaccumulation of TiO_2 NPs in *D. magna* (ng organism^{-1}) when exposed to different concentrations of the heavy metals Cd and Pb in 20-fold diluted Elendt M7 medium without EDTA for 48 hours (Paper III).

In addition, the bioaccumulation of TiO_2 NPs in *D. magna* was also influenced by Cd and Pb (Figure 7). The presence of Cd or Pb did not affect the suspended TiO_2 NPs in the test medium when compared to the control, but it caused a reduction in the bioaccumulation of TiO_2 NPs in *D. magna*. This could be explained by the moulting disturbance. When Cd or Pb was present, the moulting behaviour was disturbed and delayed as discussed earlier, which resulted in reduced amounts of new attached TiO_2 NPs. There was an increase in the body TiO_2 NPs at the Cd concentration of 200 µg L^{-1}

(Figure 7). This increase could be explained by the non-shedding surface attached TiO_2 NPs due to the unsuccessful moulting at higher Cd exposure concentrations.

NPs always co-exist with other pollutants in a natural environment and may thus interact with these pollutants by competitive adsorption, which in turn modify their occurrence and bioavailability, and ultimatly change their toxicity to water organisms. Therefore, the evaluation of the toxicity of individual pollutant has limited scientific value in the context of a naural water ecosystem. It is therefore necessary to evaluate the toxicity of NPs by considering the potential interactions with other pollutants in the environmental risk assessments.

5. CONCLUSIONS

This study revealed which natural water physicochemical properties can affect the aggregation and deposition of TiO_2 NPs and how these were probably mediated. Six out of 14 physicochemical properties were identified as being major determinants. Thus, the study contributes knowledge to the eventual prediction of TiO_2 NPs fate and behaviour in natural waters under typical Nordic conditions. Deposition takes place slowly in lake waters that have low conductivity, alkalinity and turbidity values. Moreover, pelagic organisms will have a higher probability of being exposed to suspended TiO_2 NPs than benthic organisms. On the other hand, a major fraction of TiO_2 NPs will settle as sediments in lake waters that have high value of pH, conductivity, alkalinity and turbidity and also in brackish waters with high salinity, assuming rapid dilution does not take place. Consequently, benthic organisms will have an increased risk of being exposed to the TiO_2 aggregates.

Testing and analysis of the colloidal stability of TiO_2 NPs in various ecotoxicological test media showed that TiO_2 NPs have a strong tendency to aggregate in the standard ecotoxicological test media. Medium dilution was found to be essential to prevent the aggregation of TiO_2 NPs and to keep organisms steadily exposed to TiO_2 NPs in test situations. Evaluation of the toxicity of TiO_2 NPs was performed on two pelagic organisms: *L. minor* and *D. magna.* Results indicated that TiO_2 NPs were non-toxic as no evidence of negative or positive effects on *L. minor* growth over a concentration range of 0.01−5 mg L^{-1} and with a long exposure time of 14 days. Even though no toxic effects on *L. minor* were observed, high accumulation may result in the transfer of the particles into the food chain, and thereby cause ecological influences in aquatic ecosystems. 2 mg L^{-1} TiO_2 NPs did not cause any death among *D. magna*, but its attachment onto the exoskeleton and also its ingestion were observed. *D. magna* also showed abnormal swimming behaviour, and this may further affect reproduction, predation, and food intake in conditions of long-term exposure. Furthermore, TiO_2 NPs could adsorb Cd or Pb from the ambient environment and therefore act as a heavy metal carrier, which resulted in an enhanced uptake of heavy metals in daphnia, and possibly in other water organisms as well. The presence of TiO_2 NPs in lake and brackish water samples changed the toxicity of these two tested heavy metals.

6. FUTURE STUDIES

Future efforts should be made towards understanding the role of ENPs properties and also water properties in contributing to the toxic effects on aquatic organisms. Due to the limitations of current toxicity standards and guidelines, these properties have not been systematically considered, and must therefore be monitored in future toxicity tests. The characterization and quantification analysis of ENPs should be performed both within the test media and test organisms to find and mimic the real organism exposure conditions by correlating the potential toxic effects with the exposed ENPs. A more comprehensive understanding of the environmental fate and biological effect of ENPs in natural waters are required. Therefore, further research is needed to quantify and characterize the aggregation and deposition of different type of ENPs such as silver and zinc oxide with different particle coatings. It would also be prudent to consider the released ENPs, which are aged/transformed ENPs during a products life cycle in addition to the pristine ENPs. In future studies, these phenomena should be also investigated at lower concentrations, since the recent development has improved the sensitivity and selectivity of the analytical methods such as FFFF-ICP-MS.

7. ACKNOWLEDGEMENTS

This thesis is based on the research carried out in the laboratory centre of the Finnish Environment Institute (SYKE). This work was financially supported by the Maa- ja Vesitekniikan Tuki ry association, and the Ministry of Environment of Finland.

I am very grateful to my supervisors, Dr. Markus Sillanpää, Dr. Eija Schultz, and Dr. Olli-Pekka Penttinen, for their inspirational discussions, enthusiastic guidance and mentorship. I have learned so much from you on my way to being a 'grown-up' scientist.

Thanks to the Minna Sepponen, Kaisa Heinonen, Maarit Risto, and Timo Sara-Aho, who have provided technical, experimental and graphical assistance in addition to being collaborators and co-authors. I would like to thank all the people that are working at the SYKE laboratory centre for the nice working atmosphere they contributed towards.

I would like to thank my friend and colleague Mrs Hannele Leskinen for all the friendship, discussions and support. My gratitude also goes to Alisdair Mclean for language check done on the doctoral thesis.

My heartfelt gratitude goes to my family and friends for all their encouragement and patience. Mom, dad, my dear sister and brother, thank you for all your encouragement and support. Special thanks to my two 'loooovely' little daughters MiJia and MiYao, it is you who really pushed me to finish this work. Finally, thank you Kai for your love and company!

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